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**RESEARCH ARTICLE**

# ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF NITRATE REDUCTASE-PRODUCING BACTERIA

**Marwa Eltarahony[1], Sahar Zaki*[2], Zainab hassan Khairalla[3] and Desouky Abd-El-Haleem[4]**

[1,2,4]Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technological Applications (SRTA-city), Alexandria, Egypt

[3]Botany Department, Faculty of Girls for Arts, Science and Education, Ain Shams University,Cairo, Egypt

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**ABSTRACT**

Due to the environmental importance of nitrate-reductase enzyme (NR) in removing nitrate overload, this study aimed to screen, isolate and characterize NR producing bacteria. To achieve this target, various soil and water samples were collected from different Egyptian ecosystems. Isolation of NR producing bacteria was performed on BTB medium. About 43 different isolates were selected. In order to reduce the large number and eliminate similar isolates 16S rDNA-RFLP approach employed toreduced them from 43 to 25.The hyper-active bacterial isolate has been identified by physiological, microscopically and biochemical tests and16S rDNA sequencing as *Achromobacter* and named *Achromobacter* sp. strain MMT. It reached the maximum enzyme activity after 12 h, total removing of nitrate after 48h and complete nitrite conversion to $N_2$ gas after 90 h of incubation. Detection, sequencing and phylogenetic analysis of *nar*G and *nap*A genes in strain MMT were carried out using nested PCR with specific primers and the presence of both genes was confirmed. Amplified *nar* G and *nap* A PCR products were TA cloned and analyzed by sequencing. In addtion, determination of NR activity in PAGE zymograme was perfomed confirming the ability of strain MMT to reduce nitrate under aerobic and anaerobic conditions, respectively.



## INTRODUCTION

Nitrate reductases belong to Oxidoreductases that catalyze biological oxidation-reduction reactions which mediated by microbes that control organic oxidations, element cycling in nature and solubilization of metals in the environment which are important concepts in pollution and pollution prevention (Okolo*et al.,* 2007). Three major types of microbial nitrate reductases can be distinguished, according to the type of nitrate utilization, cellular localization, structure, enzymatic function, biochemical properties and organization/regulation gene. They are the assimilatory cytoplasmic nitrate reductase (NAS), membrane bound respiratory nitrate reductase (NAR), and periplasmic dissimilatory nitrate reductase (NAP).

NAS occurs in eukaryotes as all plants, in most fungi, algae and in many bacteria. It is located in the cytoplasmic compartment, ammonium repressible, and participates in nitrogen assimilation by reduction of nitrate to which nitrite is further reduced to ammonia as the final reduction product which is then incorporated into the biomass. In this way, nitrate functions as a source of nitrogen for biosynthetic purposes in the assimilatory pathway. The process may occur under anaerobic conditions and requires energy (Conrado *et al.,* 1999).

Membrane-bound nitrate reductases (NAR) are contributed mainly in anaerobic nitrate respiration and denitrification in which nitrate and nitrite serve as terminal electron acceptors instead of molecular oxygen and are reduced to nitric and nitrous oxides, or further up to gaseous molecular nitrogen. NAR is a three-subunit enzyme composed of Nar GHI, where the catalytic subunit Nar G and the one [3Fe-4S] and three [4Fe-4S] NarH subunit are located in the cytoplasm and associate with NarI whose N-terminus faces the periplasm. Nar proteins are encoded by genes of a *nar GHJI* operon, *nar GHI* genes encode the structural subunits and *nar J* codes for a cognate chaperone required for maturation and membrane insertion of Nar. The Nar GH subunits are on the outside rather than the inside of the cytoplasmic membrane in some archaea

**Corresponding author:* **Sahar Zaki**
Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technological Applications (SRTA-city), Alexandria, Egypt

and bacteria (Galeote *et al.,* 2013).The dissimilatory nitrate reduction is coupled to the generation of the electrochemical proton gradient across the membrane and to generation of ATP. NAR is inducible enzyme and inhibited by $O_2$ and unaffected by ammonium (Alena *et al.,* 1998). The NAP was reported firstly for phototrophic and denitrifying bacteria, is widespread among many Gram negative bacteria including *Alcaligeneseutrophus* (Siddiqui *et al.,* 1993), *Paracoccus pantotrophus* and *Wolinella succinogenes*. NAP systems are not influenced by ammonium or oxygen and are expressed during growth on highly reduced substrates. Nap is a two-subunit enzyme composed of the NapAB complex located in the periplasm and a transmembrane NapC component. Up to eight different genes have been identified as components for operons that encode perisplasmic nitrate reductases in different organisms. Most bacteria studied have the *nap ABC* genes in common, while, *napDEFKL* genes encode for different proteins that are not directly involved in the nitrate reduction but in functions required for proper functioning of the enzyme (Galeote *et al.,* 2013).

Multiple nitrate reductase enzymes are found in various microorganisms, including certain nonsulfur photosynthetic bacteria and the aerobic denitrifier *Thiosphaerapantotropha*, and *Alcalgensiseutrophus* H16 (Siddiqui *et al.,* 1993). NR-producing microorganisms participate largely in clean up environment by eco-friendly management of waste in a process termed as bioremediation. When bacteria acting as biogeochemical agents and consume waste, they reduce, eliminate, contain, or transform the waste into safe by products. Such process involves the production of energy in a redox reaction within microbial cells (Adams *et al.,* 2015). Nitrate reductase is the first enzyme in aerobic/anaerobic denitrification stages. In addition, it was reported in several literatures to be responsible for nanoparticles production especially AgNPs (Silambarasan and Abraham, 2013; Zaki *et al.,* 2014). In all the organisms that synthesize silver nanoparticles nitrate reductase might be an integral part of it. The main objective of this study is to isolate, characterize and identify NR-producing bacteria from different Egyptian ecosystems.

