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पुणे विद्यापीठ

श्रीयुत पाटील शामराव गणपतराव यांनी शास्त्र विद्याशाखांतर्गत रसायन-
ास्त्र विषयाच्या एम्.एस्सी. पदवीसाठी अंशत: संशोधनद्वारा घेतल्या जाणाऱ्या परीक्षेकरिता
ादर केलेल्या "STUDY OF NITRITE OXIDIZING BACTERIA" या शीर्षकाच्या
ंधाचे मूल्यमापन करण्यासाठी नियुक्त केलेल्या परीक्षकांचा अभिप्राय विद्यापीठाने मान्य
ा असून श्रीयुत पाटील शा.ग. उपरोक्त पदवीस पात्र आहेत आणि एम्.एस्सी.
ीक्षा उत्तीर्ण झाले आहेत असे माननीय कुलगुरूंच्या आदेशानुसार जाहीर करण्यांत
आहे.

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(ग.ज. अस्यांकर)
कुलसचिव.

STUDY OF NITRITE OXIDIZING BACTERIA

A Thesis submitted
to the
UNIVERSITY OF POONA
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by

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Chapter I

INTRODUCTION

INTRODUCTION

Nitrogen compounds such as proteins and nucleic acids are essential constituents of living cells. Both oxidized forms of nitrogen, such as nitrate as well as reduced forms such as ammonia, are utilized by microorganisms. Ammonia is oxidized to nitrite by the genus Nitrosomonas and nitrite is further oxidized to nitrate by the genus Nitrobacter. These two genera, Nitrosomonas and Nitrobacter, which are autotrophic organisms and convert ammonia to nitrate are called "nitrifying bacteria" and the whole process is termed "nitrification". These two genera play a vital role in the nitrogen cycle.

HISTORY

Pasteur suggested that the oxidation of ammonia to nitrate may take place by the action of microorganisms, but did not demonstrate this. In 1877 Schloessing and Muntz observed that when sewage water was passed through a column of sand and chalk for a period of twenty days ammonia was converted to nitrate quantitatively. When the column was treated by heat or with chloroform the conversion was stopped. They observed the conversion of ammonia to nitrate again when the same column was treated with fresh non-sterile water. Schloessing and Muntz tried to isolate the microorganisms on organic media containing glucose etc. as energy source and solid gelatin media but failed to isolate the organisms. They observed that nitrification was favoured

by alkaline conditions. In 1879 Warington noted the inhibitory action of glucose on nitrification but he too was unable to establish the nutritional requirements of these organisms.

In 1890 Winogradsky succeeded in isolating the microorganisms using a simple medium containing potassium phosphate, magnesium sulfate, potassium carbonate, ammonium chloride and 0.1% tartrate. By omitting each of the constituents it was established that only inorganic constituents were needed and that nitrification could be consistently obtained. By repeated subculture into inorganic media a culture was obtained which had nitrification activity. These microorganisms could not be grown on gelatin medium. Winogradsky introduced for the first time inorganic solid media using silicic acid, "silica jelly" on which colonies of nitrifying bacteria were obtained free from other bacteria. Schloessing, Muntz and Warington had observed that the nitrification was a two-step reaction i.e., ammonia to nitrite and nitrite to nitrate. But they were not able to isolate two separate organisms for each step. Winogradsky constituted a medium having only ammonia but not nitrite and the other medium having only nitrite but not ammonia and by solidifying these media with silica jelly two types of organisms were separated. These organisms were isolated from soil. The first was termed <u>Nitrosomonas</u> and the other <u>Nitrobacter</u>. Winogradsky was able to maintain these organisms on a liquid media by

periodic transfer for a few months. Warington compounded a medium in which precipitation of inorganic compounds was prevented.

Meyerhof (1916) defined optimum pH, concentration of ammonia etc. for Nitrosomonas and concentration of nitrite for Nitrobacter. He calculated the free energy available by the oxidation of ammonia and nitrite to these two types of organisms. Meyerhof also studied the inhibitory action of organic compounds on nitrification and the effect of potassium cyanide.

Identification:

According to Bergey's Manual of Determinative Bacteriology, 8th Edition (1975) the organisms oxidizing ammonia or nitrite are included in the family Nitrobacteraceae. There are three genera of nitrite-oxidizing bacteria and four of ammonia oxidizers. These bacteria are rod shaped, ellipsoidal spherical, spirillar, without endospore, flagella subpolar, they are gram negative and derive their energy by oxidizing ammonia or nitrite. They satisfy their carbon need by fixation of carbon dioxide from the atmosphere. These are not parasitic and are found in the soil, fresh water and sea water. They are obligate, aerobes and do not require organic growth factors. These cells are rich in cytochromes but no other pigments have been reported. They are usually non-motile and grow at $25^\circ C$ to $30^\circ C$. The pH requirement is 7.5 to 8.0.

The three genera of nitrite oxidizers are:

I. Nitrobacter winogradskyi, 1892. II. Nitrospina watson and

waterbusy 1971 and III. Nitrococcus watson and waterbusy 1971. The habitat of the first in soil, fresh water or sea water, while that of the other two is sea water only. The multiplication of the first is by budding while the other two genera multiply by binary fission.

All the nitrifying bacteria which are chemolithotrophs have been studied by Watson and Mandel (1971) for their morphology and DNA composition. The three genera of nitrite oxidizers were broadly separated by minor differences in DNA composition. Nitrobacter sp. are 0.8 μm in diameter and 1.0 to 2.0 μm long and are rod shaped. They multiply by the budding process as stated earlier. The DNA composition (G + C) is 60.7 to 61.2%. They are obligate aerobes and show slow growth on organic medium.

The second genus Nitrospina, 0.3 μm to 0.4 μm wide and 2.7 to 6.5 μm long straight, slender rods multiply by binary fusion and are non-motile. The (G + C) percentage is about 57.7%.

The third genus Nitrococcus is spherical with one or two flagella, about 1.5 to 1.8 μm in diameter with reddish colour. They show (G + C) composition of 61.2%. They multiply by binary fission. The cells show spinning action.

The habitat of genus Nitrospina and Nitrococcus is only sea water and for their growth the presence of sea water is essential. While for the first genus Nitrobacter habitat is soil, fresh water etc. and it shows growth in the absence of sea water. All these genera oxidize nitrite to nitrate and fix carbon dioxide from the atmosphere.

According to Watson and Mandel *Nitrobacter* sp. include *N.winogradsky*, *N.agilis*, *N.mobilis* and *N.gracilis*. All are short rods pear shaped 0.8 μm wide and 1.0 to 2.0 μm long having a polar cap of cytomembranes. Murray and Watson (1965) described *N.agilis* as having highly organized membrane intrusion at the poles of the cells and have dense layer on the inner side of the plasma membrane where metallo-enzymes are concentrated. The (G + C) composition varies between 60.2, 60.9, 61.2 and 61.7%. Morphologically all the strains are similar.

Watson and Mandel further consider that a revision of the taxonomy of nitrifying bacteria is necessary. Biological properties such as utilization of substrates or oxidation of substrates have so far not been used for the taxonomy of these cultures. Electron microscopic observations on their morphology and differences in DNA composition may offer a basis to distinguish these from each other. They studied the morphology and DNA composition of 27 strains but found that DNA composition is not yet adequate for classifying the bacteria. The ammonia oxidizers have shown (G + C) composition between 47.4 to 54.6% while nitrite oxidizers have shown a (G + C) composition between 57.7 to 61.7%.

Nelson in 1931 differentiated motile-strain as *N.agilis* from non-motile strain *N.winogradskyi*. But according to Zavarzin and Legunkova (1959) motile *Nitrobacter* species were observed only during the logarithmic phase of growth. At the narrow end of the cell there was a small swelling which made the cell pear-shaped. At the time of multiplication oneof the terminal ends increased in size to form a bud and when the bud reached its maximum size a single flagellum attached laterally

having a length of about 20 µm was present in the daughter cell. The motility was observed only with the separated bud while the mother cell was non-motile.

Nutritional Requirements:

Winogradsky's inorganic medium contained $(NH_4)_2SO_4$ 20.0 g, K_2HPO_4 7.5 g, KH_2PO_4 2.5 g, $FeSO_4.7H_2O$ 0.1 g, $MnSO_4.7H_2O$ o.1 g, $MgSO_4.7H_2O$ 0.3 g, $CaCl_2$ 0.2 mg, in 100 ml and then diluted one to hundred for liquid medium for Nitrosomonas. $NaNO_2$ was added instead of $(NH_4)_2SO_4$ for Nitrobacter. The same medium was solidified with silicic acid for the isolation of pure colonies of nitrifying bacteria. By this process he was able to maintain the cultures by repeated subculturing for a few months.

One of the difficulties in the growth of these pure cultures was their slow multiplication rate. Kingma Boltjes (1935) added $CaCO_3$ (1 mg/ml) to the medium which helped probably to prevent fall in pH and also provided CO_2 for their carbon need. Lees (1950) isolated the nitrifying bacteria by using the following medium: $CaCO_3$ 2.0 g, NaH_2PO_4 (1.0%) 1.0 ml, dialyzed iron 5 drops, $(NH_4)_2SO_4$ 1.0 g in 100 ml. He added 1.0 g of garden soil. Subculturing was carried out by inoculating 1.0 ml to 100 ml of medium. After five subcultures pure cultures of nitrifying bacteria were claimed to have been obtained. Engel and Alexander (1958) further suggested that solid $CaCO_3$ is required as a support on which bacteria can grow. Aleem and Alexander (1958, 1960) used a medium containing KNO_2 2.4 g, $MgSO_4.7H_2O$ 1.5 g, NaCl 1.5 g, $CaCl_2.2H_2O$ 0.1 g, K_2HPO_4 4.0 g, KH_2PO_4 4.0 g, $FeSO_4.7H_2O$ 80 mg, and $KHCO_3$ 12.0 g in 8.0 l for the growth Nitrobacter.

They found that nitrite-N at 130 ppm to 500 ppm was suitable for optimum growth, while less than 40 ppm and more than 1100 ppm was not favourable. Once the lag period was over, nitrite-N could be added stepwise upto 2000 to 5000 rpm. The yield of cells was 30,000-45,000 for 1 μg for nitrite-N.

Aleem and Alexander (1960) worked out the minimum requirement of phosphorus was 0.05 ppm, the maximum 3000 ppm and the optimum 5 ppm. The magnesium requirement was also important. In the absence of this ion oxidation of nitrite was negligible while 5 ppm gave full growth. Ferrous ions enhanced nitrification. A concentration of ferrous ions of 0.001 ppm increased nitrification two-fold, and 0.007 ppm three-fold compared to that with no Fe addition, but there was probably Fe also present as impurities in the other constituents of the medium. It is unlikely that there could be any nitrification in the absence of any iron salts since these organisms are aerobic and contain cytochromes. Aleem and Alexander observed no change in nitrification by the addition of copper and manganese, but the amounts present as impurities were not determined.

They studied the aeration rate on shakers and under stationary conditions and found that aeration rate of 0.038 mM oxygen per litre per min gave maximum oxidation. The effect of pH was tested over the range of 5.5 to 9.8 and at pH 8.0 the nitrification was optimum.

Krulwich and Funk (1965) introduced biotin in the mineral medium along with KNO_2 0.7 g, $CaCO_3$ 10.0 g, $MgSO_4.7H_2O$ 0.14 g, $FeSO_4.7H_2O$ 0.03 g, $MnSO_4.H_2O$ 0.01 g, NaCl 0.3 g,

K_2HPO_4 0.14 g, $NaCO_3$ 0.25 g, and biotin 150 mμg/ml at pH 8.1 in one litre. By the addition of biotin it was estimated that there was 2 to 4-fold increase in the rate of nitrification and more than 100-fold increase in the cell mass.

Finstein and Delwiche (1965) showed that molybdenum stimulated the growth of Nitrobacter culture and observed 11-fold increase in the cell mass as well as nitrite oxidation.

Fischer and Laudelout (1965) omitted $CaCO_3$ from the Nitrobacter medium and used a medium of the following composition: $NaNO_2$ 1.38 g, $Na_2HPO_4.12H_2O$ 12.88 g, KH_2PO_4 0.544 g, $FeSO_4.7H_2O$ (EDTA di-Na salt), 10 mg, $MgSO_4.7H_2O$ 20 mg, $ZnSO_4.7H_2O$ 20 μg, $CuSO_4$ 20 μg, Na_2MoO_4 20 μg in 100 ml and the pH was adjusted to 7.6. They obtained 30-40 mg dry weight of cells per litre. Solid $CaCO_3$ for the attachment of cells was thereby shown to be unnecessary.

Walker (personal communication) used $(NH_4)_2SO_4$ 0.5 g, KH_2PO_4 0.2 g, $CaCl_2$ 0.04 g, $MgSO_4.7H_2O$ 0.04 g, Fe (as chelate) 0.5 mg, in 1 l and adjusted the pH after sterilization to 7.5 to 8.0 by sodium carbonate for Nitrosomonas sp. The medium used for Nitrobacter contained: $NaNO_2$ 2.0 g, NaCl 0.5g, K_2HPO_4 0.1 g, $CaCO_3$ 0.01 g $(NH_4)_2SO_4$ 50 mg, Fe (as chelate) 250 μg, in 1 l. The pH after sterilization was adjusted to 8.0 with KOH solution. Both these cultures were maintained by 2-3 weekly transfer to a fresh medium.

