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**ORIGINAL ARTICLE**

# A New Facultative Chemolithotrophic Nitrifying Bacteria: *Nitrobacter iranicum* sp .v Nov.

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**ABSTRACT**
*Phenotypic and genetic studies were performed on a Gram-negative, Oxidizing bacterium isolated from soil of a Potato field in Hamadan,Iran. The Cells were 0.4 – 0.5 X 0.8 -1.2µm in size with short rods spindle shaped and polar caps of interacytoplasmic membranes. Carboxissomes were also present .The organism grew chemolithotrophically, hetrotrophically or mixotrophically. It grew at pH range from 6.7 to 8.3 with optimum at 7.6 .The growth rate under hetrotrophic condition is slower than under mixotrophic condition, but faster than under lithotrophic condition. The DNA content was 57mol %. In the absent of oxygen growth was possible by dissimilatory nitrate reduction .The sequence of nearly complete 16S rRNA gene of the strain is recorded in the Gen Bank under number AY578913. Generation times varied from 7to 14houre. The new isolates from the soil were described as a new species of the genus Nitrobacteria,N.iranicum on the basis of their substantial morphological, physiological and genetic differences from the recognized neutrophylic representatives of this genus.*
*Key words: Nitrobacteria Nitrification– denitrification –Genetical- morphological-Biochemical investigation*

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## INTRODUCTION

Nitrifying bacteria of the genus *Nitrobacter* play an important role in biological nitrogen cyclin by converting reduced inorganic nitrogen compounds to nitrate [1].They are pleomorphic rod to pear shaped and polar caps of cytomembranes .Their growth is in heterotrophic condition with acetate [2,3] even under anaerobic condition [4,5]. It is possible Nitrite –oxidizing bacteria are ubiquitous in terrestrial and aquatic natural environments under moderate condition Bock and [6].There are some indications that nitrifying may also be present in extreme environments such as acid soils [7-9].These bacteria might be found in alkaline environments such as saline soda lakes and soda soils [10] . Except for one report on the positive enrichment from soda lakes samples in wadi Natur [11], so far five species of Nitrobacter,and Nitrobacteria, Nitrobacter winogradskyi;[12,13].*Nitrobater hamburgensis* . *Nirobacter vulgaris* ,*Nitrobacter alkalicus* and Nitrobacteria hamadaniensis [14] have been described. In this article the cultural and biochemical characteristic of this bacterium and the result of a phylogenetic analysis based on 16S rRNA gene sequencing were reported.

## MATERIAL AND METHODS

Isolation: *Nitrobacteria iranicum* have been isolated in pure cultur. The type strain was isolated from soil of a potato field in Hamadan, Iran. The basal culture medium was mineral Salt medium [15].The culture were incubated at $28^0$C for 2 weeks.The cell suspension was inoculated into agarmedium(basic mineral medium plus 18 g agar-agar for total Vol.of 1 liter)(Merk,Germany).The purity was checked according to Steinmüller and Bock [14].

Culture condition

For heterotrophic growth Na-acetat.yeast extract and peptone(aerobic)or nitrate (anaerobic)growth.For mixotrophic growth the basic mineral medium Drews (1968) was supplemented with Na-acetat (410mg),yeast extract (Difco,USA ,1500mg) and Peptone (Difco,USA 1500mg)were taken 30 days old

culture inoculate culture volumes from 1. [9]. Stock cultures were maintained in mineral nitrite medium with or without organic medium. The culture media essentially prepared as described by Zare et al. [14]. Master plates (stock culture)were prepared under mixotrophic condition.

Batch cultivation

Batch cultures were grown in 50 ml liquid media described by Zare et al. [9]. All experiments were done at pH 6.7 ,7.6 and 8.3 and repeated at least 3 times.

Analytical procedures

Protein purification:

The amount of nitrite, nitric oxide production were measured as described by Heubült ,Bock et al ,Zare et al. [9,14]. *Nitrobacteria* strian 101 was sub cultured in mixotrophic broth at 28°C . Stationary phase cells were collected from the growth medium by centrifugation .The pellet was washed twice with 0.9% Nacl buffer.The protein was purfied fromenzyme extract and measured as previously described by Bradford and Davie (16,17). Nitrite oxidoreductase were isolated and purified according to Sundermeyer-Klinger (18).Cytochrome spectra of previously explained by Sorokin et al. [19].Crude cell –free extracts were subjected to (SDS-PAGE) according to Francis and Becker (20).