## MATERIALS AND METHODS

### *Sampling*

Several water and soil samples were collected from different Egyptian ecosystems during December 2011 to February 2012. For water samples: (fresh, marine water and salt mine), wastewater samples (industrial, agricultural and municipal) were collected in sterile plastic bottles. While soil samples were collected from surface, subsurface, sandy and contaminated soil in sterile polyethylene bags that are closed tightly. The collection of samples occurred using sterile instruments. Each sample was collected in triplicate. All samples were transferred in an ice box to the laboratory within 6 h of collection for further analysis.

### *Isolation and screening for presence/absent of NR-producing bacteria*

One ml of each water sample and 1 gm of fine homogenized soil sample were serially diluted in 0.8 % saline then plated in duplicates on BTB media contains (g/l): $KNO_3$ 5.0, $Na_2HPO_4{\cdot}7H_2O$ 7.9, $KH_2PO_4$ 1.5, $MgSO_4{\cdot}7H_2O$ 0.1, disodium succinate 15.0, agar 10.0, BTB (1% in ethanol) 1 ml/l, trace element solution 2 ml/l at pH 7.0. Trace element solution components: EDTA 50.0, $ZnSO_4$ 2.2, $CaCl_2$ 5.5, $MnCl_2{\cdot}4H_2O$ 5.06, $FeSO_4{\cdot}7H_2O$ 5.0, $(NH_4)6Mo_7O_2{\cdot}4H_2O$ 1.1, $CuSO_4{\cdot}5H_2O$ 1.57, $CoCl_2{\cdot}6H_2O$ 1.61 at pH 7.0and incubated aerobically at inverted position at 30$^{O}$C for 1-3 days. This method was based on the increase in the pH of the medium due to consumption of $NO_3^-$(Wang *et al*., 2007; Wu *et al*., 2013)

### *Assay of NR activity*

The reaction mixture contained (in order of addition): 450 µl of distilled water; 200 µl of 0.5 M phosphate buffer, pH 7.0; 100 µl of 0.2 M $KNO_3$ ; 100 µl of 40 mM benzyleviologen; 50 µl of crud enzyme; and 100 µl of 0.1 M $Na_2S_2O_4$ (prepared in 0.3 M NaHCO3). The two blank reactions included; one of them contains inactive enzyme "boiled at 95$^{O}$C for 10 min" and another contains distilled water. After 30 minutes at 37$^{O}$C, the reactions were stopped by vigorous vortex (to oxidize the viologen). 50 µl of 2 M $ZnSO_4$ and 50 µl of 2 M NaOH were added to each mixture, to remove the green debris that could interfere with the spectrophotometric determinations, and the tubes were centrifuged at 10000 rpm for 1 min. The supernatant was transferred to clean tubes, and then 1 ml of 58 mM sulfanilamide (4-aminobenzenesulfonamide) and 1 ml of 0.77 mM N-1 naphthylethylenediamine (NNEDA) were added to each mixture to determine the formation of nitrite. After 10 min to allow the appearance of the pink color, absorbance at 540 nm was determined in T60 UV/VIS Spectrophotometer, to calculate nitrite concentration. One unit of NR activity is the amount of enzyme that catalyzes the formation of 1 µmol of nitrite per minute (Antonio *et al*., 2002; Kenji *et al*., 1981).

### *16S rDNA restriction fragment length polymorphism (16s rDNA- RFLP)*

PCR amplification of the 16S rDNA genes was performed on a Biometra PCR Thermocycler. Genomic DNA was prepared from overnight cultures using AMSHAG - DNA Extraction Kit (Elrashdy and Abd-El-Haleem, 2005). PCR was conducted using genomic DNA (0.1 µg) as the template and the commercially synthesized 16Suniversal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GATTACCTTGTTACGACTT) (Wang *et al.,* 2007). The PCR conditions were 30 cycles at 94 for 5 min94$^{O}$C for 1min, 55 $^{O}$C for 1 min, 72 $^{O}$C for 1 min, and extension at 72 $^{O}$C for 10 min. A volume of 10-µl of amplified 16S rDNA (~1500 bp) was digested for 5 min at 37°C with 2 U of *Eco* RI, *Hind* III, *Hae* III, *Hinc* I I and *Hin fI* fast digest restriction enzyme (Thermo Scientific) (El-Kerdany and Abd-El-Haleem, 2003). The fragments were separated by electrophoresis on 2% agarose gels (FMC, Rockland, USA) containing ethidium bromide at 0.1 µg/ml. Gels were run at 100 V in 1X TBE buffer and then visualized and photographed in the MultiImage light cabinet. 1

Kb ladder mix (GeneRuler™ Fermentase) was used as molecular weight marker.