Stanier used simple inorganic compounds like $MgSO_4.7H_2O$ 0.2 g, K_2HPO_4 1.0 g, $FeSO_4.7H_2O$ 0.05 g, $CaCl_2$ 0.02 g, $MnCl_2.4H_2O$ 0.002 g, $Na_2MoO_4.2H_2O$ 0.001 g as a basal medium for the autotrophic cultures and used $NaNO_2$ 3.0 g for Nitrobacter sp.

and NH_4Cl 1.5 g and $CaCO_3$ 5.0 g for Nitrosomonas sp. The pH was adjusted for both the media to 8.5. The cultures were incubated at 25°C to 31°C in the dark. The transfers were done monthly in liquid media.

Maintenance:

All the earlier workers faced the difficulties of isolation and maintenance of nitrite oxidizing bacteria and maintenance of these still remains a difficult task. The technique used by Winogradsky (1890) was followed by all the workers upto 1929 to isolate these nitrifying bacteria. Heubult (1929) used enrichment medium and dilution method to isolate pure cultures, but these methods were far from perfect. Kingma Boltjes (1935) isolated nitrifying cultures which were maintained by subculture every two months on "Nahrstoff Heyden" (a preparation which is not now available). He isolated Nitrosomonas and Nitrobacter on ammonia and nitrite media respectively. But he was reported to have lost all these cultures during the second World War. Jensen (1949) isolated these cultures and was able to maintain them upto a period of three years by liquid to liquid transfer. Meiklejohn (1950) tried to isolate them by enrichment and then plate them on silica gel but was not able to maintain for longer periods. Gundersen (1957) noticed that nitrifying bacteria maintained by periodic transfer to fresh liquid media last longer. He tried to maintain these nitrifying cultures by different methods such as,(i) monthly subculturing in liquid medium, (ii) preservation under paraffin oil, (iii) preservation under soil and (iv) freeze drying. He too lost all these

cultures within a period of 13 months by the first two methods while the last two methods were not found useful at all.

Loveless and Painter (1968) maintained activated sludge cultures for 18 months but their viability for longer periods has not been reported. Wallace and Nicholas (1969) concluded that no one has presented satisfactory data for maintenance of these nitrifying cultures. Gundersen stated that the toxicity of the end product might be responsible for the loss of the cultures. But there is no satisfactory evidence regarding the factors involved in the loss of these cultures during maintenance and suitable media and other conditions for their continued viability.

The maintenance of nitrifying bacteria is still a difficult task which has not been solved satisfactorily. Lees and Simpson (1957) used impure cultures for the study of biochemistry of nitrifying bacteria. It is evident from this that the continuous maintenance of these cultures is of great importance.

Gould and Lees (1960) were able to isolate Nitrobacter colonies by enrichment technique even in dilute medium containing 70 µg/ml nitrite-N and found growth of tiny colonies within a period of 10 days. They used for the first time antibiotics like terramycin, matromycin to remove heterotrophic contamination. Transfer of a loop of the grown culture to nutrient broth, tryptone water and 0.1% glucose and microscopic observation showed the purity of these cultures. Lees was able to isolate pure cultures by subculturing five times in the enrichment medium.

Matulewich, Strom and Finstein (1975) found that the conversion of nitrite to nitrate was complete within a period of

10 days when the concentration of KNO_2 was 0.1 mg/ml for <u>Nitrobacter</u> while 0.5 mg/ml was the concentration of $(NH_4)_2SO_4$ for <u>Nitrosomonas</u>. They also observed that <u>Nitrobacter</u> even when left in the incubation medium for 103-120 days at 29°C were viable.

<u>Effect of organic compounds on nitrification</u>

The nitrifying bacteria are autotrophs and they fix atmospheric carbon dioxide. The culture media contain only inorganic compounds and they derive their energy by oxidizing ammonia or nitrite ions. Till 1950 it was believed that the presence of organic compounds would inhibit the growth of nitrifying bacteria. <u>Nitrosomonas</u> and <u>Nitrobacter</u> species did not survive in the presence of organic compounds like glucose, glycerol etc. Jensen (1950) studied the effect of organic compounds on these nitrifying bacteria. The growth of these bacteria was inhibited by organic compounds above a certain level but at lower concentration nitrification was observed. He showed that 0.37 M of Na-formate 0.44 M of Na-acetate and 0.3 M Na-succinate, 0.56 M glucose, glycerol and sucrose had no effect of nitrification. It was also found that 0.022 M asparagine, 0.11 M aspartic acid, 0.17 M glutamic acid and 0.58 M urea permitted nitrification.

Smith and Hoare (1968) studied acetate assimilation by <u>Nitrobacter agilis</u>. Acetate upto 10 mM had no effect on nitrite oxidation and observed that acetate itself was oxidized to carbon dioxide. They used C^{14} acetate and showed that it was assimilated into cell protein. The assimilation was observed even in the absence of nitrite or bicarbonate but

the rate of assimilation was very low while in the presence of nitrite it was normal. The cultures which were grown on acetate had the ability to grow on inorganic media. Smith and Hoare studied the DNA composition of the cultures grown on acetate medium and nitrite medium and found that both had the same composition of bases. They also possessed the enzymes of the TCA cycle.

O'Kelley (1971) further observed that formate was also oxidized by autotrophic cultures to carbon dioxide but the generation time of these bacteria was increased. He used glucose in continuously dialyzed culture and came to the conclusion that cultures such as Nitrobacter species are not obligate autotrophs. Nitrobacter species have the ability to fix atmospheric carbon dioxide but at the same time can utilize organic carbon sources. But when grown on organic carbon source the generation time increased from 10 to 90 h. Smith, Hoare and Kelley came to the conclusion that cultures such as Nitrobacter species are facultative autotrophs whereas other nitrifying bacteria such as Nitrosomonas species are obligate autotrophs.

Steinmuller and Bock (1976) used the filtrates from heterotrophic organisms for the growth of nitrifying bacteria and found that the filtrate at 10% level increased the rate of nitrite oxidation and cell growth of N.agilis while 20% filtrate was required for N.winogradskyi for maximum stimulation. The filtrate from Ps.fluoresens gave approximately 4 times higher activity while M.phei filtrate gave double the activity compared to controls with no added culture filtrates. The other heterotrophic filtrates gave two to four times higher activity.

Steinmuller and Bock used three strains of nitrifying bacteria out of which two strains, N.agilis and N.winogradskyi showed higher activity in the presence of heterotrophic filtrates while Nitrobacter K4 was not activated at all. Morphological observations did not show any clear differences. The ability to respond to organic and other factors may be considered for the classification of nitrifying bacteria. The cultures which have the property of both autotrophs as well as heterotrophic are called Mixotrophs or Chemoorganotrophs.

Bock (1976) adapted the cells of N.agilis on acetate, formate and pyruvate and observed that pyruvate gave the highest growth and formate the least. With regard to oxygen consumption it was observed that with pyruvate it was double that with acetate. The nitrite oxidation activity of this adapted culture was not affected but cytochrome a_1 was not detected when compared with tne normal cultures. The adapted cultures took 2-3 weeks to regain the nitrite oxidation activity. The morphologically important observation was that the adapted cells possessed double cell membrane and s torage of polyphosphate and glycogen compared with the normal cells.

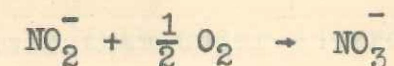
Inhibitors:

Lees and Simpson (1957) tested compounds such as cyanate, chlorate, fluoroacetate and nitrourea and observed that cyanate was a strong inhibitor of nitrite oxidation while chlorate also decreased the rate of nitrite oxidation. The other two compounds were not inhibitory. O'Kelley and co-workers (1970) confirmed that cyanate is a good inhibitor of nitrite oxidation. Butt and Lees (1960) tested 2,4-dinitrophenol on nitrite oxidation and

observed that this compound showed a good inhibitory action. The other compounds like "N-serve" [2-chloro-6-(trichloro-methyl)pyridine] used in the United States and 2-amino-4-chloro-6-methyl pyrimidine in Japan are valuable as inhibitors of nitrification. These two were used to conserve ammoniacal fertilizers. Natural inhibitors such as fatty oils, neem oils and neem-cake were found to be inhibitors of the nitrification process. (Ketkar, 1976).

Nitrite Oxidation:

The main energy source for Nitrobacter species is the oxidation of nitrite to nitrate where oxygen is the electron acceptor.



$$NO_2^- + \frac{1}{2} O_2 \rightarrow NO_3^-$$

Lees and Simpson (1957) first observed the reduction of cytochromes c and a_1 by Nitrobacter cells. They showed that cyanate inhibited nitrite oxidation directly. Aleem and Nason (1969) isolated a particulate fraction designated nitrite oxidase which contained these cytochromes and they considered that the electron flow from NO_2^- takes place via cytochrome c, cytochrome a_1 and then to O_2. The work of Kiesow, Aleem, Lees and Nicholas (1963) showed that cytochrome a_1 gets reduced first and then cytochrome c. They reported that cytochrome c is the site of energy generation. The path of electron flow was given as NO_2^- to cytochrome a_1 → cytochrome c → O_2. Aleem (1968) working with N.agilis confirmed the above path.

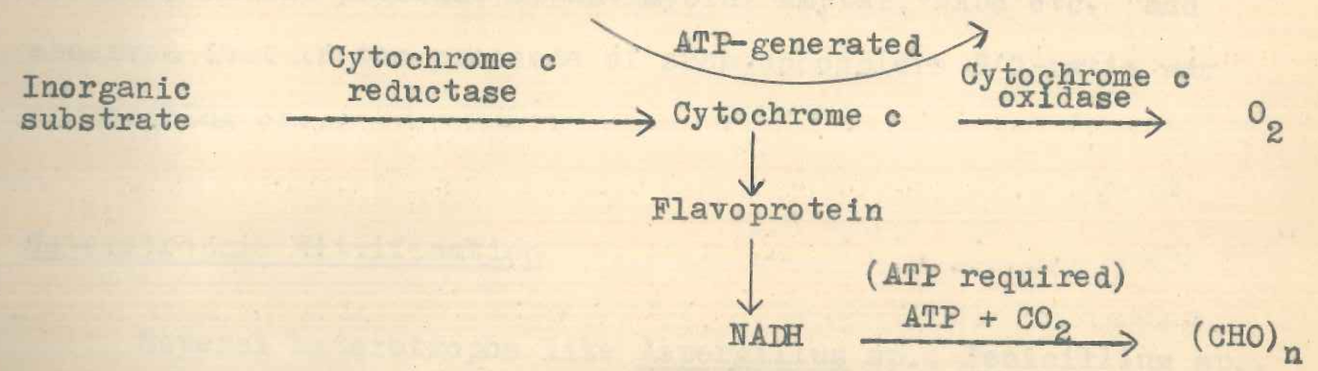
O'Kelley, Backer and Nason in 1970 studied the particulate nitrite oxidase in detail and observed the reduction of cytochrome by succinate and to some extent by NADH. They showed the presence of NADH oxidase, succinate oxidase, formate oxidase and traces of NADPH oxidase in N.agilis. The activity of these enzymes was maximum at alkaline pH. By the use of inhibitors such as cyanate they confirmed the presence of cytochrome oxidase.

Gool and Laudelout in 1966 showed that formate was oxidized by N.winogradskyi at pH 8.0. The energy available by the oxidation of formate was utilized for carbon dioxide fixation and cell growth. Further O'Kelley and Nason in 1970 confirmed the activity of formate oxidase in another strain of N.agilis along with nitrite oxidase. They showed that under anaerobic conditions NO_3^- or formate can act as electron donor in place of oxygen. Straat and Nason in 1965 showed nitrate reductase in N.agilis. They showed that cyanate and azide inhibited both nitrate reductase as well as cytochrome oxidase activities.

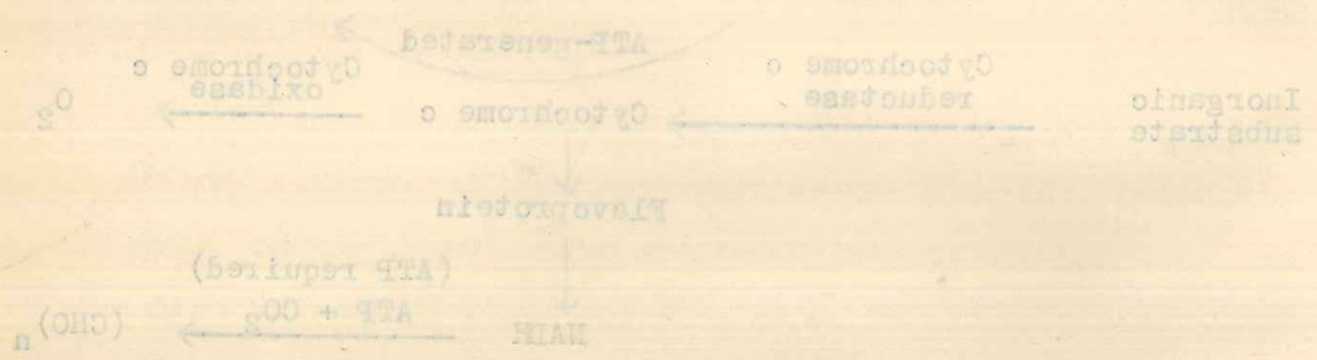
Oxidative Phosphorylation by Nitrobacter species

Nitrite oxidation is a single step reaction with a change in valency of N from +3 to +5. The change in ΔF energy is -17.8 K cal per mole. The reaction is carried out by the enzyme nitrite oxidase. The free energy change is utilized to carry out reactions such as fixation of carbon dioxide, growth of cells and synthesis of polyphosphate etc. Aleem and Nason in 1960 showed that the particulate cytochrome-containing nitrite oxidase from N.agilis is linked to phosphorylation. The presence of ADP and nitrite increased the phosphate uptake

while Mg^{++} was stimulatory to the phosphorylation rate. They observed that IDP and GDP also can serve as phosphate acceptors. The phosphorylation was inhibited by several inhibitors such as dinitrophenol, dicoumarol etc. Aleem and Lees (1963) further showed that cell-free extracts from N.agilis in the presence of nitrite, ADP and NAD increased the rate of carbon dioxide fixation and omission of any one of the compounds resulted in decreased carbon dioxide fixation. Aleem and Lees (1963) worked on cell-free extracts of N.agilis and suggested the following system of energy generation and its utilization towards carbon dioxide fixation etc.