DNA and 16S rRNA analysis

Cell lysis was performed according to Kraft and Bock (1984). Determination of the G+C content of the DNA according to standard procedures were done Marmur, Deley [21,22]. DNA isolation and PCR amplification were performed as described by Brinkhoff and Muyzer [23] . and Muyzer et al.[24].

Sequences were compared using the ARB software (Ludwig 2004).The16S rRNA gene sequences of the isolates were automatically aligned to sequences stored in the ARB database.

Gel electrophoresis

SDS-PAGE (Merk,Germany) was performed as described by Sundemeyer-Klinger et al.(18) and Zare et al.(14) The cytochrome spectra of cell-free extract 0f the new strain (101) was determined as previosly explained by Sorokin et al.[10]and Zare et al. [14] . protein were identified from polyacrylamide gels by the method of Francis and Backer [20].

Electron microscopy:The electron microscopically methods were those described by Bock and Heinrich [3] . Section was stained with Uranil acetate and leadcitrate (Electron microscopy sciences USA).Electron microscopywere taken with transmission elctron microscope (Carl Zeiss EM -900;Zeiss ,Germany) at 80 KV accelerating voltage .Then negatives were scanned at 1200 dpi resulation,by CanoScan 8800F (Canon ,Japan).and pictures were processed in Adobe Photoshop software (CS4 Extended.middle East Version)

Phylogenetic analysis

In order to establish the precise taxonomic position of unknown bacteria the entire 16S rRNA sequences of the strain [10] was determined.

## RESULTS

G+C analysis

G+C content of strain (101) DNA is (57mol%.this volue is differnt from Nitrobacteria hamadaniensis strain 104(59 mol%).the derived 16S rRNA consists of 1417 nucleotides the determined sequences were compared with those of other 16S rRNA sequences available in the GenBank.Nitrobacteria iranicum with 97.8 genetic homology with Brevendimonas,and 96.1% withNitrobacteria hamadaniensis Table 1,has complete biochemical and physiological similarities.It has 86.6 ,86.9% genetic homology to genus ,Nitrobacter.This is agreement with the physiological Variability of strains of the new Nitrobacteria species,perhaps resulting from adaptation to special environment such as desent soil and natural buildingstones.Fig1. In protein analysis of cell-free extracts of the strain 101 grewn at PH 7.6 we observed α band (437 and 589 nm in size).In addition cytochrome c 550 and cytochrome C oxidase were identified Nitrobateria iranicum grew optimally at 28 C and pH 7.6. Colonies an agar plates formed within 3 ,4 and 12 days due to mixotrophic, heterotrophic and lithoauthotrophic conditions. Clonies on mineralsalt agar plates sized 0.1mm in diameter were yellowish ,circular and smooth ,optimum growth rateswere obtained in mixotrophic medium containing nitrite ,sodium acetate ,yeast extract and peptone.

Cell morphology

Cell of the *Nitrobacteria iranicum* had a similar shape ,size and ultra structure as described for the species of the genus Nitrobacter and Nitrobacteria .They were pleomorphic ,rods 0.4-0.5 X 0.8-1.2µm.The cytoplasmic membrane in folds in to cytoplasmic formig a po lar caps of interacytoplasmic membranes composed of one layers of paired menbranes .species of genus Nitrobcter have characteristic peripheral multilayer's of interacytoplasmmic Lamellar membranes ,Sorokin et al.(1997).Carboxisomes were numerus in mixotrophically grown cells,other typical inclusion bodies were Poly- - hydroxybutyrate and polyphosphate granules. The cells membranes in mixotropic conditios formed spiral shaped Fig.2.

SDS PAGE analysis:

The results of SDS-PAGE of cell-free extracts, based on phenotypic criteria ,showed that Nitrobacteria iranicum is composed of 3 bands ,2 strong and prominent bands of 115,116 KDa,and one 14 KDa band respectively Fig 3.