***Selection of the powerful NR-producing bacteria***

Based on RFLP results, 25 different isolates were selected for further screening. About 0.5McFarland (equivalent to about $10^8$ CFU/ml) of each strain was cultivated in SM medium containing in g/l: $KNO_3$; 5.0, disodium succinate; 9.3, $KH_2PO_4$;3, $MgSO_4.7H_2O$; 0.12, $K_2HPO_4$; 1, NaCl; 0.5 and 1ml of trace element solution (includes in g/l: $CuSO_4.5H_2O$; 0.06, $MnCl_2 \cdot 4H_2O$; 0.03, $ZnSO_4 \cdot 7H_2O$; 0.31, $CoCl_2 \cdot 6H_2O$; 0.04, $Na_2MoO_4 \cdot 2H_2O$; 0.03, $H_3BO_3$; 0.57, $FeCl_3 \cdot 6H_2O$; 0.24)(Al-Rajhi *et al*., 2010; Wu *et al*., 2013) A small inverted Durham tube was added to each 10 ml tube in order to detect gas formation. The cultures were incubated aerobically for 3-7 days at 30$^O$C. The presence of gas indicating the formation of nitrate and nitrite were checked (Koreishit and Hinds, 1984; Ramdhani and Bux, 2007).

***Characterization of the isolate MMT and its NR***

Isolate MMT exhibited the strongest NR-production activity among the 25 assayed isolates was selected for further characterization of both the isolate itself and its produced NR enzyme as follow:

***Molecular characterization of isolate MMT***

About 1500 bp 16S rDNA gene of MMT was identified by sequencing using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 373 DNA Sequencer (Perkin-Elmer, Foster City, Calif.). The sequence was analyzed using N-BLAST program (National Centre for Biotechnology Information) to determine the similarities to available database sequences and its phylogenetic tree that was constructed by neighbor joining method.

***Morphological and biochemical characterization of isolate MMT***

Cell status to gram staining of the isolate was observed by a light microscope (Olympus BH-2, Japan). The morphology and dimensions of isolate were determined from photomicrographs using scanning electron microscopy (SEM) (JEOL JEM-1230, Japan- Faculty of Science- Alexandria University), and transmission electron microscopy (TEM)(JEOL JSM 6360LA, Japan -Faculty of Science- Alexandria University). The widths and lengths given represent the averages of measurements of several cells. For colony characterization, the cells were cultivated on NB agar and incubated both aerobic and anaerobic at 30$^O$C. Biochemical assays of Catalase, Oxidase, DNase, Lipase and Amylase were performed as reported by (DebRoy *et al.,* 2012). In addition, micro-identification system [Microbact-24E (Oxoid)] was used to examine the biochemical capabilities. The optimum pH (5, 6, 7, 8 and 9), temperature (4, 10, 20, 30, 40, 50 and 60$^O$C) (Mahmood *et al*., 2009; Yamamoo *et al*., 1988) and NaCl tolerance(1%, 3%, 5%, 7% and 10%) were determined in SM medium.

***Antibiotic sensitivity***

The antibiogram was performed by a disk diffusion method on Mueller Hinton agar. The culture was inoculated and incubated at 30$^O$C for 24h. The examined antibiotics in concentration of 30µg were; Chloramphenicol, Ampicillin, Gentamycin, Neomycin, Polymyxin-B (300 unit), Penicillin, Streptomycin, Tetracycline, Erythromycin, kanamycin, Rifamycin and nalidixic acid (Uyanika *et al*., 2009)

***PCR detection of NR genes in MMT***

Nitrate reductase genes *narG* and *napA* were detected using nested PCR as follow:

***PCR detection of narG gene***

Genomic DNA was used as the template in the first round of the nested PCR of *narG*primed by 10 µl of (20 pmol / µl) T37 (5' CAYGGNGTNAAYTGYACNGG 3') and T39 (5' TARTGNGGCCCANCCNCCNCC 3') in a 50 µl reaction volume also containing 0.15 µl Dream Taq (Thermo Scientific), 15 µl of green buffer containing 1.5 mM $MgCl_2$ and 2 µl of 10 mM deoxynucleotide triphosphate (dATP, dCTP, dGTPand dTTP) mix (Thermo Scientific). Thermocycling was in 0.2-ml thin walled tubes in a Biometra-thermocycler under the following conditions: 5 min at 94$^O$C, 30 cycles of 1 min at 94$^O$C, 1 min at 58$^O$C, 1.5 min at 72$^O$C, 10 min at 72$^O$C. The first round product was about 1690 bp and used as the template in the second reaction primed to give ~500 bp by 10 µl (20 pmol / µl) of W9 (5' MGNGGNTGYCCNMGNGGNGC3') and T38 ( 5' ACRTCNGTYTGYTCNCCCCA) (Gregory *et al*., 2000) in a 50 µl reaction volume. The same second round cycling conditions was as the first but differ only in annealing temperature to be 53$^O$C for 1 min. Amplicons were rune on 1.5% (w/v) agarose gels stained with ethidium bromide (0.1 µg/ ml) and photographed as described above.