Aleem (1965) observed that CO_2 fixation in cell-free extracts and oxidation of nitrite were similar to those of the whole cell. He studied the carboxydismutase and other Calvin cycle enzymes. The pentose phosphate enzymes were localized in the 144,000 x g supernate while nitrite oxidase was associated with cytochrome-containing particulate preparation. Aleem (1968) confirmed that the phosphorylation is coupled to nitrite oxidation in N.agilis by studying the effect of inhibitors like N-oxides, 2-n-hydroxyquinoline, rotenone, amytal and antimycin.



Malavolta, Delwiche and Burge in 1960 studied whole cells and cell extracts from Nitrobacter species and observed that 1 μmole of nitrite when oxidized esterified 0.5 μmoles of phosphate. Aleem and Nason (1960) also reported a P/O ratio of 0.14 to 0.2 which is quite low. Fischer and Laudelout in (1965) used cell-free extracts of N.winogradskyi and observed that the P/O ratio was 0.17 and reported that the purification of crude extracts further lowered the P/O ratio. In 1968 Aleem worked on cytochrome electron transport particulates from N.agilis and showed that the P/O ratio was close to 1.0. The oxidation of nitrite coupled to phosphorylation was not affected in the presence of antimycin, amytal, NADH etc. and observed that in the presence of such uncouplers P/O ratio was approaching one.

Heterotrophic Nitrification

Several heterotrophs like Aspergillus sp., Penicillium sp., Arthrobacter globiformis, Pseudomonas sp. are also known to carry out nitrification, but the rate of nitrification was very much less than that of nitrifying bacteria. Heterotrophic nitrification is outside the scope of this survey.

PRESENT INVESTIGATION

The present work was carried out to study the isolation of nitrifying bacteria from soils, their growth, maintenance and biological activity with special reference to Nitrobacter species which oxidise nitrite to nitrate. Several Nitrobacter strains were isolated. As stated earlier nitrifying bacteria are difficult to preserve. A method of preservation was found by which these nitrite oxidizing bacteria would be preserved for four years. The optimum conditions of pH, temperature, incubation period, concentration of nutrients in the medium for nitrite oxidation were determined. Organic and inorganic compounds were tested for their effect on nitrite oxidation by these cultures.

Chapter II of this thesis deals with the Materials and Methods used in the work.

Chapter III presents the experimental results of this work.

Chapter IV deals with a discussion of these results and a summary of the conclusions of these studies.

A bibliography of the references cited in this thesis is given in Chapter V.

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Chapter II

MATERIALS AND METHODS

Materials:

Chemicals and Biochemicals:

All the chemicals used were of analytical grade from British Drug Houses Ltd. or from E. Merck. Silica gel was prepared in this Laboratory. Dowex-50-X, 200-400 mesh cation exchange resin was from Dow Chemical Co.(U.S.A.). Biotin, folic acid and other vitamins were from B. D. H. except vitamin B_{12} (cyanocobalamin) which was from Themis Pharmaceuticals.

Carbohydrates and other compounds were from the source shown in brackets. D(-) Fructose (E. Merck), D-Glucose (B.D.H.), Pyruvic acid (Riedel), citric acid, formate and malic acid (B.D.H.).

Media such as Malt Extract, Yeast Extract, peptone, beef extract, noble agar, bacto agar were from Difco Laboratory. Sulphanilamide and brucine were from B.D.H. and N-1-naphthylethylene diamine HCl from E. Merck. Penicillin (sodium salt) was from Alembic Chemicals Works Ltd.

Methods

A modified Stanier's medium was used both for the isolation and maintenance of Nitrobacter species.

Basal Medium I		Addition to the Medium II	
$MgSO_4.7H_2O$	0.2 g		
K_2HPO_4	1.0 g	$NaNO_2$	3.0 g
$FeSO_4.7H_2O$	0.05 g		
$CaCl_2$	0.02 g		
$MnCl_2.4H_2O$	0.002 g		
$Na_2MoO_4.2H_2O$	0.001 g		
Biotin	1 µg		
Folic acid	1 µg		
B_{12}	1 µg	Final volume 1 litre	

The final pH of the medium was adjusted to 8.5 with sodium hydroxide. The basal medium was steam sterilized at 15 lbs. per sq. inch for 15 min. The additional medium nitrite solution was seitz filtered and then mixed under aseptic conditions with the basal medium. The modified Stanier's medium will be termed as 'Nitrobacter medium'. 20 ml Nitrobacter medium were distributed in 500 ml conical flasks and 1 ml inoculum added to each flask. The Nitrobacter cultures were incubated at 30°C to a stationary condition unless otherwise stated in the dark. Subculturing was carried out every month by inoculating 1 ml of the culture to 20 ml of fresh liquid medium. Care was taken not to

agitate the flasks after inoculation.

Noble agar plates were prepared to isolate Nitrobacter strains by using 2% special agar-noble and double strength basal medium which were steam sterilized separately. Sodium nitrite was seitz filtered and the three solutions were mixed together prior to modification and then poured in 20 ml lots into petri dishes under aseptic conditions. Then 1:1 diluted suspension of organisms grown in a suitable enrichment medium was streaked on these plates. These plates were kept in a desiccator containing water instead of desiccant at the bottom to maintain high relative humidity. These plates were incubated at $30^{\circ}C$. After a period of 20 to 25 days single colonies developed on these plates which were transferred to liquid medium.

A second method used to isolate single cell colonies of Nitrobacter species was with silica gel plates. These plates were prepared by the procedure of Sommers and Harris (1968). The potassium silicate was prepared by suspending 24 g of silica gel in 250 ml of 7% potassium hydroxide solution and by keeping in boiling water bath till the silica gel was completely dissolved. To this was added 93 g of Dowex 'H' form to bring the pH to between 9.5 to 9.7. The mixture was stirred for 20 min and then filtered to get a clear solution of silicic acid.

100 ml of Nitrobacter medium of triple strength (3X) having a pH of 6.4 to 6.7 and 200 ml of silicic acid at pH 9.5 to 9.7 were mixed thoroughly. The final pH of this mixture was adjusted to 8.4 to 8.5 by orthophosphoric acid (6N). 20 ml of this mixture were distributed into petri dishes. The solidification took place in 3 h. These plates instead of being steam sterilized were exposed to ultra violet light for 20 min. Then the culture suspension without dilution was streaked on these plates with a glass rod. These plates were kept in a desiccator under a moist atmosphere at $30^{o}C$. After a period of 10 to 15 days colonies appeared on these plates. These colonies were aseptically transferred to the Nitrobacter liquid medium.

Ammonia was estimated by Nessler's method. The samples to be estimated were diluted to an ammonia level of 50-100 μg/ml. 0.2 ml sample was taken and to this 9.4 ml water was added. Then 0.2 ml of Nessler's reagent prepared according to Vogel (1964) was added and final volume made to 10 ml. After 15 min the intensities of the colour developed were measured in a Beckman spectrophotometer at 500 mμ with corex glass cuvettes of 10 mm light path.

Nitrite was estimated by the sulphanilamide method [Snell and Snell (1970)]. The reagents were:

1) sulphanilamide 1.0% in one to four dilution of conc. hydrochloric acid, 2) N-1-naphthylethylene diamine HCl 0.02%, 3) standard solution of sodium nitrite, 100 μg/ml. The range of nitrite estimation was set between 1 μg to 10 μg/ml. The reagents sulphanilamide, 1 ml and N-1-naphthylethylene diamine, 1 mk, were added and after 10 min. The volume was adjusted to 6 ml with water. The colour developed was read at 540 mμ in a Klett Summerson colorimeter.

Nitrate was estimated with the Brucine reagent [Benjamin, Wolf and Bridgeton (1944)]. The reagents were: 1) Brucine 1% in conc. sulphuric acid prepared fresh before use, 2) urea 50%, 3) sulphuric acid 6M and 4) standard solution of nitrate as sodium nitrate. 100 μg/ml samples were diluted to give 10 μg to 150 μg/ml of nitrate were taken in test tubes and 0.1 ml of 50% urea was added. After mixing thoroughly 0.5 ml of 6 M sulphuric acid was added. The final volume of this mixture was adjusted to 5.6 ml with water. The tubes were shaken for 2 min and then kept in a boiling water bath for 8 to 10 min. The tubes were cooled to room temperature by keeping in water. 2.5 ml of 1% brucine in conc. sulphuric acid was added slowly with slow shaking and cooling in water. The colour intensities were then read at 425 mμ in a Klett Summerson colorimeter.

For all estimations, reagent blanks and standards of known amounts of NH_3, nitrite and nitrate were taken. Recovery experiments were also run in all cases with known amounts of standards added to the unknown to ensure that there were no interfering substances in the culture broths etc.

The media for the growth of heterotrophic cultures of bacteria, fungi and yeasts were prepared by the following methods. Potato-dextrose-agar (PDA) slants or plates for the fungal cultures were prepared using 200 g of peeled potatoes out into small pieces in 1 l of distilled water and steamed for 30 min. The supernatant liquid was collected and the volume was made up to 1 l. To this 2% dextrose and 2% agar were added. The pH of the medium was adjusted to 6 by HCl or NaOH. After melting the agar the required quantity of the medium was distributed in boiling tubes or test tubes. Steam sterilization was carried out at 15 lbs for 20 min. The slants were then made by keeping these tubes at the required slope.

The medium for yeast cultures was compounded by the addition of malt extract 0.3%, glucose 1%, yeast extract 0.3% and peptone 0.5%. The pH of the medium was adjusted to 6.4 to 6.8 with HCl and then 2% agar was added. After melting the agar the medium was distributed in tubes and then steam sterilized at 15 lbs for 20 min.

The nutrient broth slants were prepared by using beef extract 0.3%, sodium chloride 0.5% and peptone 0.5%. The pH of the medium was brought to 7.0 to 7.2 with sodium hydroxide. For the preparation of slants 2% agar was used and after melting, 7 to 8 ml were added to the test tubes and then steam sterilized at 15 lbs for 20 min. All these media were incubated for 1 to 2 days at $30^{\circ}C$ to check for any contamination before use. For the nutrient broth medium agar was omitted.

For Nitrosomonas species isolation and maintenance the above modified Stanier's medium was used with the following changes. To the Nitrobacter medium $CaCO_3$ 5 g, NH_4Cl 1.5 g per litre were added instead of $NaNO_2$. The procedure for media preparation, isolation, incubation etc. were otherwise the same.

For antibiotic assay, the seed and base agar medium having the following composition was used: Peptone 0.6 g, yeast extract 0.3, beef extract 0.15 g, agar 1.5 g in 100 ml. The pH was adjusted to 6.5 and after melting the agar 20 ml medium was distributed in boiling test tubes. The tubes were autoclaved at 15 lbs. for 20 min. The medium was poured in petri dishes under aseptic conditions. These dishes were incubated for 24 h before use.

Chapter III

EXPERIMENTAL RESULTS

The following experimental conditions were used for the studies on Nitrobacter. 1) The modified Stanier's medium termed as "Nitrobacter medium" was used. 20 ml of medium was taken in 500 ml conical stoppered flasks. 2) Nitrite-N concentration was 608 $\mu g/ml$. 3) The pH of the medium was adjusted to 8.5 with NaOH or HCl. 4) The incubation temperature was 30°C. 5) All the experimental flasks were left stationary. 6) The flasks were kept in dark. 7) All experiments were run in duplicate and values of nitrate presented in the tables are the mean values.

Any change in these conditions will be indicated in the text.

Section I-A

1) Isolation of *Nitrobacter* species from soil

The isolation of *Nitrobacter* species, which convert nitrite to nitrate, was carried out from the soil by enrichment techniques. The modified Stanier's media described in Chapter II were used for the isolation of *Nitrobacter* as well as *Nitrosomonas*. The soil samples were collected from different regions such as Poona (National Chemical Laboratory garden and Manjari Farm), Phaltan and Rajgurunagar (sugarcane fields which are heavily fertilized with ammonium sulfate or urea). Soil samples (approximately 0.2 g) were added to 20 ml Nitrobacter medium containing nitrite in 500 ml conical flasks in duplicate. One of these was kept on a shaker while the other was left stationary at 30°C.

At weekly intervals the estimation of nitrate formation was carried out by the brucine method. After a period of 20 days nitrate formation was detected in some of these flasks. The flasks which showed nitrate formation were used for subculture by transferring 1 ml to fresh Nitrobacter medium. These flasks were again incubated and tested for nitrate as before after 20 days.