## DISCUSSION

The new species of bacteria was identified that was different from cell fiv known species of the genus *Nitrobacter* winogradky [12].*Nitrobacter hamburgensis* Bock et al [3].*Nitrobacter vulgaris* Bock et al.(3),*Nitrobacter alkalikus* sorokin et al (19),and *Nitrobacteria hamadaiensis* Zare et al, [14]. Optimum growth was too close to their upper pH limit (around 7.6). The most rapid growth on medium culture used for active cultivation of the strain 101with a starting pH7.6 and a nitrite concentration about 1.g/L. The strain 101 was able to grew in nitrite limited culture within broad pH range from 6.7 to 8.3 with an optimum pH 7.6 Fig 4. The doubling times of autotrophically and mixotrophycally growth of *Nitrobacteria iranicum* was 14 and 7 hours at pH 7.6 respectively .They are higher than the rate described for neatrophilic species grown litoautotrophically with nitrite [9,14] . Our study shows that organic compounds influence on the growth of nitrite-oxidizing strain 101 from soil, at pH 6.7 to 8.3. During 120 hours incubation, there were no significant differences between the bacteria activity in hetrotrophic with 1000mg /l nitrate under anaerobic conditions at different pH values in batch culture tabel2.

The pH profile in the kinetics of oxidation batch culture was significantly different for cells grown at different pH values. The profile for the rate of nitrite condition Fig 4, measured with cells grown at pH 6.7was similar to that measured for *Nitrobacter* species [25], *Nitrobacteria hamadaniensis* Zare et al .[14]. The curve had its maximum at pH 7.6 and dropped abruptly at pH higher than 8 the nitrite oxidizing activity measured with cells grown at pH7.6,6.7 and 8.3 that was maximal at pH 7.6 respectively, and incubations time was 192 hours. The strain 101 was able to grew on nitrite –oxidizing lithoautotrophic ,mixotrophic and heterotrophic conditions at pH 6.7to8.3,with stoichiometry analysis conversion of nitrite to nitrate in batch culture was 96%-99% and nitrate to nitrite under anaerobic condition was (1-1.5%).

The organism grew on mineral medium supplemented with organic compounds such as natrium acetate, yeast extract and peptone as sources of energy and carbon. Batch cultivation at different pH values clearly demonstrated that the nitrite-oxidizing strain 101 isolated from soil, belong to facultative species at could grew within a wide range of pH value 6.7to 8.3 Fig.4 ,the growth rate n the pH level below and above 7.6 extremely slow .During growth at pH value above 7.

Cells started to branch. Identification of strain by SDS-PAGE: This identfication was under taken by visula comparison of the electrophoresis patterns Fig .3.protein profil of the sausage isolates were compared with those of the respective type strain .There producibility of the SDS-PAGE techniques was estimated by including duplicate run of a single protein extract on one gel on separate gels. Based on phenotypic criteria ,a very good correlation witch the SDS-PAGE results was observed strain 101 Fig.3 gave three stronger bands,2strong and prominent bands of 11 5 and 116 KDa ,one 14 KDa band respectively Fig 3.A very good correlation was found between the results of the phenotypic analysis and comparison of protein patterns of the strain 101 examined ,it was confirmed that all isolation belong to strain Nitrobacter and Nitrobacteria. Respectively. The fact that some isolates lacked characteristic protein bandes from their profile did not affect the given identify .The phenotypic criteria are complatly similar to recent findings obtained by Samelis et al. [26]. Another form of strain 101 identified, that was different from the Nitrobacter *winogradskyi* Bock [11],*Nitrobacter* hamburgensis(3 ),Nirobacter vulgaris [9] and Nitrobacteria hamadaniensis (14),it grew at the all different conditions,and growth rates in mixotrophic media could be used for taxonomic purposes.

Our observations show ,this new organism, like *Nitrobacteria hamadaniensis* [14] grows by dissmilation and reduces nitrate in which nitrate is present as on alternative electron acceptor the cells of Nitrobacteria strains 101had similar shape size and ultrastructure,this is a further evidence of the existent a new Nitrobacteria species .Although the isolates from the soil are similar to genus *Nitrobacteria*, they show clear ultra structural, physiological. Genetically differences from the *Nitrobacteria hamadaniensis*,these different are sufficient to described the new isolates as a new spices of genus *Nitrobacteria* we propose the name *Nitrobcteria* strain 101 with *Nitrobacteria iranicum* .

Species description

Description of *Nitrobacteria iranicum* sp.nov.

The cells are Gram negative,pleomorphic short rods to pear shaped with a size of 0,4-0,5x0,8-1,2$\mu$ms . colonies on agar plates are yellowish 0.1 mm in diameter. Cell division occurs by budding intracytoplasmic membranes are forming caps of flattened vesicles, composed of one layers of paired membranes ,carboxysoms are present cells produce extra cellular polymers at all growth conditions,cousing the formation of a biofilm. Facultative lithotrophic that oxidized nitrite to nitrate

under aerobic condition and reduce nitrate to nitrite under anaerobic conditions.altough,lithotrophic growth was slower than heterotrophic, the best growth was observed in mixotrophic conditions. Optimum lithotrophic growth was 28 C.and PH values 7.6.