***PCR detection of napA gene***

For *napA* the same PCR reaction conditions were performed as described above. While, the first PCR reaction was performed using the primers V16 (5' GCN CCN TGY MGN TTY TGY GG 3') and V17 (5' RTG YTG RTT RAA NCC CAT NGT CCA 3') with a total PCR product of 1040 bp. Subsequently, the amplicon was used as a template for the second PCR round using the primersV66 (5' TAYTTY YTN HSNAAR ATH ATG TAY GG 3') and V67 (5' DATNGG RTGCATYTC NGC CAT RTT 3') to give a final PCR product about 385 bp (Nogales *et al.,* 2002)

***Clone library construction and sequencing***

Amplicons of *narG* and *napA* were purified using the NEBRAS-PCR purification kit (Elrashdy and Abd-El-Haleem, 2005) and cloned using the TOPO TA cloning kit (Invitrogen, Paisley, United Kingdom) according to the manufacturer's instructions. Transformants were selected on Luria-Bertani agar plates containing ampicillin (100 µg/ ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (20 µg/ ml)

and IPTG (Isopropyl β-D-1-thiogalactopyranoside) (20 µg/ ml). White colonies were screened and plasmids were isolated from the transformed colonies containing inserts of the expected size by using a Qiaprep & miniprep kit (Qiagen). Inserts were amplified by PCR using the primers M13F (5' GTA AAACGA CGG CCA G 3') and M13R (5' CAG GAA ACA GCT ATG AC 3') (Invitrogen). Nucleotide sequences of amplified amplicons were performed as described above. Cloned *nar G* and *napA* nucleotide sequences were compared to entries NCBI sequences to determine the GenBank accession numbers (Nygren *et al.,* 2008; Smith *et al*., 2007).

### *Phylogenetic analysis of narG and napA genes*

Phylogenetic affiliations of the *napA* and *narG* partial nucleotide sequences were initially translated into protein sequences using the Translate tool on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://us.expasy.org /tools/ dna. html). Protein sequences were compared to entries in NCBI/ GenBank using Blast-P. Protein sequence alignments were constructed using ClustalX (Gomila *et al*., 2011). Phylogenetic trees were created from the distance matrices by using the neighbor-joining method and Kimura substitution algorithm using PHYLIP. Consensus trees were calculated after bootstrapping (100 replicate trees).

### *Zymography detection of NR activities*

Native electrophoresis was performed with 11x11 cm plates using a gradient polyacrylamide gel (6% polyacrylamide) in Tris-HCl buffer, pH 8.8. The current employed was 40 mA in the separating mode. To determine reductase activity in PAGE, the reaction mixture containing 0.2 M sodium phosphate buffer, pH 7.0, 20 mM $KNO_3$, 5 mM Benzyl Viologen and 10 mM sodium dithionite was used. The gels were immersed in this mixture and incubated until transparent bands appeared against the blue background of the gel as a result of reductase activity. Thereafter, the gels were fixed first in 0.05% (w/v) triphenyltetrazolium chloride and subsequently in 5% (v}v) acetic acid. After fixation NR activity appears as transparent band against red background. With respect to negative control samples, inactive enzyme (denatured by boiling 10 min) was used (Aantipov *et al.,* 2003; Ridley *et al.,* 2006).

## RESULTS AND DISCUSSION

### *Isolation and prescreening for NR-producing bacteria*

As illustrated in Table 1, 43 different blue colonies and/or halos were selected on BTB agar plates. Subsequently, pure colonies were obtained by repetitive streaking on the same medium and confirmed for their NR activity. Isolation data reported that colonization of municipal sewage; contaminated soil and Mariout Lake (basin 3) were equipped with the highest density and diversity of NR-producing microbial niches. However, no NR-producing microorganisms were detected in the samples collected from Alzaafaran sand, Alkantaraa and Waddy Elmlok Plant for electroplating, respectively.

It is known that the microbiota composition and physiological role varies as a function of ecosystem structure, composition, nature and location (Seenivasagan *et al.*, 2014). Due to the variation in nutrients availability, salt and organic compound concentration, turbidity, temperature, and heavy metal contaminants leads to variation in bacterial density and community that found in the seawater, freshwater ( rivers and lakes) and in the soil. Also, microbial niches that present in municipal water differ from that in agricultural or industrial wastewaters. In addition this may select specific microbial populations to dominate in the environment that characterized with unique properties to adapt and cope with its environmental conditions.

**Table 1** NR producers according to sampling sites

| Sample Name | Sample Type | Governorate | Isolated Number |
|---|---|---|---|
| soil from the city of scientific research garden | Soil | Alexandria | 4 [ $S_6$, $S_7$, $S_{15}$, $S_{16}$ ] |
| Alarish Shore | Salt Mine Soil | North Sinai | 1 [$S_{17}$] |
| Brolros Lake Shore | Salt Mine Soil | Kafe- Elshikh | 2 [ $S_{32}$, $S_{42}$] |
| Alzaafaran Sand | Sand Soil | Red-Sea | Negative |
| Beer Alabd | Sand Soil | North Sinai | 2 [$S_{19}$, $S_{20}$] |
| Alkantaraa | Soil | Alesmaelia | Negative |
| Contaminated Soil with engine | Soil | | 6 [ $S_8$,$S_{10}$, $S_{22}$, $S_{25}$, $S_{27}$, $S_{39}$] |
| Mariot Lake basin 3, Alexandria | lake Water | | 5 [$S_5$, $S_{13}$,$S_{35}$,$S_{36}$, $S_{43}$] |
| Mariot Lake basin 6 | Lake Water | | 3 [$S_{14}$, $S_{24}$,$S_{29}$] |
| Al malahat | Salt mine | | 1 [$S_{11}$] |
| Mahmoudia canal | Fresh Water | | 3 [$S_3$, $S_4$, $S_{28}$] |
| Mediterranean Sea "Al-max" | Sea water | | 3 [ $S_1$, $S_{18}$, $S_{26}$ ] |
| Sewage Treatment Plant "Borg El-Arab" before treatment | Municipal Waste Water | Alexandria | 8[$S_9$, $S_{21}$, $S_{23}$, $S_{30}$, $S_{31}$, $S_{33}$, $S_{34}$, $S_{38}$] |
| Mennas Factory for Electroplating | Industrial Wastewater | | 2 [$S_{40}$, $S_{41}$] |
| Egyptian Spanish for Mining | Industrial Wastewater | | 1 [ $S_{37}$ ] |
| WaddyElmlok Plant for Electroplating | Industrial Wastewater | | Negative |
| Bahige sluiceway | Agriculture waste water | | 2 [ $S_2$, $S_{12}$] |

