

## ISOLATION AND CHARACTERIZATION OF FREE-NITROGEN FIXER BACTERIAL STRAINS (*Azotobacter* sp.) AND THEIR PHAGES FROM MAIZE RHIZOSPHERE-SOIL AT TAIF

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Article received 02.11.2015; Revised 06.01.2016; Accepted 12.01.2016

### ABSTRACT

We are focusing on isolation of bacteriophage(s) specific to some free N<sub>2</sub>-fixer bacteria from rhizosphere and non-rhizosphere soils of Taif region of KSA as a first record for such study in Taif area (Makkah, Jeddah and Taif). A number of 10 bacterial isolates grown on the nitrogen-free specific medium (Waksman base No.77 Medium) were purified and separately used as hosts for bacteriophage(s) isolation. The spot test and turbidity tests were used to detect the presence of the phage of interest in the prepared phage suspensions. The level of lysis revealed the presence of turbid zones (plaques), as weak (+) lysis (for isolates # 07) and (# 09); moderate lysis (++) (for isolate # 05), and high lysis (+++) (for isolates # 01 and # 03). The phage(s) was propagated and partially purified for determining the morphology of viral particles *via* electron microscopy. Sperm shape virus-like particles with long tail and icosahedral head were shown in the electron micrographs of partially purified phages specific to the two selected bacterial isolates ((# 01 and # 03). These two bacterial isolates were then morphologically and molecularly identified. The nucleotide sequences of 16S rRNA gene of the two bacterial isolates was determined and final sequences of 927 and 873 nts for the 16S rRNA gene of two isolates of this study ((# 01 and # 03), respectively, were recorded. Data of the phylogentic trees show that the two bacterial isolates (# 01 and # 03) could be strains of *Azotobacter* sp. (LC054002.1 and LC054003.1).

**Keywords:** Bacteriophage, N<sub>2</sub>-fixer bacteria, plaque-forming assay, electron microscopy, 16S rRNA gene.

### INTRODUCTION

Biological nitrogen fixation is carried out only by prokaryotes, which may be symbiotic or free living in nature. It is well documented that biological nitrogen fixation mediated by nitrogenase enzymes is a process important to the biological activity of soil (Matthew, *et al.*, 2008 and Bagali, 2012). Nitrogen fixing free living microorganisms have frequently been reported as plant growth promoters (Requena, *et al.*, 1997). Several microorganisms such as *Rhizobium* (Nghia and Gyurjan, 1987), blue-green algae (BGA) or *cyanobacteria* (Rodrigo and Novelo, 2007), *Azolla* (Galal, 1997), *Azotobacter* (Gonzalez-Lopez, *et al.*, 2005), *Azospirillum* (Gonzalez-Lopez, *et al.*, 2005 and Emtiazi, *et al.*, 2007) and seven species of family Acetobacteraceae (Saravanan, *et al.*, 2007) can play significant role in agriculture as N<sub>2</sub>-fixing microorganisms.

*Azotobacter* is a genus of usually motile, oval or spherical bacteria that form thick-walled cysts

and may produce large quantities of capsular slime. The first representative of the genus *Azotobacter chroococcum*, was discovered and described in 1901 by the Dutch microbiologist and botanist Martinus Beijerinck. *Azotobacter* species are Gram negative bacteria found in neutral and alkaline soils (Gandora, *et al.*, 1998 and Martyniuk and Martyniuk, 2003) in water, and in association with some plants (Tejera, *et al.*, 2005 and Kumar, *et al.*, 2007). *Azotobacter* is an obligate aerobe, although it can grow under low oxygen concentration. The ecological distribution of this bacterium is a complicated subject and is related with diverse factors which determine the presence or absence of this organism in a specific soil (Gonzalez-Lopez, *et al.*, 2005).