The G+C content of DNA is 57 mol% the sequence of the nearly complete 16S rRNA gene of strain 101 is stored in the Gene Bank database under accession no.AY578913 and JCM 14787 respectively. Nitrobacteria iranicum is deposited in Persian type culture collection under the number PTCC1680.Nitrobacteria iranicum strain 101 were isolated from a potato field in Hamadan Iran.

Table 1.Similarity matrix of 16S rRNA sequences

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ***Afipia felis*** | | | | | | | | | | | |
| 2 | ***Blas tobacter denifricans*** | 96.5 | | | | | | | | | | |
| 3 | ***Bradyrhizobium japonicum*** | 97.3 | 98.2 | | | | | | | | | |
| 4 | ***Rhodopseudomonas palustris*** | 96 | 97.3 | 98.3 | | | | | | | | |
| 5 | ***Nitrobacter winogradskyi* ATCC 25381** | 96.9 | 97.1 | 98.2 | 97.1 | | | | | | | |
| 6 | ***Nitrobacter winogradskyi* ATTCC 14123** | 96.7 | 96.5 | 98.9 | 96.9 | 98.7 | | | | | | |
| 7 | ***Nitrobacter hamburgensis* strain x 14** | 96.3 | 96.8 | 98.7 | 97.2 | 98 | 98 | | | | | |
| 8 | ***Nitrobacter alkalicus* strain AN1** | 96.9 | 97.2 | 98.3 | 97.2 | 99.1 | 99.2 | 98.4 | | | | |
| 9 | ***Nitrobacter alkalicus* strain AN2** | 97.5 | 97.1 | 98.3 | 96.6 | 99 | 991 | 98.3 | 99.9 | | | |
| 10 | ***Nitrobacteria hamadaniensis* strain 104** | 85.3 | 86 | 86.3 | 88.1 | 86.2 | 87.8 | 88.1 | 87.4 | 87.5 | | |
| 11 | ***Brevundimonas diminuta*** | 87.1 | 87.8 | 87.6 | 87.8 | 87.7 | 87.1 | 88 | 87.7 | 87.7 | 96.3 | |
| 12 | ***Nitrobacteria iranicum* strain 101** | 86.3 | 86.5 | 86.8 | 86.9 | 86.8 | 86.6. | 86.6 | 86.9 | 86.1 | 96.1 | 97.9 |

Table 2. Influence of organic compounds on growth of the nitrite –oxidizing strain 101 under anaerobic conditions at different PH values in batch culture

| Growth condition | strain 101 | Amounts of No2 Concentration (mmol) |
|---|---|---|
| PH 8.3 | 5 days | 0.16 |
| PH 7.6 | 5days | 0.17 |
| PH 6.7 | 5days | 0.1 |

Specific activity of the strain 101 was in 5 day hetrotropic condition

Fig.1.A phylogenetic tree derived from 16 S rRNA gene sequences .The tree was created by using the neighbor-joining method and Kunc values, showing the phylogenetic interrelationships between *Nitrobacteria iranicum* and other close relatives .The bootstrap values are indicated**.**