To check the purity of these cultures one or two loops of these cultures were transferred to the

nutrient broth tubes and after incubating them at 30°C were observed for the growth of heterotrophic organisms.

Nitrobacter species free from other organisms were obtained by the streaking method. The suspension from these flasks either without dilution and after 1:1 dilution was streaked on noble-agar plates as well as on silica gel plates. These plates were kept in a desicoator under moist atmosphere at 30°C to avoid drying of these plates. After 10-15 days small tiny colonies appeared on these plates and they were transferred under aseptic conditions to liquid Nitrobacter medium in duplicate in 500 ml conical flasks. One set was kept on a shaker and the other kept stationary.

Four to five subculturings were carried out after incubating for a period of a month at 30°C each time to eliminate heterotrophs. Each time the nitrate formation was recorded. After five subcultures the conversion of nitrite to nitrate was observed within 20 days. Microscopic examination revealed that these organisms were gram-negative and all of them were of the same morphology.

The maintenance of these cultures was carried out by monthly transfer to fresh Nitrobacter medium. Initially for the first few months one set was left stationary and the other on a shaker at 30°C. Since the stationary cultures showed steady nitrate formation, the set of the cultures kept on the shaker was omitted and the maintenance of these

Nitrobacter species was carried out only under stationary conditions at 30°C.

Section- I B

Identification of isolated strains as Nitrobacter species

The microorganisms used in the present investigation were isolated by enrichment technique from the soil. The microorganisms of interest were made to grow on a specific substrate, nitrite, with no organic compounds except the vitamins. These cultures after four to five subcultures in Nitrobacter medium at pH 8.5 and at 30°C were plated twice on silica gel or on noble agar plates. By these methods 29 strains were isolated.

1) These 29 isolated cultures did not show growth on ammonium medium (see Section I-D). 2) These cultures did not show oxidizing activity under nitrogen gas atmosphere (Section II-C). 3) They did not require light for growth (Section II-E). 4) These cultures survived on simple inorganic salt. 5) Morphological observations showed that all these 29 strains from different regions have similarity in size. 6) Habitat is only from soil. 7) The growth rate was slow.

Microscopic examinations also showed [Plate 1] that they were not filamentous but were small single rods and gram-negative. The colonies on plates were light buffy coloured. Nitrite in the form of sodium or potassium was oxidized to nitrate. All these characteristics according to Bergey's Manual of bacteria proved that these cultures are *Nitrobacter* species only. It is however not known whether all the 29 strains are different or not. Further characterization was not carried out.

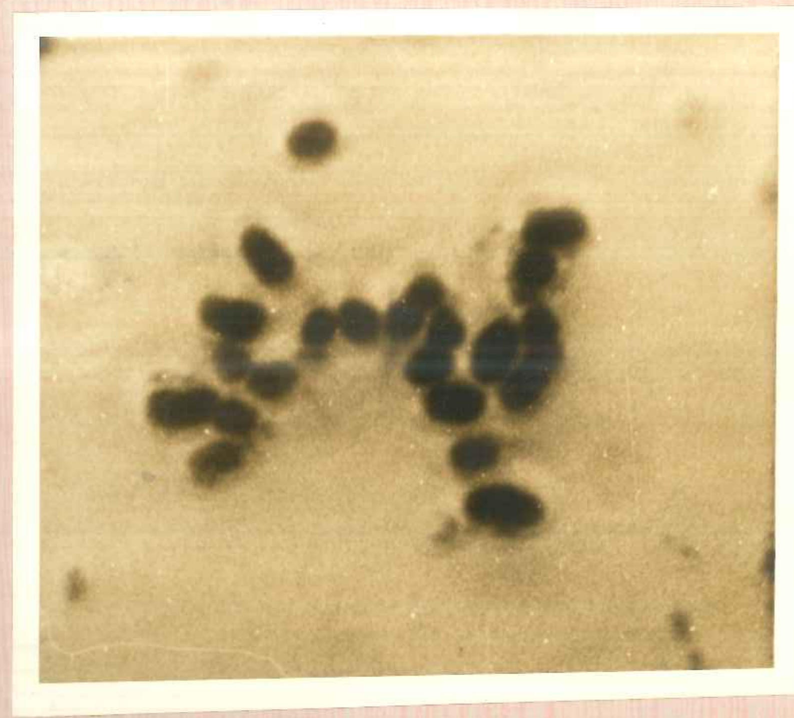


Plate 1: PHOTOGRAPH OF *Nitrobacter* species

Culture No.$25N_1$ X 6000

Section I-C

Maintenance of isolated Nitrobacter species

The Nitrobacter species isolated from 45 different soil samples have been maintained by the following method. Out of the isolated cultures a few were lost after four to five subcultures to fresh Nitrobacter medium. The remaining were purified by enrichment method and by isolating single cell colonies on silica gel or on noble agar plates.

The Isolated Nitrobacter cultures were numbered in order like $4N_1$, $5N_1$, $5N_2$, $6N_1$, $10N_1$, $12N_1$ and $12N_2$ and so on. A total of 29 Nitrobacter species are now being maintained. The cultures were tested for their purity by transferring a loop to tryptone water, nutrient broth and glucose yeast extract liquid media. They did not show heterotrophic growth after 2 days' incubation.

These cultures were maintained by liquid to liquid subculturings in Nitrobacter medium described earlier using 20 ml in 500 ml glass stoppered conical flasks and kept in the dark at $30^{\circ}C$. Incubation was carried out generally for one month. Nitrate formation was estimated for all the cultures regularly. [Table 1]

With this procedure the 29 Nitrobacter species have been maintained for over 4 years. There was no diminution in the rate of nitrate formation after such prolonged subcultures.

Since these are long-term experiments continued over 4 years it was unfortunately not possible to carry out controls in which different factors such as the macro and micro nutrients etc. were varied in order to determine which of them is responsible for continued viability of the Nitrobacter species. It is therefore not known whether these vitamins etc. were essential for the viability of these Nitrobacter cultures. It should however be emphasized that this is one of the few reports, possibly the only one, on the maintenance of several Nitrobacter strains for such a long period on a well-defined medium without loss of viability. One of the main objects of this work, the isolation and maintenance without loss of viability of Nitrobacter strains, was successfully achieved.

Seven strains of Nitrobacter species were also maintained on Nitrobacter-agar slants using "noble-agar". The cultures were transferred every month and incubated at 30°C. At the end of 5 subcultures, the activity of the cultures remained unaltered. The maintenance of these cultures for longer periods on agar is under investigation.

TABLE 1

MAINTENANCE OF Nitrobacter species AND THEIR NITRATE FORMATION ACTIVITY

[Data for two months of each year are presented though nitrate formation was determined for all subcultures.]

Medium	Nitrobacter medium
Volume	20 ml in 500 ml stoppered conical flask
pH	8.5
Temperature ..	30°C
Nitrite-N added	608 μg/ml

Nitrate-N formed in μg/ml

Culture No.	1974 May	1974 June	1975 May	1975 Aug.	1976 May	1976 June	1977 May	1977 June
4 N-1	564	605	627	623	612	613	627	615
5 N-1	614	602	640	618	620	621	620	590
5 N-2	624	595	592	576	600	590	613	624
6 N-1	632	564	626	608	609	613	628	630
7 N-1	600	608	620	637	618	627	592	610
8 N-1	-	492	612	617	617	633	654	613
9 N-1	590	613	597	579	612	618	640	624
10 N-1	613	670	620	627	627	619	608	618
12 N-1	564	591	613	592	598	601	632	600
12 N-2	570	670	618	600	543	612	614	594
13 N-1	670	613	615	451	492	613	617	627
14 N-1	590	570	529	540	570	614	620	618
15 N-1	613	608	570	589	595	600	594	624
16 N-1	492	564	490	590	600	590	570	600
17 N-1	600	614	597	537	627	612	618	610
17 N-2	608	624	620	451	598	619	615	620
18 N-1	564	600	620	461	515	570	640	633
19 N-1	595	595	627	527	670	580	653	627
19 N-2	602	608	618	460	-	590	627	608
20 N-1	605	593	623	650	633	618	615	618
21 N-1	615	615	620	590	670	617	608	627
25 N-1	627	571	598	603	600	541	617	620
27 N-1	613	627	591	601	612	632	612	623
31 N-1	594	619	620	618	605	542	610	627
32 N-1	597	574	633	637	609	587	613	608
34 N-1	609	627	525	587	608	519	627	626
35 N-1	613	591	525	587	627	587	608	594
39 N-1	617	612	527	600	615	642	640	634
39 N-2	628	624	518	600	610	625	600	615

Section I-D

Ammonium nitrogen for the growth of *Nitrobacter* species

The growth of N_8, $15N_1$, $16N_1$, $17N_1$ and $17N_2$ was determined on ammonium nitrogen. Both *Nitrosomonas* and *Nitrobacter* media were used for the growth experiments. Growth was checked by microscopic examination and activity was observed by measuring the formation of nitrate.

TABLE 2

AMMONIUM-NITROGEN FOR THE GROWTH OF *Nitrobacter*

Nitrobacter medium ... 20 ml
Nitrosomonas medium ... 20 ml
pH of the media 8.5
Temperature .... 30°C
Inoculation nitrate-N.. 31-35 μg/ml

Flask No.	Type of medium	Initial Ammonium-N	Initial Nitrite-N	Culture	Nitrate-N formed	Final Ammonium-N
		μg/ml	μg/ml		μg/ml	μg/ml
1	NS	392	-	$15N_1$	68	370
2	"	"	-	$16N_1$	73	384
3	"	"	-	$17N_1$	76	377
4	"	"	-	$17N_2$	67	380
5	NB	-	-	$15N_1$	47	---
6	"	-	-	$16N_1$	42	---
7	"	-	-	$17N_1$	53	---
8	"	-	-	$17N_2$	43	---
9	"	-	608	$15N_1$	658	---
10	"	-		$16N_1$	640	---
11	"	-		$17N_1$	633	---
12	"	-		$17N_2$	627	---

NS = Nitrosomonas medium
NB = Nitrobacter medium

It was observed that the culture did not utilize nitrogen supplied in the form of ammonium ions. There was no nitrite formation from ammonium ions. The slight apparent increase in nitrate in the ammonium media was partially due to experimental error since there was no corresponding decrease in ammonium ions. There was also no bacterial growth in these flasks. The results show that the cultures were specific for nitrite oxidation and proved that cultures were pure and had no contamination of <u>Nitrosomonas</u> organisms.

Section II-A

Optimum pH for the growth of *Nitrobacter* species

Nitrobacter species $5N_1$ and $12N_1$ were used for the determination of optimum pH. The initial pH of the media was from 5.5 to 11.5. The medium was adjusted with 0.1N NaOH or 0.1N HCl to the required pH. A month old inoculum was used in this experiment. The conversion of nitrite to nitrate was estimated.

TABLE 3

OPTIMUM pH FOR THE GROWTH OF *Nitrobacter* species

Nitrobacter medium ...... 20 ml
Nitrite-N added .... 608 μg/ml
Inoculum nitrate-N ...... 32 μg/ml for $5N_1$
34 μg/ml for $12N_1$
Temperature .... $30^{o}C$

Flask No.	Initial pH of the medium	Nitrate-N formed after 7 days
1	5.5	70
2	6.0	80
3	7.0	502
4	8.5	622
5	9.5	577
6	10.0	85
7	11.5	72

Results with culture $5N_1$ were similar to those of culture $12N_1$.

[Fig. 1]

An initial pH of the medium between 7.0 to 9.5 gave good nitrite oxidation and pH 8.5 appears to be the optimum pH. At a pH lower than 7.0 or above 9.5 there was a marked fall in the activity. In routine practice pH 8.5 was used for the maintenance of *Nitrobacter* species.