Bacteriophages are measurable component of the natural microflora in the food production continuum from the farm to the retail outlet. Phages are remarkably stable in these environments and are readily recovered from soil, sewage,

water, farm and processing plant effluents, feces and retail foods (Kevin, *et al.*, 2003). Previous attempts by Jones (unpublished data) have shown that azotophage are difficult to isolate, even though enrichment of naturally occurring *Azotobacter* in samples of soil, water, and sewage was performed by methods described by Duff and Wyss (1961). Hegazi and Leitgeb (1976) studied *Azotobacter* bacteriophages using liquid as well as solid enrichment cultures in 16 samples representing the soils of Ruzyně, Prague. They also reported that the phages were belonging to the groups with long non-contractile, contractile, and short contractile tails. Host specificity assay showed that the majority of the strains of *A. chroococcum*, *A. vinelandii*, and one strain of *A. beijerinckii* were lysed by phage, while none of *A. macrocytogenes*, *A. agilis* and *A. insignis* strains were lysed.

In Saudi Arabia, the presence of bacteriophages in some rhizosphere soil samples (*Argemone*, rose and *Datura*) collected from Taif region was detected by Sadik, *et al.*, (2014). The first direct counts of soil bacteriophage and show that substantial populations of these viruses exist in soil (grand mean =  $1.5 \times 10^7 \text{ g}^{-1}$ ), at least 350-fold more than the highest numbers estimated from traditional viable plaque counts was reported by Kevin, *et al.*, (2003).

The goal of this study could be summarized in isolation, purification, and molecular identification of a nitrogen-fixing bacterium occurred in some rhizosphere soil of Taif region. The presence of bacteriophage(s) specific to the nitrogen-fixing bacterial strain(s) and their electron microscopy was also aimed.

## MATERIALS AND METHODS

### Collection of soil samples and their analyses:

Some soil samples were randomly collected from some cultivated and non-cultivated soils as described by Mohamed (1998). Soil bags were then transferred to the laboratory and kept at 4°C until used. Physical properties were determined in the Central Laboratory, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The total bacterial count was determined using the plate count method as described by Jacobs and Gerstein (1960).

**Isolation of free N<sub>2</sub>-fixer bacteria:** To isolate free nitrogen fixer bacteria, a nitrogen-free

specific medium (Waksman base No.77 Medium (0.5 gm K<sub>2</sub>HPO<sub>4</sub>, 0.2 gm MgSO<sub>4</sub>, 0.2 gm NaCl, trace of MnSO<sub>4</sub>.4H<sub>2</sub>O, trace of FeCl<sub>3</sub>.6H<sub>2</sub>O, 1000 mL distilled water (H<sub>2</sub>O)) supplemented with 1 mL of each of 10% mannitol solution and benzoate solution was used) (WB77) was used. From each soil sample, one gram of soil was dissolved in 10 mL d.H<sub>2</sub>O and loopful suspension was inoculated on WB77 and incubated at 28°C for three days. Single colonies were picked up, purified (by streaking technique) and maintained on WB77 or nutrient agar medium at 4-5°C until used (Mohamed, 1998).

### Preparation of phage suspension from collected soil samples:

The method of Othman (1997) was applied for enrichment, and preparation of phage suspensions using the selected N<sub>2</sub>-fixer bacterial isolates as host range. The plaque forming technique was used to detect the presence of the phage(s) of interest as reported by Stenholm, *et al.*, (2008). Turbidity test was also used as a confirmatory tool as recommended by Dr. Othman B.A. (Personal communication). For phage propagation the method of Othman (1997) was applied.

### Phage purification and electron microscopy:

Dextran sulfate-polyethylene glycol two phase liquid system as described by Othman (1997) was followed in a trial to partial purifying of the azotophages. For electron microscopy, the phage suspensions were filtrated and negatively stained as described by Nugent and Cole (1977). Photographs were taken with a Model Beckman 1010 transmission electron microscope at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

### PCR isolation of 16S rRNA gene and its

**analysis:** Two copies of each of the two free N<sub>2</sub>-fixer bacterial isolates were sent to Macrogen Inc. in Korea for molecular identification by determining their nucleotide sequences of 16S rRNA gene. Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). NCBI blast tool is used for blast search result. The DNA sequences were analyzed using BLASTN

2.2.23+software (<http://www.ncbi.nlm.nih.gov/blast/>) against the strains and/or isolates collected from the database for genotyping.