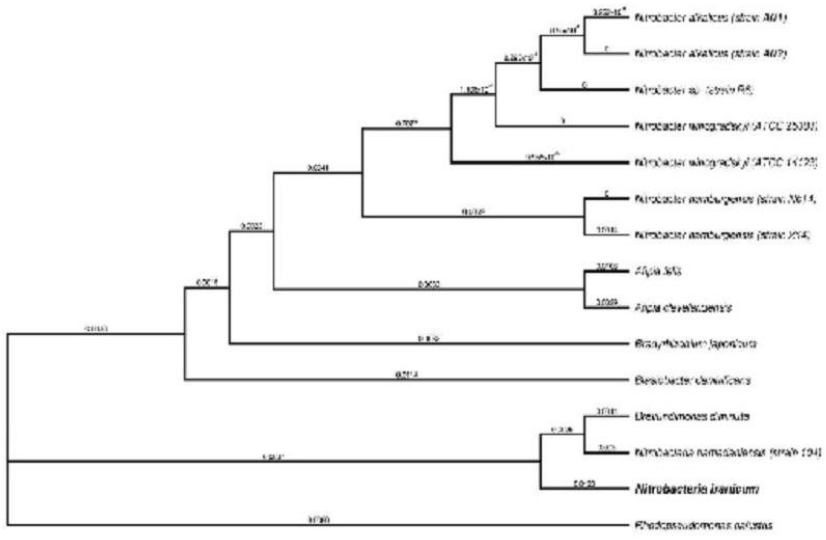
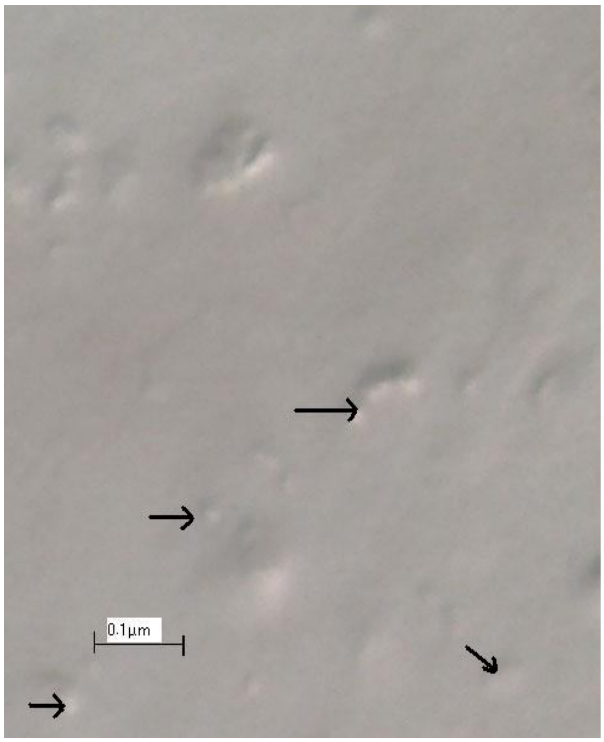


Fig.2 Macro- and micro morphology of Nitrobacteria iranicum grown at PH 7,6 a two week old colonies b ,.Electron micrograph of a thin sectiond flock of Nitrobacteria iranicum . Cell are embeded in and sectioned by extracellular polymers.PHB=Poly-B-hydroxybutyrate granules, ,PP pyrophosphate granules ICM=intracytoplsmic membranes bar=1micron

A:




B:

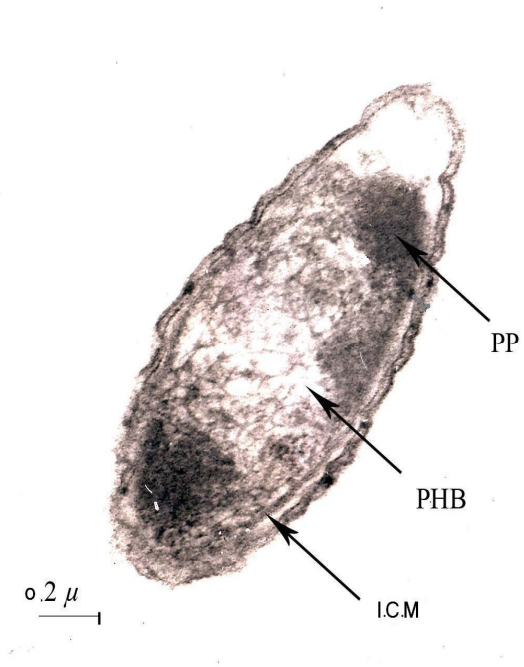
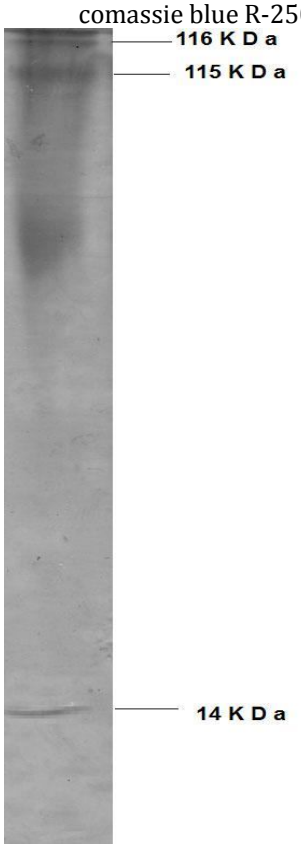
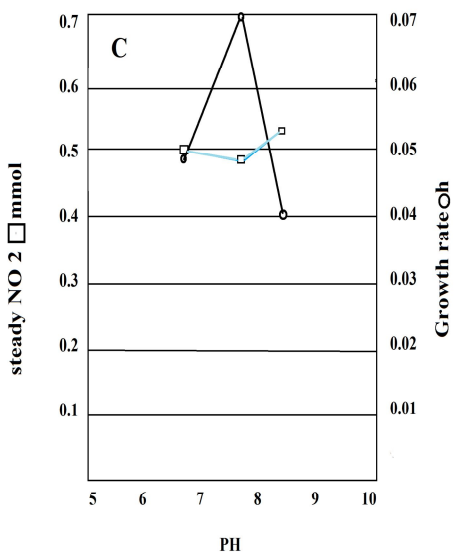
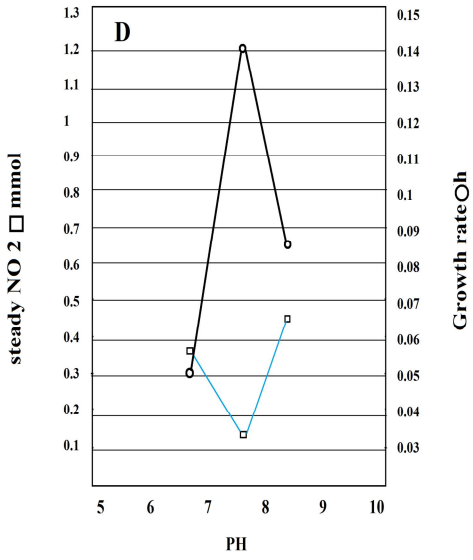