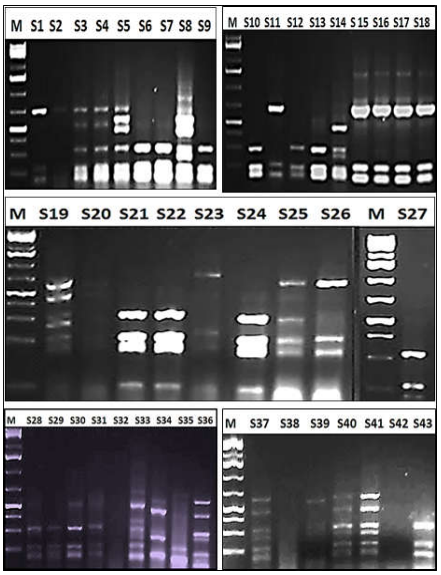


**Figure 1** *HaeIII* - 16s-rDNA RFLP digestion pattern for bacterial isolates from different Egyptian ecosystems

### *16S rDNA- RFLP*

In order to reduce the large number and eliminate similar isolates, the rapid and highly discriminative 16S rDNA-RFLP approach was employed. Among all examined restriction enzymes, *HaeIII* was the most discriminative enzyme (Fig.1).

It generates polymorphic banding pattern among the isolates. It was able to divide strains into 21 groups including $S_1$, $S_2$, and $S_{26}$ (group 1), $S_3$, $S_4$, and $S_{25}$ (group 2), $S_6$, $S_7$, $S_9$, $S_{10}$, $S_{12}$ and$S_{13}$, (group 3) that they have identical bands with identical size, While group 4 contains $S_{11}$, $S_{15}$, $S_{16}$, $S_{17}$ and$S_{18}$ as having monomorphic pattern. $S_{14}$, $S_{29}$ and $S_{43}$ (group 5), $S_{21}$, $S_{22}$ and $S_{24}$ (group 6), $S_{19}$ and $S_{20}$ (group 7), $S_{28}$ and $S_{30}$ (group 8). $S_{37}$, $S_{40}$ and $S_{41}$ (group 9), $S_{32}$ and $S_{42}$ (group 10), and each of the following groups contains one strain with unique banding pattern for each. $S_5$ (group 11), $S_8$ (group 12), $S_{23}$ (group 13), $S_{27}$ (group 14), $S_{31}$ (group 15), $S_{33}$ (group 16), $S_{34}$ (group 17), $S_{35}$ (group 18), $S_{36}$ (group 19), $S_{38}$ (group 20) and $S_{39}$ (group 21).

According to *Hea III*-RFLP banding patterns, municipal sewage, contaminated soil and Mariout Lake (basin 3) contain 8, 6 and 5 distinct genotypes exhibiting high diversity and heterogeneity within each sample. Phenomena of strain specificity with unique genotype banding pattern which inhabit certain sampling site could be observed in this study. The patterns of bacteria isolated from Brolosse lake shore, Beeralabd and Egyptian Spanish for Mining and Mennas Factory for Electroplating were unique and differ totally from other isolates.

In the same direction, Durham tube assay indicated that among the 25 examined isolates, 11 (44%) was scored as putative/true denitrifiers (D) and 14 isolates (56%) were scored as nitrate reducers. Among the 11 true denitrifiers isolates, 45 %were from municipal sewage ($S_{30}$, $S_{31}$, $S_{33}$, $S_{34}$ and $S_{38}$), 18 % in both contaminated soil ($S_{27}$ and$S_{39}$) and agricultural wastewater ($S_2$ and$S_{12}$) and 9 % in both garden soil ($S_7$) and lake water ($S_5$). Such conclusions confirming that eco-physiological role is ecosystem dependent. At which ecosystem constituent compels microbial communities to behave manner that dissimilates nitrate in preferred way either partial or complete reduction (Koreishit and Hinds, 1984; Ramdhani *et al*., 2007).

### *Selection of the powerful NR-producing bacteria*

Isolate **S5**that was isolated from Mariout Lake Basin 3 recorded the highest NR activity among the 25 isolates selected by 16S rDNA-RFLP approach and reached the maximum NR activity after 12 h, total removing of nitrate after48h and complete nitrite conversion to gaseous after 90 h of incubation.