**Phage stability:** The dilution end point (DEP) of partial purified phage suspension was determined using a serial two fold dilutions up to 1/32. Spot test was used to detect the presence of phage in diluted suspension. Regarding phage longevity at 4°C, a number of five fresh tubes containing one mL of the enriched phage suspension was prepared, and stored at 4°C in a refrigerator. The phage was detected after 0, 2, 4, 6, 8, 10, 12, 14 and 16 weeks post storage at 4°C by spot test technique.

## RESULTS AND DISCUSSION

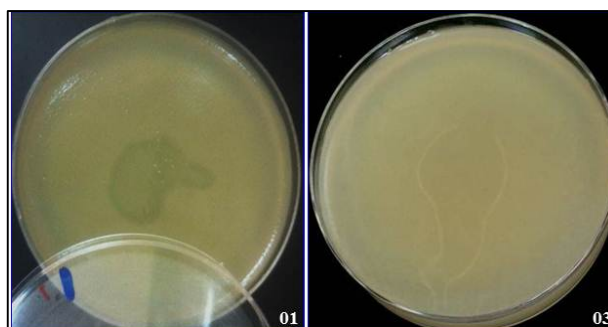
Bacteriophages are viruses that kill bacteria. They are plentiful in nature; are safe, having no known activity to human or animal cells; and are an attractive alternative to antibiotics (El-Gohary, *et al.*, 2014). Studies on bacteriophages in Saudi Arabia showed that no works were recorded on *Azotobacter* sp. (Al-Nakhli, *et al.*, 1999, Almajhdi, *et al.*, 2009, Al-Arfaj, *et al.*, 2012, Eifan, 2012, Al-Sum and Al-Dhabi, 2014 and Sadik, *et al.*, 2014). Therefore, we are focusing on isolation of bacteriophage(s) specific to some free N<sub>2</sub>-fixer bacteria (*Azotobacter*, *Azomonas*, etc) from rhizosphere and non-rhizosphere soils of Taif region of Kingdom of Saudi Arabia. *Azotobacter* are the free living bacteria, which grow on a nitrogen free medium. The importance of the bacterium *Azotobacter* due to its ability to fix atmospheric nitrogen, secrete growth substances and antifungal antibiotics, which improve plant stands in inoculated fields by inhibiting root pathogens (*shodhganga.inflibnet.ac.in/bitstream/./11\_chapter3.pdf*).

The physical properties of collected soil samples were: sandy loam or loamy sand textures, alkaline, and electric conductivity (EC) values ranged from 1.125 to 1.39 ds/m. At the level of microbiological analysis, the bacterial total count was ranged from  $2.8 \times 10^4$  to  $4.0 \times 10^4$  cfu/mL, and a number of 10 free N<sub>2</sub>-fixer bacterial isolates were purified and separately used as hosts for bacteriophage(s) isolation. These results were in harmony with that reported by Martinez-Toledo, *et al.* (1985) who showed that maize root populations were  $1.4\text{--}6.0 \times 10^4$  microorganisms g<sup>-1</sup>. They also showed that there was an association between *A.*

*chroococcum* strains and roots of maize planted in some Spanish soils.