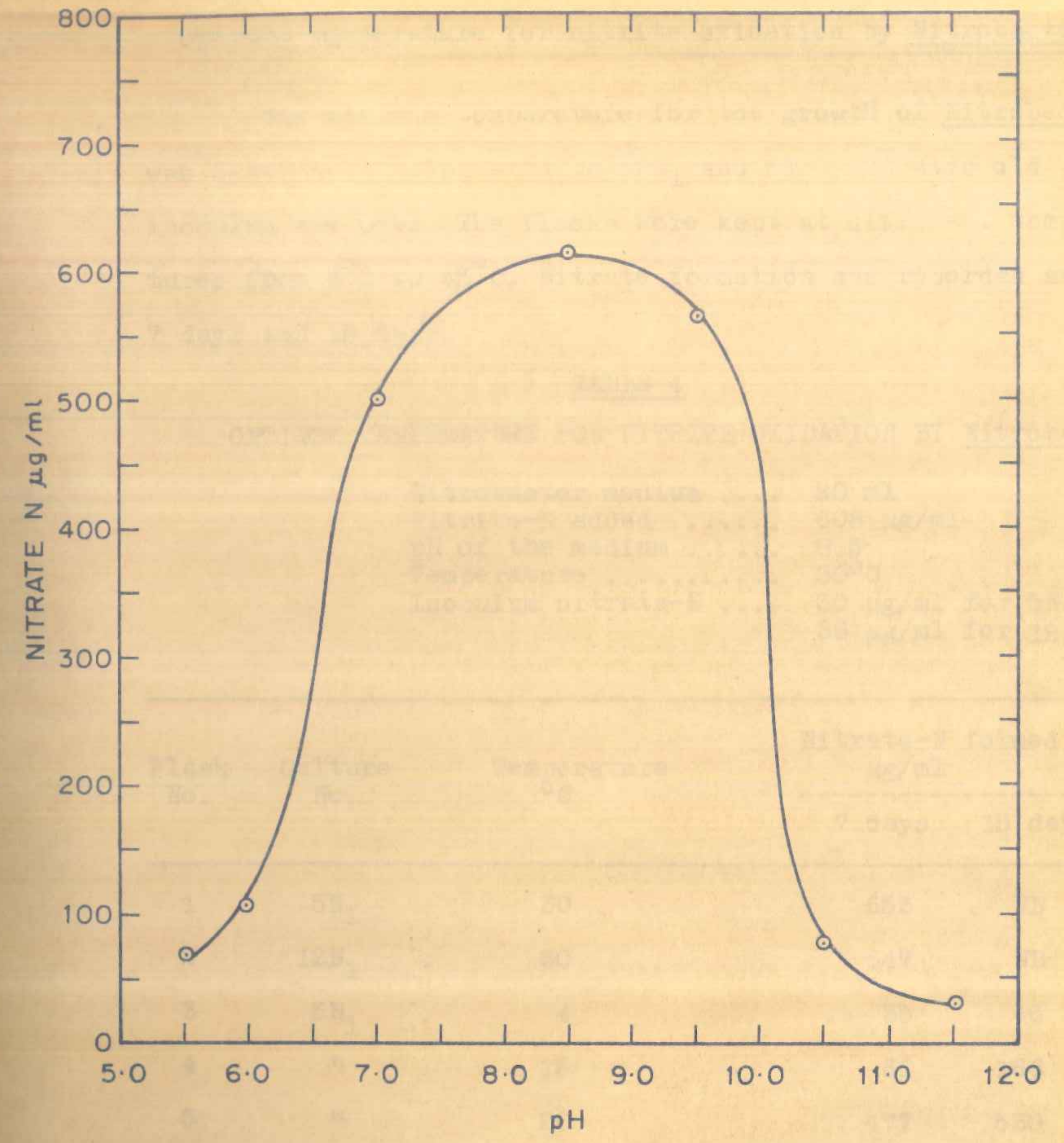


FIG. 1: OPTIMUM pH FOR GROWTH OF NITROBACTER

Section II-B

Optimum temperature for nitrite oxidation by Nitrobacter

The optimum temperature for the growth of Nitrobacter was determined using strains $12N_1$ and $5N_1$. 20 days old inoculum was used. The flasks were kept at different temperatures from 4°C to 45°C. Nitrate formation was recorded after 7 days and 15 days.

TABLE 4

OPTIMUM TEMPERATURE FOR NITRITE OXIDATION BY Nitrobacter

Nitrobacter medium .... 20 ml
Nitrite-N added .... 608 μg/ml
pH of the medium 8.5
Temperature .... 30°C
Inoculum nitrate-N .... 30 μg/ml for $5N_1$
38 μg/ml for $12\ N_1$

Flask No.	Culture No.	Temperature °C	Nitrate-N formed μg/ml	
			7 days	15 days
1	$5N_1$	30	653	ND
2	$12N_1$	30	647	ND
3	$5N_1$	4	35	46
4	"	15	61	166
5	"	20	477	530
6	"	24	541	595
7	"	30	649	655
8	"	37	69	72
9	"	45	28	ND

ND = not determined

Culture No.$12N_1$ gave similar results

Nitrite oxidation activity of **Nitrobacter** was observed to be the highest at 24°C to 30°C. The optimum temperature was found to be 30°C in this experiment. Above 30°C and below 15°C nitrite oxidation was very poor, while at 45°C there was no activity. Since all the 29 strains were maintained at 30°C for over 4 years without any diminution in nitrite oxidation. These cultures are quite stable at 30°C.

[Fig.2]

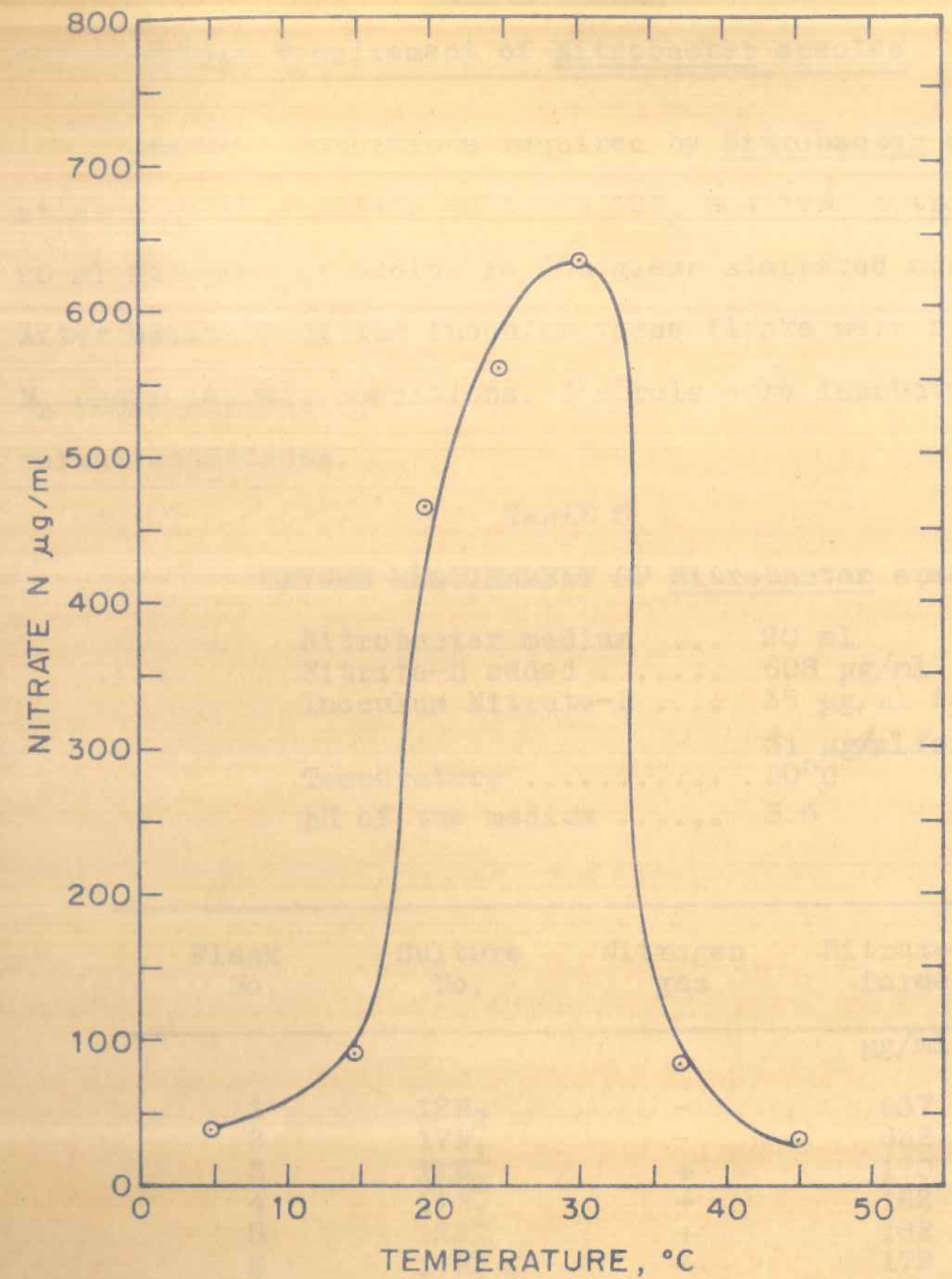


FIG. 2: OPTIMUM TEMPERATURE FOR NITRITE OXIDATION BY <u>NITROBACTER</u>

Section II-C

Oxygen requirement of Nitrobacter species

Aerobic conditions required by Nitrobacter species was studied by inoculating cultures $12N_2$ and $17N_1$ Nitrobacter into 20 ml Nitrobacter medium in 500 glass stoppered conical flasks. After addition of the inoculum these flasks were flushed with N_2 under aseptic conditions. Controls were incubated under normal conditions.

TABLE 5

OXYGEN REQUIREMENT OF Nitrobacter species

Nitrobacter medium ... 20 ml
Nitrite-N added .... 608 μg/ml
Inoculum Nitrate-N .... 35 μg/ml for $12N_2$
31 μg/ml for $17N_1$
Temperature .... 30°C
pH of the medium 8.5

Flask No.	Culture No.	Nitrogen gas	Nitrate-N formed
			μg/ml
1	$12N_2$	-	657
2	$17N_1$	-	632
3	$12N_2$	+	170
4	$17N_1$	+	162
5	$12N_2$	+	182
6	$17N_1$	+	172

It will be seen from Table 5 that there was very little nitrite oxidation under N_2 compared to the controls. The nitrate formed under N_2 was probably due to a small amount of dissolved oxygen in the medium for the growth of Nitrobacter species oxygen requirement appears to be essential.

Section II-D

Optimum concentration of nitrite

The effect of different concentrations of nitrite on nitrite oxidation was determined. Flasks containing basal medium with different amounts of sodium nitrite were inoculated with culture $25N_1$. The flasks were incubated stationary at 30°C. The samples for nitrate estimation were taken every 72 h. In Table 11 only the period required for nearly complete oxidation of the initial nitrite are presented. (An initial period of 72 h was subtracted from the total incubation period. (See also Section II-E for nitrite oxidation.)

(Table 6)

It was observed from the Table that nitrate formation was complete when the initial concentration of nitrite was 608 μg/ml within 8 days while it was not completed at an initial nitrite concentration of 3040 μg/ml even after 30 days. The average nitrite oxidation rate was 5.2 μg per h per ml at an initial nitrite concentration of 608 μg/ml and only 3.3 μg/h at an initial concentration of 3040 μg/ml. This may be compared with the maximum rate of 13 μg nitrite oxidation per h per ml (Section II-F) between 96 and 120 h).

TABLE 6

TO DETERMINE OPTIMUM CONCENTRATIONOF NITRITE

Nitrobacter medium ... 20 ml
Inoculum nitrate-N ... 33 μg/ml
pH of the medium 8.5
Temperature .... 30°C

Flask No.	Nitrite-N added	Period of oxidation	Nitrate-N formed	Rate of oxidation
	μg/ml	h	μg/ml	μg/h
1	608	120	632	5.2
2	1216	240	1196	5.0
3	1826	360	1603	4.5
4	2435	504	2030	4.0
5	3040	720	2393	3.3

[The initial lag period of 72 h was subtracted from the total period of incubation - column 3.]

Section II-E

Effect of light on nitrite oxidation by Nitrobacter species

To study the effect of light on Nitrobacter species two Nitrobacter species $12N_1$ and $15N_1$ (15 days old) were used. Controls were in the dark and experimental flasks illuminated with fluorescent tube lights (800 x 1.3 to 850 x 1.3 lux). The conversion of nitrite to nitrate was measured after 23 days.

TABLE 7

EFFECT OF LIGHT ON NITRITE OXIDATION BY Nitrobacter species

Nitrobacter medium ...... 20 ml
Nitrite-N added .... 608 μg/ml
pH 8.5
Temperature .... 30°C

Culture No.	Inoculum Nitrate-N	Dark or illuminated	Nitrate-N formed
	μg/ml		μg/ml
$12N_1$	31	Control	615
$15N_1$	33	"	616
$12N_1$	31	Dark	627
$15N_1$	33	"	614
$12N_1$	31	Light	602
$15N_1$	33	"	577

The conversion of nitrite to nitrate was the same with the cultures in the dark or with illumination. Light is not essential for nitrite oxidation by these strains.

Section II-F

Rate of nitrate formation

To study the rate of nitrite oxidation and nitrate formation the culture *Nitrobacter* $5N_1$ was used 20 ml Nitrobacter medium were used. A month-old inoculum of *Nitrobacter* $5N_1$ was used in this experiment. Three flasks were left stationary at 30^oC and samples from the flasks were removed every 24 h for the estimation of nitrite and nitrate.