### *Characterization of selected isolate*

### *Molecular characterization of the selected isolate*

The sequence of 16S rDNA of isolate S5 (~727 bp) was subjected for homology search in NCBI/GenBank database. The data revealed about 99% sequence similarity with all species of the genus *Achromobacter.* In addition, the sequence was homologues in about 98 % with *Bordetella*, 97% with *Kerstersia* and less than that with other genera as illustrated in (Fig. 2). Subsequently, strain S5 designated MMT (M for Mariout lake, M for Mutual and T for nitrate treatment) was grouped with the smallest major cluster including *Achromobacterdenitrificans and Achromobacteranxifer* which confirming their phylogenetically proximity with them. Also, it located in vicinity with the second major cluster that including several species of *Achromobacter.* Presently, the acceptable positional standard is that if the similarity of strain under investigation and a reference strain sequences is higher than 97–98%, they are regarded as belonging to the same (Mahmood*et al*., 2009). Therefore, the result of this phylogenetic analysis of isolate MMT was identified as*Achromobacter* sp.strain MMT. The nucleotide sequence of strain MMT was submitted to GenBank under accession number KT735046.

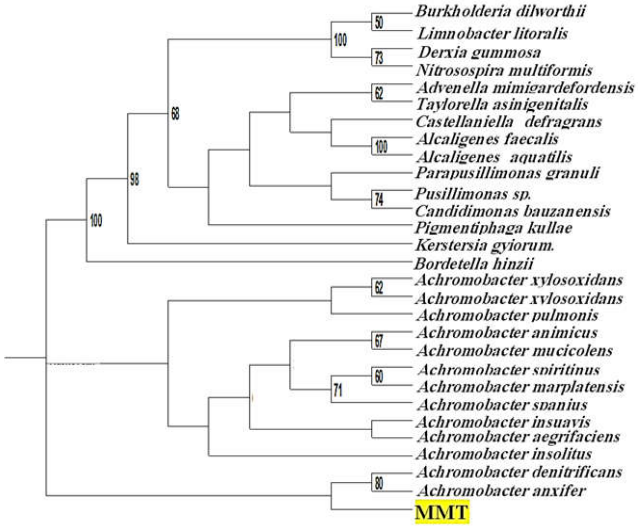


**Fig 2 Neighbor**-joining dendrogram of strain MMT. Bootstrap values of more than 50 (from 100 replicates) are indicated at the nodes.

### *Cultural and Morphological characterization*

Colonies of MMT on nutrient agar were translucent non pigmented, round, flat or slightly convex with smooth margins and white in color. It was facultative anaerobic as having the ability to grow aerobically/anaerobically but with higher rate in aerobic conditions. Cell morphology characterized by: non-spore forming, Gram-negative, non-motile, small and rod shape cells with rounded ends that occur as a single unit, in pairs or in short chains. The average dimensions were 0.6 µm in length and 0.27 µm in width as measured by SEM & TEM (Fig.3).

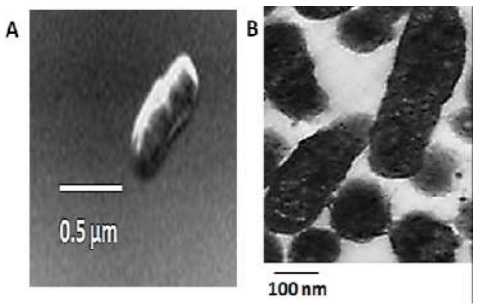


**Fig 3** Morphological characters of MMT cells, A) SEM, B) TEM analysis.

### *Biochemical and Physiological characteristics*

As illustrated in Table 2, cells of MMT showed various biochemical activities. The strain was able to grow over a wide range of temperature (10 $^{O}$C to 50 $^{O}$C) with optimum at 30$^{O}$C, below and above this range growth adversely affected. In addition, MMT was grown under pH from 5.0 to 9.0 with optimal at 7.0. For NaCl tolerance, MMT was grown well at 1% and 3% of NaCl, while inhibition of growth was observed at the remaining examined concentrations (Vandammea *et al*., 2013).

### *Antibiotic assay*

The response of strain MMT towards examined different antibiotics was indicated in Table 3. The antibiogram effects of

**Table 2** Biochemical reactions of *Achromobacter sp.* KT735046

| Biochemical Test | Result | Biochemical Test | Result |
|---|---|---|---|
| oxidase | + | Gelatin liquefaction | - |
| catalase | + | Malonate inhibition | + |
| DNAse | + | Inositol fermentation | - |
| lipase | - | Sorbitol fermentation | - |
| lysine decarboxylase | - | Rhamnose fermentation | - |
| ornithine decarboxylase | - | Sucrose fermentation | - |
| $H_2S$ | - | Lactose fermentation | - |
| Glucose fermentation | + | Arabinose fermentation | - |
| Mannitol fermentation | + | Adonitol fermentation | - |
| Xylose fermentation | + | Raffinose fermentation | - |
| ONPG | + | Salicin fermentation | - |
| Indole production | - | Arginine dihydrolase | - |
| Urease | + | Motility | - |
| V.P | - | haemolysis | - |
| Citrate utilization | + | Growth on acetamide | + |
| TDA | - | amylase | - |

all examined antibiotics were in agreement with (Chester and Cooper, 1979; Gomila *et al*., 2011; Lambert, 2012; Pienfn and Higa, 1978; Walsh and Duffy, 2013).