**Fig.3.**SDS-PAGE (12.5% acrylamide ) of cell free extract from *Nitrobacteria iranicum* protein stained with comassie blue R-250.



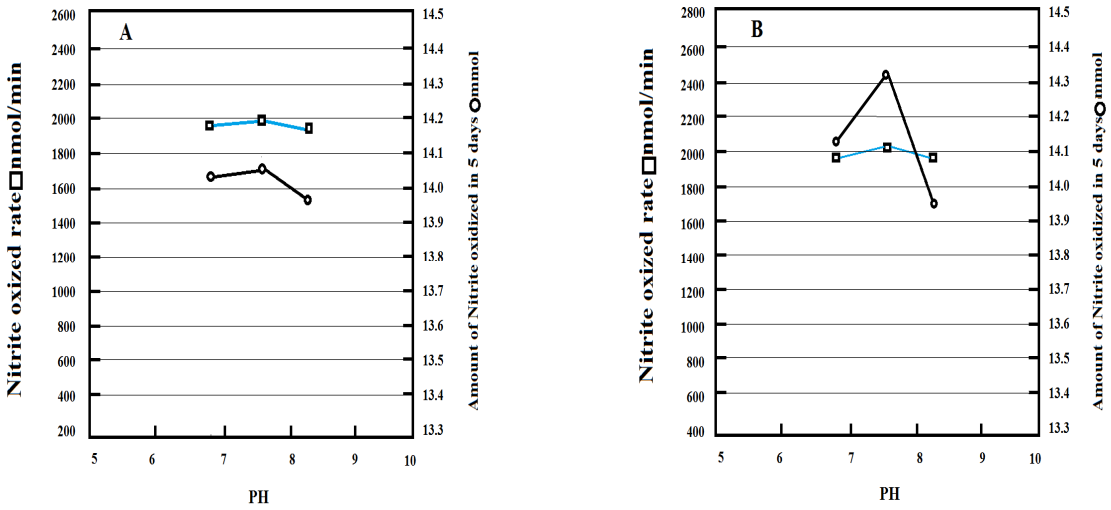



**Fig.4.A-B .**Influence of PH and culture condition on dynamic of nitrite oxidation of Nitrobacteria iranicum strain 101 in Batch culture and influence of PH on oxidation activity of washed cells- grown in Batch culture at PH 6.7 -8.3.The culture started to wash out at D=0.007 $h^{-1}$
A-Lithoautotophic condition
B-Mixotrophic condition
C-D. Influence of PH on the growth of Nitrobacteria iranicum strain 101 in Batch culture with 14.5 mmol nitrite at PH 6.7 ,7.6 and 8.3 (culture wash out at D=0.007 $h^{-1}$
C.Nitrite oxidizing activity of cell cultivated at lithotrophic with nitrite
D.Nitrite oxidizing activity of cell cultivated at mixotophic condition

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