TABLE 8

RATE OF NITRATE FORMATION

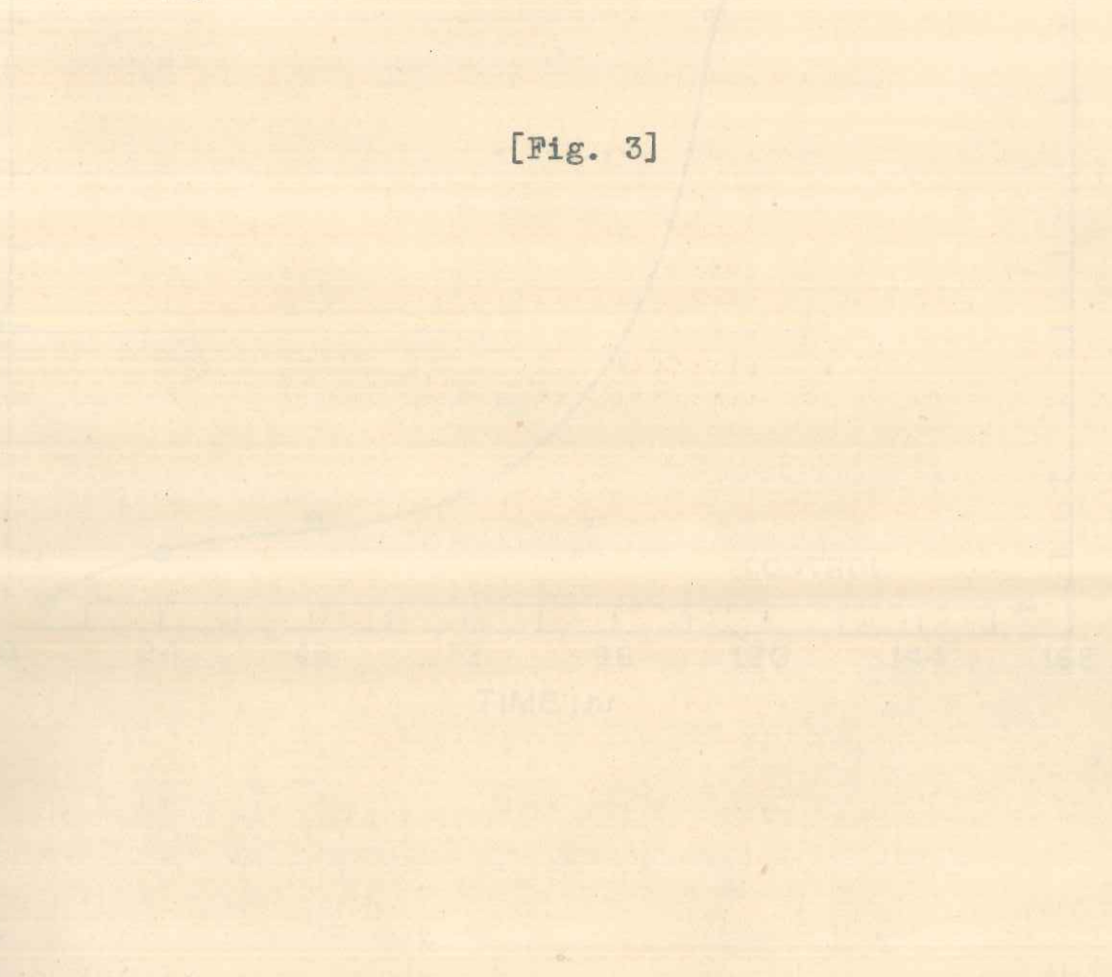
Nitrobacter medium ..... 20 ml
Nitrite-N added .... 608 µg/ml
Inoculum nitrate-N ...... 33 µg/ml
pH of the medium 8.5
Temperature .... 30^oC

Flask No.	Nitrate-N formed: Period in h	24	48	72	96	120	144	168	Nitrite-N remaining after 168 h
	µg nitrate N/ml								
1		52	52	52	52	52	52	52	611
2		57	59	67	170	496	587	652	0
3		57	61	90	180	515	618	650	0
4		57	67	78	180	541	627	687	0

Flask 1 was not inoculated

It was observed that the oxidation of nitrite to nitrate was negligible upto 72 h. The rate of oxidation began to increase between 62 h and was maximum between 96 h to 120 h. It then began to decrease between 120 to 168 h. After 168 h there was no nitrite left and the nitrate formation showed no further increase. Between 96-120 the rate of nitrite oxidation was 13 μg/h (c.f. Section II-D).

[Fig. 3]



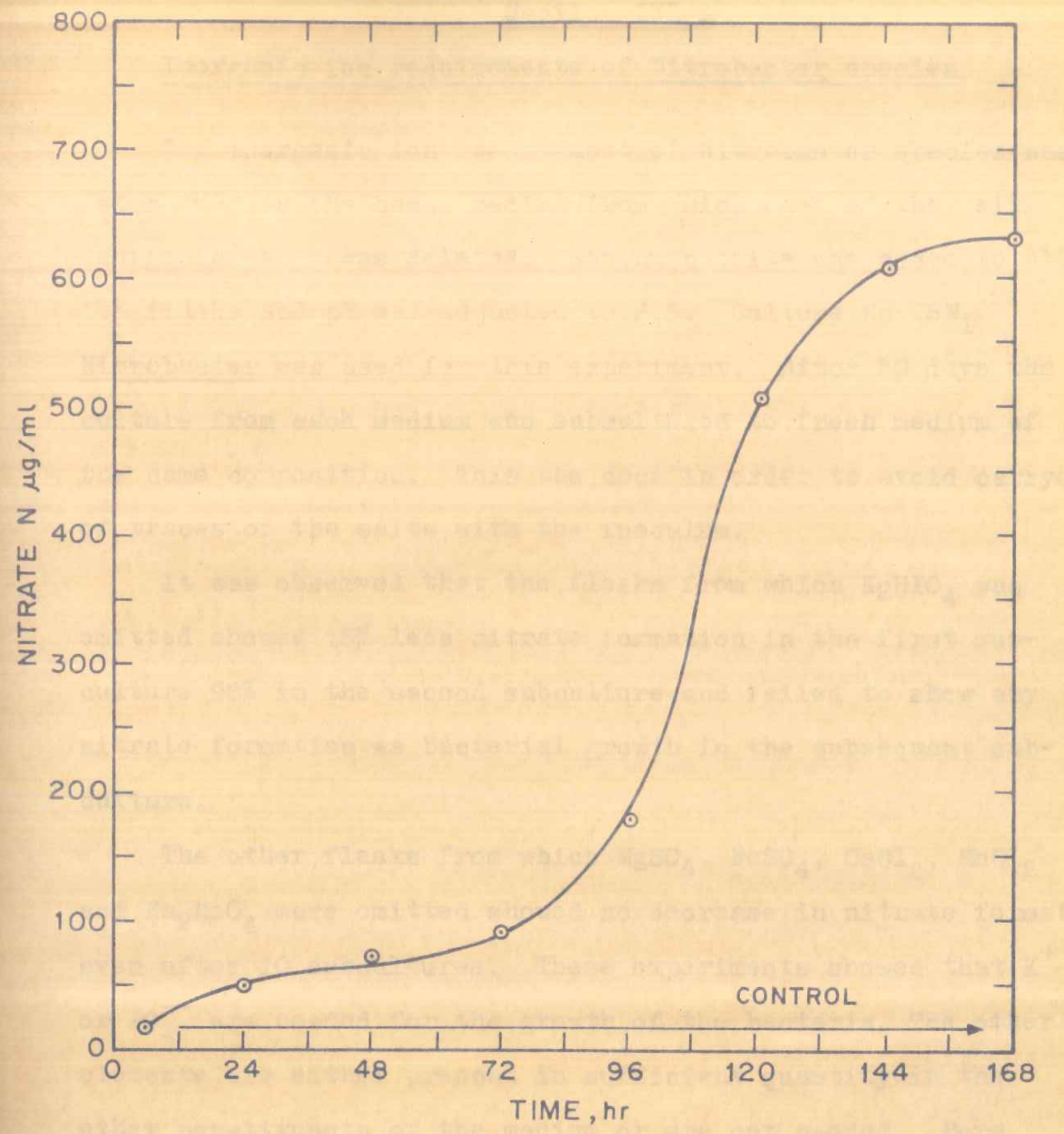


FIG. 3: RATE OF NITRATE FORMATION

Section III-A

Inorganic ion requirements of *Nitrobacter* species

The inorganic ion requirement of Nitrobacter species was determined in the basal medium from which each of the six inorganic salts was deleted. Sodium nitrite was added to all the flasks and pH was adjusted to 8.5. Culture No.25N_1 *Nitrobacter* was used for this experiment. After 30 days the culture from each medium was subcultured to fresh medium of the same composition. This was done in order to avoid carryover of traces of the salts with the inoculum.

It was observed that the flasks from which K_2HPO_4 was omitted showed 15% less nitrate formation in the first subculture 98% in the second subculture and failed to show any nitrate formation as bacterial growth in the subsequent subculture.

The other flasks from which $MgSO_4$, $FeSO_4$, $CaCl_2$, $MnCl_2$ and Na_2MoO_4 were omitted showed no decrease in nitrate formation even after 10 subcultures. These experiments showed that K^+ or PO_4^{---} are needed for the growth of the bacteria. The other elements are either present in sufficient quantity in the other constituents of the medium or are not needed. More vigorous experiments with highly purified reagents are needed to establish the minimum requirements of inorganic ions for these organisms. In the absence of $CaCl_2$ it was observed that tiny floating motiles containing bacteria appeared though there was no loss of nitrate formation activity.

Section III-B

Nitrobacter growth in the absence of vitamins

In the regular maintenance medium of the Nitrobacter cultures, three vitamins, biotin, folic acid, cyanocobalamin, were added at the level of 1 $\mu g/l$. The requirement of these vitamins was determined by growing cultures on a medium containing none of these vitamins. The composition of the medium was otherwise the same. Subculture was made into the same vitamin-free medium. So far 15 subcultures have been made using Nitrobacter Nos. $5N_1$, $12N_1$ and $17N_1$.

During these subcultures none of the cultures showed any diminution in growth or ability to oxidize nitrite. These vitamins do not appear to be essential in the medium for these Nitrobacter cultures. Long term growth studies on vitamin-free medium are however being continued in order to determine whether they are required for viability of these cultures over a period of several years.

Section III-C

Effect of acetate on *Nitrobacter* growth in nitrite medium

The culture Nos. $5N_1$, $12N_1$ and $17N_1$ were tested for their ability to grow on nitrite medium in the presence of acetate. These cultures were maintained by monthly sub-culture in media containing both nitrite as in standard Nitrobacter medium and an equimolar amount of sodium acetate. The pH and other conditions were kept constant.

15 subcultures were carried out and it was observed that there was no decrease in nitrate formation in the presence of acetate even on prolonged subculture. Acetate is, therefore, not inhibitory to these organisms. The ability of these cultures to grow on acetate in the absence of nitrite will be determined separately with these acetate-adapted cultures.

Section III-D

Utilization of organic compounds by Nitrobacter species

D-Glucose, sodium acetate, sodium citrate, malic acid, pyruvate and formate were tested for their utilization by the Nitrobacter cultures. Four flasks two containing the organic compound in 20 ml medium along with nitrite and two flasks without nitrite were used for each of the compounds. The moles of the organic compounds added were equal to the moles of sodium nitrite in the basal medium. The pH of the medium was 8.5. 25 days old inoculum of $5N_2$ was used.

TABLE 9

UTILIZATION OF ORGANIC COMPOUNDS BY Nitrobacter species

Nitrobacter medium ...... 20 ml
Nitrite-N added .... 608 μg/ml
Inoculum nitrate-N ...... 32 μg/ml
pH 8.5
Temperature .... 30°C
Nitrobacter culture ..... $5N_2$

Flask No.	Compound	Quantity added	Nitrate-N formed	Nitrite Oxidation
		moles	μg/ml	%
1	-	-	638	100
2	D-Glucose	0.044	632	100
3	Acetate	0.05	643	100
4	Citrate	0.042	495	77
5	Malate	0.045	485	75
6	Pyruvate	0.05	46	0
7	Formate	0.04	320	50

D-Glucose and sodium acetate had no inhibitory effect on nitrite oxidation while citrate and malate showed inhibition upto 25% and formate 50%. Pyruvate on the other hand showed complete inhibition of nitrite oxidation. In the flasks with nitrite the bacterial growth was not observed by microscopic examination compared to growth in controls.

Section III-E

Effect of heterotrophic filtrates on Nitrobacter species

The heterotrophic cultures, *Escherichia coli* B, *Escherichia coli* NCIM 8879 and *Pseudomonas fluorescens* NCIM 2315 were grown on nutrient broth for 24 h at 30°C. The yeast cultures *Candida utilis* NCIM 3055 was grown in MGYP liquid medium at 30°C for 24 h while *Gibberella saubinetti* NCIM 851 and *Gibberella fujikuroi* NCIM 665 were grown in potato dextrose liquid medium at 30°C for 48 h. All these cultures were kept on a shaker.

After checking for growth and purity the culture fluids were centrifuged at 3000 rpm for 30 min and the supernatant liquids were collected under aseptic condition and 1 ml of each added to Nitrobacter medium. The *Nitrobacter* No.$17N_1$ was used as inoculum. After incubating these flasks for 7 days nitrite formation in these flasks and the controls were determined.

TABLE 10

EFFECT OF HETEROTROPHIC FILTRATES ON Nitrobacter species

Nitrobacter medium	20 ml
Nitrite-N added	608 µg/ml
Inoculum nitrate-N	32 µg/ml
pH of the medium	8.5
Temperature	30°C
Culture used	$17N_1$

Flask No.	Filtrates of heterotroph cultures (1 ml to 20 ml)	Nitrate-N formed µg/ml
1	Control	638
2	E.coli B	90
3	E.coli NCIM 8879	87
4	Ps.fluorescens 2315	94
5	C.utilis 3055	91
6	G.saubinetti 851	90
7	G.saubinetti 665	90

It was observed that the filtrates from all these heterotrophic cultures instead of stimulating the activity of Nitrobacter culture were strongly inhibitory to nitrate formation.

TABLE 10

EFFECT OF HETEROTROPHIC FILTRATES ON Nitrobacter species

Nitrobacter medium	20 ml
Nitrite-N added	608 μg/ml
Inoculum nitrate-N	32 μg/ml
pH of the medium	8.5
Temperature	30°C
Culture used	$17N_1$

Flask No.	Filtrates of heterotroph cultures (1 ml to 20 ml)	Nitrate-N formed μg/ml
1	Control	638
2	E.coli B	90
3	E.coli NCIM 8879	87
4	Ps.fluorescens 2315	94
5	C.utilis 3055	91
6	G.saubinetti 851	90
7	G.saubinetti 665	90

It was observed that the filtrates from all these heterotrophic cultures instead of stimulating the activity of Nitrobacter culture were strongly inhibitory to nitrate formation.