**Table 3** Antibiogram of strain MMT against various antibiotic classes

| Mode of Action | Antibiotic | Antibiotic (30µg) | Antibiotic Resistance (R) Susceptibility Profile (S) |
|---|---|---|---|
| Cell Wall Inhibition | β-Lactam | Ampicillin | R |
| | | Penicillin | R |
| | Polypeptides | Polymyxin-B "300 UNIT" | S |
| Protein Inhibition | Macrolides | Erythromycin | S |
| | Tetracyclines | Tetracycline | R |
| | Chloramphenicols | Chloramphenicol | R |
| | Aminoglycosides | Streptomycin | R |
| | | kanamycin | S |
| | | Gentamycin | S |
| | | Neomycin | S |
| Inhibition of Nucleic Acid synthesis | Quinolones | Nalidixic acid | S |
| | Rifamycins | Rifamycin | S |

### *Molecular detection of NR genes and enzyme*

### *Detection of NR genes*

As shown in (Fig. 4) A and B, both *narG* and *napA*genes were detected in the genomic DNA of strain MMTin both first and second PCR rounds without any artifacts or nonspecific bands (Nogales *et al*., 2002).However, Zhu *et al*., (2012) speculated that *Achromobacter* that was isolated from oligotrophic ecosystem could express *napA* in an aerobic denitrification process, while, *Achromobacterfischeri* expressed only *narG*.

### *Phylogenetic analysis of cloned napA genes*

Approximate 353 partial sequenced nucleotides of cloned *napA* gene of MMT was compared with NCBI sequence databases verified that this clone potentially encoded periplasmic nitrate reductase sequences, showing the varied levels of identity with catalytic unit of NAP in the NCBI database. The FASTA values ranged from 42% identity with*Pasteurellamultocida*, 38-41% different species of *Pseudomonase*, 40% with

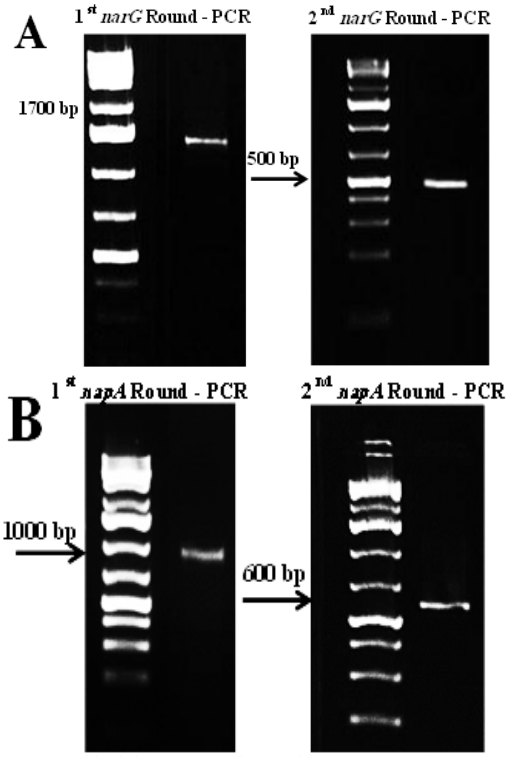


**Fig 4** Agarose gel of the PCR products of *narG* (A), and *napA(B)* genes of strain MMT.

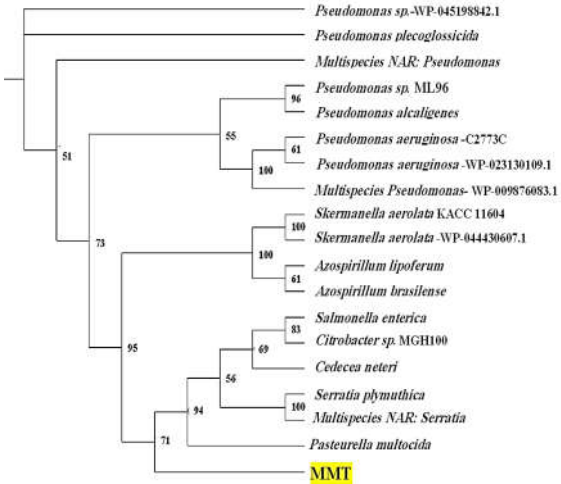


**Fig 5** Neighbor-joining dendrogram derived from the deduced protein sequences encoded by cloned *napA*sequences of MMT and periplasmic nitrate reductase sequences from NCBI database. Bootstrap values of more than 50 (from 100 replicates) are indicated at the nodes.

Rhodospirillaceae that includes *Azospirillum* and *Skermanella*, 40-43% with Enterobacteriaceae (*Salmonella, Serratia*, *Citrobacter* and *Cedecea*).

The sequences were conceptually translated to 117 corresponding amino acids used to construct *napA* dendrogram by assembling an alignment of *napA* sequence of MMT with NCBI related NR sequences. The nucleotide sequence of *napA*gene was submitted to the GenBank under accession number (KT877347).

The overall topology of *napA* dendrogram indicated in (Fig. 5), it was classified into 3 main cluster; MMT is located in the dominant subcluster within the major cluster. MMT is grouped with Pasteurellaceae and Enterobacteriaceae that were classified in α proteobacteria which indicate the vicinity of their aligned sequence. As observed from napA dendrogram, there is wide variation and diversity of*napA* genes indicated by comprising genera fromα, ß and ϒproteobacteria. This variation reflects the diversity in physiological roles of periplasmic nitrate reductases than those of the assimilatory and membrane associated nitrate reductases (Klatte *et al*., 2011).The NAP is probably a functionally versatile enzyme according to the organisms. In some organisms, NAP clearly has a catalyzing role in anaerobic denitrification. For some others, it may provide the biochemical apparatus for aerobic nitrate respiration as in *Pseudomonas* strains (Roussel-Delif *et al.,* 2005). In others microorganisms, it participate in maintenance of redox balance during growth on reduced carbon sources and during photosynthetic growth in addition to scavenging low concentrations of nitrate (Klatte *et al*., 2011).

### *Phylogenetic analysis of cloned narG genes*

Partial nucleotide sequence of 397 bp was compared with NCBI sequence databases conforming that this clone potentially encoded *narG* exhibiting the varied levels of identity to membrane bound nitrate reductase/catalytic-alpha subunit of narG. The FASTA values ranged from 99% for*Ochrobactrum*and94% *Brucellaas* genera. However, 91 % for *Agrobacterium*, 87 -89% for *Martelella,* 87% for *Pseudovibrio* and 82% dissimilatory narG [uncultured bacterium].

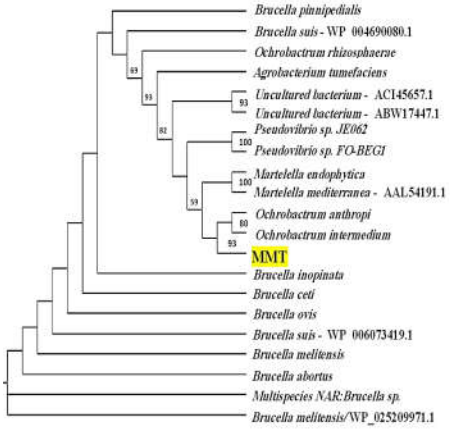


**Fig 6** Neighbor-joining dendrogram derived from the deduced protein sequences encoded by cloned *narG*sequences MMT and membrane bound nitrate reductase sequences from NCBI database. Bootstrap values of more than 50 (from 100 replicates) are indicated at the nodes.

About 132 unambiguous amino acids were translated and aligned with other deduced protein sequences of *narG*of GenBank related sequences to construct the phylogenetic relationship between them. The nucleotide sequence of *narG* sequence was submitted to GenBank under accession number (KT884546).

(Fig. 6) illustrate the phylogenetic tree of *narG*. It was classified into 3 main clusters, all were belong to α-protobacteria, while MMT related to ß -protobacteria . MMT-*narG*were grouped in the main subcluster within the major cluster in the tree. It presents in vicinity with *Ochrobactrum* and *Martelella* as a genera which both belong to the same order (Rhizobiales). The dendrogram contain also other genera as *Brucella* and *Agrobacterium* that were also classified along with the same order in addition to *Pseudovibrio* which belongs to order Rhodobacterales. All mentioned genera were reported previously in nitrate reduction and denitrification. The presence of several divergent lines as 2 main cluster and about 6 from major one suggest reflecting presence of wide variation within *narG* encoding genes. NAR is the key enzyme in anaerobic denitrification (Roussel-Delif *et al.,* 2005).

### *Determination of NR activity in PAGE zymograme*

The crud NR complex (narG and napA) was resolved as single bands on native PAGE when stained with reduced benzyleviologen (Fig.7). Lane A show membrane bound NR that produced and expressed under oxygen limitation, while lane B indicates periplasmic NR that was in-sensitive to oxygen and expressed under aerobic conditions. For both negative lanes, they loaded with inactive NAR and NAP, respectively which representing negative control. Apparent variation in molecular weights of NAP and NAR was observed. At which NAP was shorter in size and so move faster and migrate farther and more easily on the gel than NAR that remain in the front with slow motion and lower migration rate. The zymograme gel and its sieving property were clear and easy to discriminate for both location and activity.

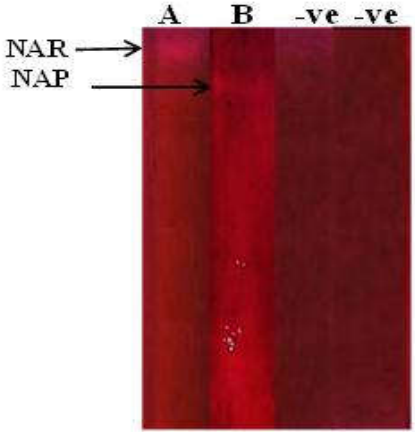


**Fig 7** NR activity of MMTon native gel, Lane A crud NAR, lane 2crud NAP and Lanes -ve represent negative controls.

NR can exist in the form of active dimers or active monomers, and in all three forms (native tetramer, dimer and monomer) it possessed nitrate reductase activities. A similar structure and properties (i.e. catalytic activity of separate subunits) have been reported previously for NRs from *Pseudomonas isachenkoii*

(Aantipov *et al*., 2003). Roussel-Delif *et al*, (2005) proved the presence of good correspondence between the nitrate reductase activity and the presence of *narG* and/or *napA* among nitrate-reducing and putative denitrifying *Pseudomonas* species isolated from three different soils.

## CONCLUSION

The powerful *Achromobacter sp*. strain MMT was selected among 43 nitrate reducing bacteria isolated from various Egyptian ecosystems**.** MMT exhibits denitrifying ability by growing on SM containing durham tube. It was subjected to morphological, physiological and biochemical characterization in addition to its antibiotic sensitivity. Both *napA* and *narG* genes were detected identified and their phylogenetic trees were constructed. Activity of both NAR and NAP were detected zymographycally under anaerobic and aerobic conditions. So it is possible to use strain MMT in bioremediation applications under various conditions.

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