Section III-F

The optimum medium depth for nitrite oxidation by *Nitrobacter* species

Preliminary experiments showed that continuous agitation resulted in the loss of some ammonia oxidizing cultures. Maintenance therefore was carried out only under stationary conditions. The optimum depth of the medium was determined so that diffusion of gases was not rate-limiting. Different volumes of media were taken in 500 ml conical flasks so as to give 1, 2, 4, 8, 10 cm depth. The experiments were carried out in duplicates. 2.5% inoculum of cultures No.$5N_1$ and $12N_1$ was added to each flasks and incubated as usual at 30^oC. Samples were removed every 72 h and nitrate formation was measured.

TABLE 11

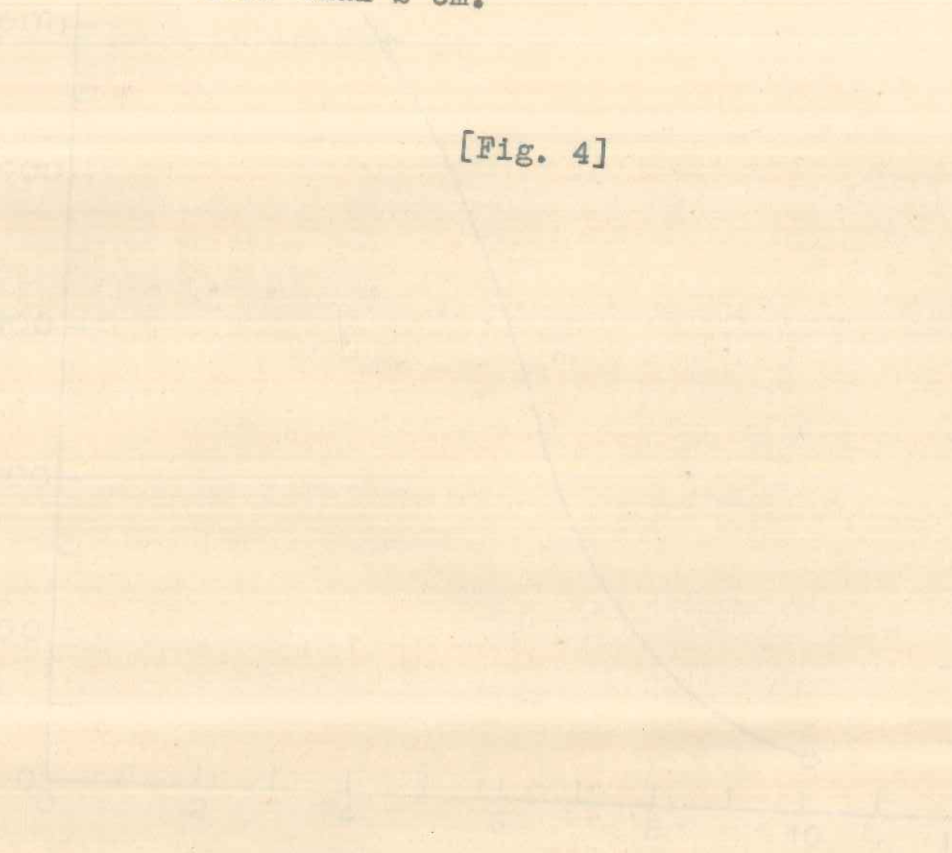
THE OPTIMUM MEDIUM DEPTH FOR NITRITE OXIDATION BY *Nitrobacter* species

Nitrite-N added .... 608 μg/ml
Nitrate-N inoculum.. 16 μg/ml
pH of the medium ... 8.5
Temperature ,....... 30^oC
Inoculum added 2.5%

Flask No.	Medium volume	Approximate height of the medium	Nitrate-N formed	Percentage
	ml	cm	μg/ml	
1	20	<1	637	100
2	100	2	599	95
3	200	4	496	75
4	400	8	93	10
5	500	10	38	-

It was observed that after 10 days the nitrite oxidation appeared to be nearly 100% at a medium depth of about 1 cm and nearly the same (95%) at 2 cm depth and 75% at a depth of 4 cm. It was only 10% at a depth of 8 cm and negligible at 10 cm depth. For optimum nitrite utilization the depth appears to be less than 2 cm.

[Fig. 4]



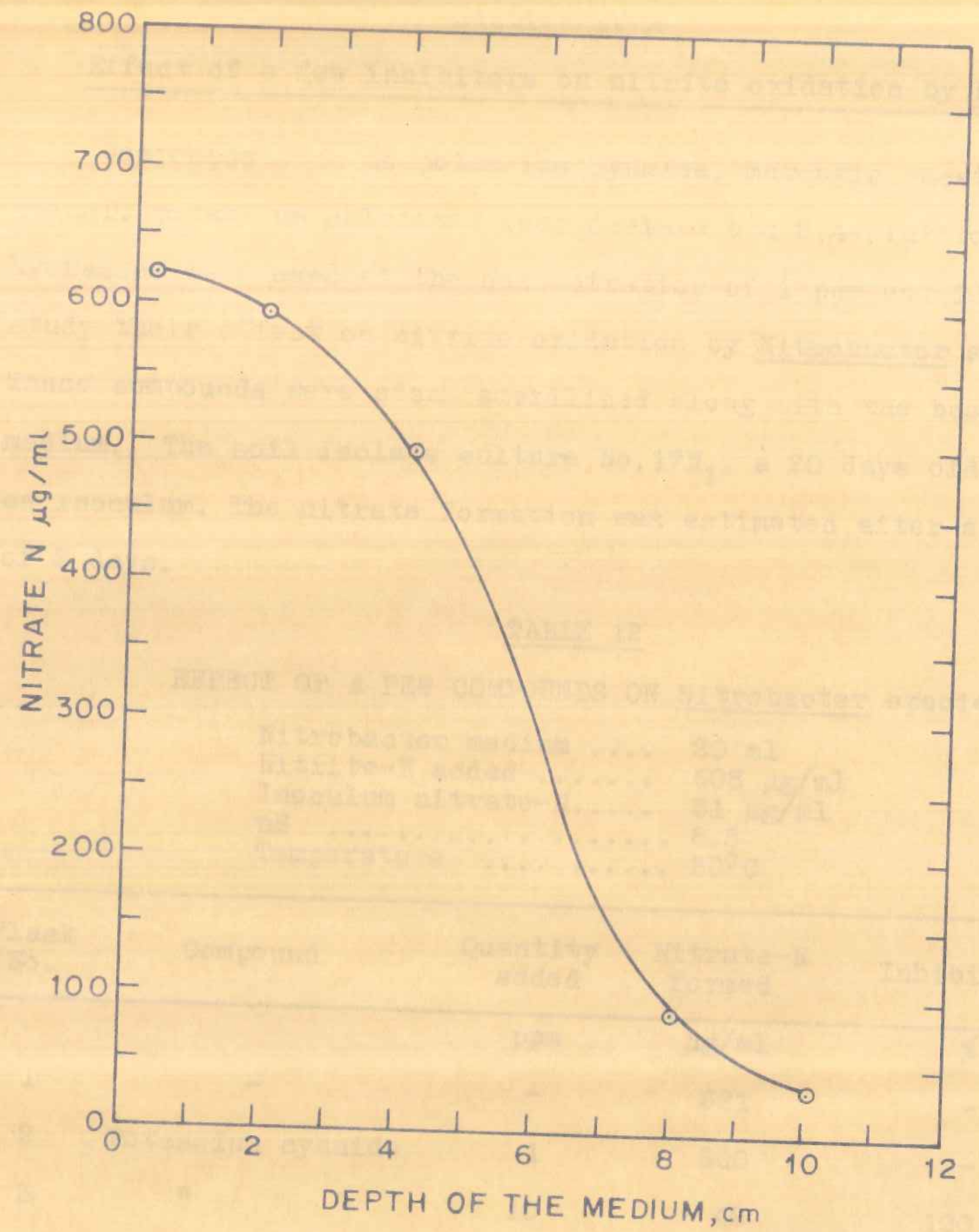


FIG. 4: THE OPTIMUM MEDIUM DEPTH FOR NITRITE OXIDATION BY <u>NITROBACTER</u> SPECIES

Section IV-A

Effect of a few inhibitors on nitrite oxidation by *Nitrobacter*

Compounds such as potassium cyanide, mercuric acetate, phenol, potassium chlorate, lead acetate and 2,4-dinitrophenyl hydrazine were used at the concentration of 1 ppm and 10 ppm to study their effect on nitrite oxidation by *Nitrobacter* species. These compounds were steam sterilized along with the basal medium. The soil isolate culture No.$17N_1$, a 20 days old used as inoculum. The nitrate formation was estimated after a period of 8 days.

TABLE 12

EFFECT OF A FEW COMPOUNDS ON *Nitrobacter* species

Nitrobacter medium .... 20 ml
Nitrite-N added .... 608 µg/ml
Inoculum nitrate-N..... 31 µg/ml
pH 8.5
Temperature .... 30°C

Flask No.	Compound	Quantity added	Nitrate-N formed	Inhibition
		ppm	µg/ml	%
1	-	-	621	-
2	Potassium cyanide	1	600	-
3	" "	10	42	100
4	Mercuric acetate	1	44	100
5	Phenol	1	520	17
6	"	10	316	50
7	Potassium chlorate	1	615	-
8	" "	10	43	100
9	2,4-Dinitrophenyl hydraz ine	1	541	15
10	" "	10	45	100

It was observed that mercuric acetate at 1 ppm showed complete inhibition, while phenol and 2,4-dinitrophenyl hydrazine at 1 ppm showed inhibition of about 15%. Phenol at 10 ppm gave 50% while the remaining compounds at 10 ppm gave complete inhibition of nitrite oxidation.

Section IV-B

Growth of Nitrobacter species in the presence of natural fatty oils

Different commercial samples of natural oils such as groundnut oil (*Arachis hypogaea*), coconut oil (*Cocos nucifera*), Castor oil (*Ricinus communis*),safflower (*Carthamus tinctorius*), sesame (*Sesamam indicum*), mustard (*Brassica nigra*) and Indian beech (*Pongamia glabra*) were tested for their effect on the growth of *Nitrobacter*. Neem cake was reported to inhibit ammonia oxidation in soil [Ketkar (1976)]. In this section the effect of the oils other than neem oil is described. 20 ml Nitrobacter medium and aliquots sterilized in 500 ml conical flasks. The *Nitrobacter* species No.19N_2 (20 days old) was used as inoculum. The experiments were in duplicate and controls were run without any addition of oils.

[Table 13]

All the oils showed inhibition of nitrite oxidation when they were used at 1 to 2% level in the Nitrobacter medium. Groundnut oil and coconut oil showed 20% inhibition, castor oil and mustard oil gave 50% and Indian beech oil 1% level showed 60 to 85% inhibition while safflower oil almost completely inhibited nitrite oxidation.

TABLE 13

GROWTH OF *Nitrobacter* species IN THE PRESENCE OF NATURAL FATTY OILS

Nitrobacter medium .... 20 ml
Nitrite-N added .... 608 μg/ml
Inoculum nitrate-N .... 32 μg/ml
pH of the medium 8.5
Temperature .... 30°C

Flask No.	Type of oil	Oil aliquot added	Nitrate-N formed	Inhibition
		ml	μg/ml	%
1	Control	–	632	[illegible]
2	Groundnut	0.4	496	22
3	"	0.2	530	17
4	Coconut	0.4	474	25
5	"	0.2	496	22
6	Castor	0.4	316	51
7	"	0.2	378	41
8	Safflower	0.4	68	91
9	"	0.2	82	88
10	Sesame	0.4	112	83
11	"	0.2	203	68
12	Mustard	0.4	299	54
13	"	0.2	338	48
14	Indian beech	0.4	90	86
15	" "	0.2	98	83

Section IV-C

Effect of neem oil on Nitrobacter species

The effect of neem oil and different fractions of neem oilobtained by extraction with acetone, alcohol, ether petroleum ether and water as well as several fatty acids and other compounds was tested on nitrite oxidation. (All the extracts and fractions were prepared in the Organic - Chemistry Division of this Laboratory under the supervision of Dr. C. R. Narayanan). The neem oil, extracts of neem oil etc. were added to the conical flasks and then basal Nitrobacter medium (20 ml was added, thoroughly mixed and then steam-sterilized at 15 lbs. for 15 min. Seitz filtered sodium nitrite was added separately under aseptic conditions.

The soil isolates Nos. $5N_1$, $5N_2$, $12N_1$, $12N_2$, $17N_1$ and $17N_2$ Nitrobacter were used in these studies. Nitrate formation was determined after incubating these cultures at 30°C. In the case of compounds found to be inhibitory different amounts were added to the medium and the quantity required to produce 50% inhibition was determined.

TABLE 14

EFFECT OF NEEM OIL ON *Nitrobacter* species

Nitrobacter media	20 ml
Nitrite-N added	608 µg/ml
Inoculum Nitrate-N ...	30-35 µg/ml
pH of the medium	8.5
Temperature	30°C
Cultures used:........	$5N_1, 5N_2, 12N_1, 12N_2, 17N_1, 17N_2$

Serial No.	Compounds	Quantity added	Percentage inhibition	Quantity as 100% inhibition
		mg		mg
1	Neem oil	0.1 ml	93	0.2 ml
2	Pet.ether ext. of neem oil	10	79	15
3	Ether " " "	10	93	15
4	Acetone ext.	10	90	15
5	Water	50	-	NT
6	Pet.ether ext. of de-oiled neem cake	44	40	10
7	Linoleic	5	80	NT
8	Undecylenic	5	89	NT
9	Crotonic	2	50	5
10	Stearic	NT	-	20
11	Methyl oleate	NT	-	20
12	Cinnamic acid	3	93	5
13	Fraction (7-9)	4	50	NT
14	" 28	8	40	20
15	" 83	8	50	NT

NT = not tested

It was observed that neem oil as well as neem oil extracts with different organic solvents were inhibitory to nitrite oxidation. Aqueous extracts were, however, ineffective as inhibitors. Fatty acids both saturated as well as unsaturated were inhibitory, though oleic acid was more inhibitory than stearic acid. Other organic acids such as cinnamic acid and crotonic acid as well as esters such as methyl oleate were also inhibitory.

Section IV-D

The effect of dichromate on nitrifying bacteria

The effect of chromium as dichromate on <u>Nitrosomonas</u> culture 11 A_1 isolated in this Laboratory and on <u>Nitrobacter</u> 12 N_1 was determined with different dichromate concentrations. <u>Nitrosomonas</u> and <u>Nitrobacter</u> cultures were grown in their respective media and other conditions of pH etc. were kept constant. Nitrite formation by <u>Nitrosomonas</u> after 15 days and nitrate formation by <u>Nitrobacter</u> after 7 days were determined

TABLE 15

EFFECT OF DICHROMATE ON NITRIFYING BACTERIA

Nitrosomonas medium ...	20 ml	Nitrobacter medium ..	20 ml
Ammonium-N added	392 µg/ml	Nitrite-N added	608 µg/ml
Nitrite-N inoc.	20 µg/ml	Nitrate-N inocul. ...	31 µg/ml
pH	8.5	pH	8.5
Temperature	30°C	Temperature	30°C

Flask No.	Type of culture	Dichromate added	Nitrite formed	Nitrate formed
		µg/ml	µg/ml	µg/ml
1	<u>Nitrosomonas</u> 11A_1	-	421	-
2	"	0.1	425	-
3	"	0.25	406	-
4	"	1.0	114	-
5	"	2.0	71	-
6	"	3.0	23	-
7	<u>Nitrobacter</u> 12N_1	-	-	631
		30	-	633

Ammonia oxidation by Nitrosomonas was completely inhibited at 3 µg/ml of chromium as dichromate and about 75% inhibited at 1 µg/ml. Nitrite oxidation by Nitrobacter was not inhibited were at 30 µg/ml of chromium as dichromate. Hence dichromate can be used for the selective inhibition of Nitrosomonas cultures in the presence of Nitrobacter. Control experiments showed that nitrite was not oxidized in the presence of 30 µg/ml of dichromate in uninoculated Nitrobacter media.

Section IV-E

Isolation of Nitrobacter species in the presence of dichromate

The selective inhibition of Nitrosomonas by dichromate and its non-toxicity to Nitrobacter was used for the isolation of Nitrobacter from the soil. 1 g of garden soil was added to Nitrosomonas and Nitrobacter media containing 20 mgωl of $K_2Cr_2O_7$. Four different soil samples from different locations were used. These flasks were then left stationary at 30°C. The other conditions of pH etc. were maintained as in earlier experiments. Nitrite and nitrate formation were determined weekly.

It was observed that in Nitrosomonas medium containing dichromate, there was no nitrite or nitrate formation in any of the flasks. Out of 4 flasks containing Nitrobacter medium and dichromate three flasks showed nitrate formation after three weeks. Subcultures were made from these three flasks to fresh Nitrobacter medium as well as to Nitrosomonas medium without chromium. After incubating these for 15 days at 30°C nitrate formation was examined in the Nitrobacter medium and nitrite formation in the Nitrosomonas. There was no nitrite in the latter whereas nitrite was oxidized in the former.

By this method the isolation of Nitrobacter species was achieved without contamination with Nitrosomonas species. These three Nitrobacter species and three more from another batch obtained by the above procedure are being maintained for the last five months by monthly transfer to liquid medium. They show only nitrite oxidation and no ammonia oxidation.

Section IV-F

To examine antibiotic activity of *Nitrobacter* species

Nitrobacter culture Nos. $4N_1$, $5N_1$, $7N_1$, $10N_1$, $14N_1$, $18N_1$, $20N_1$, $21N_1$, $35N_1$ and $39N_1$ grown on the *Nitrobacter* maintenance medium were tested for antibiotic activity against the following test organisms.. *Staphylococcus aureus* NCIM 2122, *Escherichia coli* NCIM 2068 and *Pseudomonas aeruginosa* NCIM 2389 and *Penicillium chrysogenum* NCIM 724 and *Aspergillus niger* NCIM 528. *S.aureus* was grown at 37°C for 24 h and the other two bacterial cultures at 30°C for 24 h. *P.chrysogenum* and *A.niger* species were collected in saline water for experimental use. 5 ml of nutrient broth agar medium was mixed thoroughly with 1 ml of bacterial suspension at 40°C and transferred to 20 ml of solidified agar medium in 20 mm x 100 mm petri dishes and allowed to solidify. The petri dishes for fungi containing 20 ml of solid potato dextrose agar medium were inoculated with 1 ml of spare suspension in saline solution spread on the surface.

Porcelain cylindrical beads 5 mm x 5 mm sterilized at 20 lbs. for 30 min were dipped in a suspension of month-old cultures of *Nitrobacter* and placed on these plates. *S.aureus* was incubated at 37°C for 24 h and other bacteria at 30°C for 24 h and fungi at 30°C for 5 days.

Antibiotic standards were also run using penicillin (1 unit/ml) for bacterial cultures. The penicillin treated beads showed clear inhibition zones. But there was no inhibition of any of the bacteria or fungi by any of the *Nitrobacter* culture media. The *Nitrobacter* cultures showed no antibiotic activity against the test organisms used in this experiment.

Chapter IV

GENERAL DISCUSSION AND SUMMARY

GENERAL DISCUSSION

Isolation

The *Nitrobacter* species were isolated from soil according to Gould and Lees (1960) by enrichment technique. After 5 subcultures on modified Stanier's medium (Nitrobacter medium) *Nitrobacter* species were obtained on silica gel as well as on Noble agar plates. After a fes subcultures on Nitrobacter medium these were checked for their purity and shown to contain no heterotrophs. 29 *Nitrobacter* cultures have been isolated by this method.

Chromium and nickel were reported by Skinner and Walker (1961) to be inhibitors of growth *Nitrosomonas* and not of growth of *Nitrobacter*. This was confirmed with the pure cultures of *Nitrosomonas* and *Nitrobacter* and only the former was shown to be completely inhibited by 3 μg/ml of dichromate whereas even at 30 μg/ml *Nitrobacter* cultures were not inhibited. This observation was used to isolate Nitrobacter species free from *Nitrosomonas* in the presence of 20 μg/ml dichromate. By this method 6 *Nitrobacter* species were isolated and shown to be free from *Nitrosomonas*.

Morphological observations showed that all the 35 cultures isolated were gram-negative,and rod shaped and

of similar size. There was no change in morphology even after 3-4 years of subcultures. None of these cultures had ammonia oxidizing ability and were free from Nitrosomonas as well as heterotrophs.

Maintenance

Maintenance of these Nitrobacter species was carried out on a modified Stanier's medium (Nitrobacter medium). One of the main changes made was in the basal medium. The sodium nitrite was sterilized separately. The basal medium was steam-sterilized at 15 lbs. for 20 min while sodium nitrite solution was seitz-filtered and then added to the basal medium aseptically. The pH was 8.5 and the concentration of nitrite-N was 600 µg/ml. Secondly the vitamins biotin, folic acid and B_{12} at 1 µg/l were added to the basal medium. Thirdly incubation was carried out at 30°C and the flasks were kept stationary in the dark. Subcultures were made after about 30 days into fresh Nitrobacter medium. 20 ml of medium in 500 ml conical flasks (about 1 cm liquid depth) was used.

Under these conditions these Nitrobacter cultures have been maintained for about 4 years

without any diminution in growth or nitrite oxidation ability. There are very few reports of the maintenance of so many cultures obtained from widely separated areas for more than 3-4 years.

But in separate experiments it was shown that the vitamins are not required even when subcultures were continued in vitamin-free Nitrobacter medium for 15 months. Only prolonged studies over several years can indicate whether these vitamins are required for viability. It is also not known whether other modifications listed above (sterilization method, keeping cultures stationary, nitrite concentration etc.) were responsible for the viability of these cultures which is unlike that of cultures isolated in other laboratories which were lost after a few years. Long term studies are needed to determine why our cultures remain stable on subcultures.

It should be noted that no solid constituents such as $CaCO_3$ are added to the medium. These are apparently not needed by <u>Nitrobacter</u> cultures.

Since bacterial growth was too low for optical density measurement and staining and counting were too time-consuming, nitrite oxidation was used as

an index of growth.

<u>Optimum conditions for growth or nitrite oxidation</u>

The cultures grew equally well in the dark or when illuminated. Whether light is harmful for long-term viability was not determined.

The optimum pH for nitrite oxidation was 8.5. Growth was negligible at pH 6.0 or less or at 10.0 or more. The optimum temperature was 30°C. There was markedly best growth at a temperature of 15°C or less and at 37°C or more.

Growth was negligible in the absence of or on ammonium salts in the absence of nitrite.

Acetate and glucose was not inhibitory to nitrite oxidation and citrate, malate and formate were only partially inhibitory whereas pyruvate (0.05 M) almost completely suppressed nitrite oxidation. Continuous growth of the *Nitrobacter* strains in the presence of both nitrite and acetate for 15 monthly subcultures showed no decrease in growth. Growth

on organic compounds in the absence of nitrite was not tested. These cultures are autotrophic with regard to their ability to grow on CO_2 as sole carbon source, but they are not inhibited by several organic compounds.

Experiments on inorganic ion requirements were inconclusive except that in the absence of K_2HPO_4 growth stopped. The other elements such as Mg, Mn, Fe, Ca and Mo are required if at all in trace quantities which are present in other inorganic compounds added.

Inhibitors

The synthetic compound "N-serve" was introduced in some countries to control the nitrifying activity of bacteria and reduce loss of ammonia fertilizers by oxidation to nitrate which is readily leached out.

The present work has shown for the first time with pure Culture of *Nitrobacter* that Indian beech oil, safflower oil and neem oil are strongly inhibitory to these organisms. Several fractions of these neem oils as well as several saturated and

unsaturated fatty acids and other compounds were found to be inhibitory. It is not yet established whether any of the constituents is very highly inhibitory or whether the action is due to several inhibitory substances including the triglycerides. These observations show that these pure cultures can be used for rapid screening of compounds with potential inhibitory activity. Through such studies should be supplemented by studies in soil.

SUMMARY

1) 29 strains of Nitrobacter species were isolated from different soil samples and purified by plating out on modified Stanier's medium. These pure cultures did not contain heterotrophs or ammonia oxidizing organisms. They grow on simple inorganic salts medium containing K_2HPO_4, $MgSO_4$, $MnSO_4$, $FeSO_4$, Na_2MoO_4 and $NaNO_2$ plus biotin, folic acid and B_{12}. They were gram-negative rods which were aerobic. These properties identify these cultures as Nitrobacter species.

2) These 29 cultures of Nitrobacter have been maintained for over 4 years by monthly subculture in the above medium at pH 8.5 and incubated at 30°C in the dark in stationary flasks. It is not certain whether the vitamins and other experimental conditions are essential for long term viability. However this is the largest known collection of Nitrobacter species maintained successfully for so long a period without loss of nitrite oxidizing ability.

3) Dichromate containing medium was used to isolated Nitrobacter species from the soil without

any contamination of Nitrosomonas species. Six Nitrobacter species isolated from the soil with the help of dichromate have remained viable for the last six months.

4) The optimum pH for nitrite oxidation was 8.5.

5) The optimum temperature was 30°C for nitrite oxidation.

6) Acetate and glucose were not inhibitory whereas 0.05 M pyruvate completely inhibited the growth of Nitrobacter in the presence of nitrite.

7) Neem oil, safflower oil and Indian beech oil were markedly inhibitory to Nitrobacter activity.

8) Oleic acid, methyl oleate, stearate, crotonate and cinnamate were also inhibitory to these cultures.

9) The optimum depth of the medium was less than 2 cm for nitrite oxidation by Nitrobacter.

10) Heterotrophic filtrates did not stimulate nitrite oxidation rate.

11) Light had no effect on nitrite oxidation by <u>Nitrobacter</u> species.

12) Hg-acetate at the level of 1 ppm, while KCN, $KClO_3$, 2'4-dinitrophenyl hydrazine at the level 10 ppm completely inhibited the nitrite oxidation.

13) In summary a method has been developed for the isolation and prolonged maintenance of <u>Nitrobacter</u> cultures without loss of viability and activity. 29 strains of <u>Nitrobacter</u> have been successfully maintained for over 4 years and utilized for biochemical studies.

Chapter V

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