In this study, *Azotobacter* isolates were successfully isolated from the maize-rhizosphere soil, and this was supported by the results of Martinez-Toledo, *et al.*, (1985) and Abd El-Ghany, *et al.*, (2015). As bacteria (*Azotobacter chroococcum* and rhizobacteria including *Azotobacter vinelandii*) isolated from maize roots collected from agricultural soils showed rapid growth on a nitrogen free medium and acetylene-reducing activity (Martinez-Toledo, *et al.*, 1985 and Abd El-Ghany, *et al.*, 2015). Based on phage ability to form plaques on the 10 indicator bacterial hosts isolated from maize-rhizosphere and non-rhizosphere soils of Taif, the phages were detected against 50% of tested hosts (four from maize-rhizosphere soil and one for non-rhizosphere soils). Our results could be supported by that reported by several investigators (Monsour, *et al.*, 1955, Bishop, *et al.*, 1977, Othman 1997, Hammad, 1998, Othman, *et al.*, 2008, Lee, *et al.*, 2011 and Sadik, *et al.*, 2014).

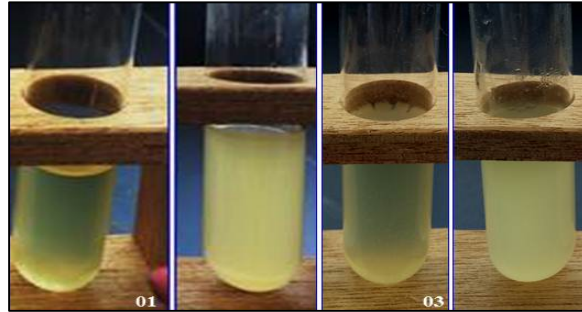
The plaques on nutrient agar medium showed three levels of lysis, *i.e.*, weak (+), moderate lysis (++) and high lysis (+++). Different phages specific to *Azotobacter* sp. were detected using enrichment technique and produced plaques (Figure-1). In 1976, Hegazi and Leitgeb studied *Azotobacter* bacteriophages using liquid as well as solid enrichment cultures. They mentioned that liquid enrichments yielded of *Azotobacter* phages in fairly high titers, and phages were found in most of the soils while solid ones only gave low titers in occasional soils.



**Figure -1:** Spot test technique shows the presence of phage specific to a Gram free N<sub>2</sub>-fixer bacteria (# 01 and # 03) isolated from maize-rhizosphere soil in Taif region.

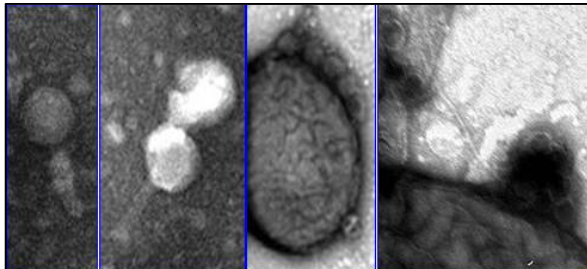
Turbidity test confirmed the phages specificity to the two free N<sub>2</sub>-fixer bacterial isolates (# 01 and # 03), as clear lysis tube was observed

compared to the control (bacterial culture non-inoculated with phage suspension) (Figure-2).

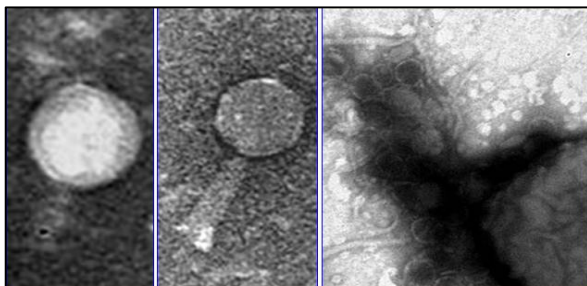


**Figure -2:** Turbidity test used for detection of phage specific to a Gram negative free N<sub>2</sub>-fixer bacteria (# 01 and # 03) isolated from maize-rhizosphere soil in Taif region.

Phage suspensions of the two free N<sub>2</sub>-fixer bacteria (# 01 and # 03) were subjected to electron microscopy, and sperm shape virus-like particles with long tail and icosahedral head were present (Figures -3 and -4). These results in agreement with that found by Hegazi and Leitgeb (1976) who reported that electron micrographs showed that the phages belong to the groups with long non-contractile, contractile, and short contractile tails specific to *A. chroococcum*, *A. vinelandii* and *A. beijerinckii*.

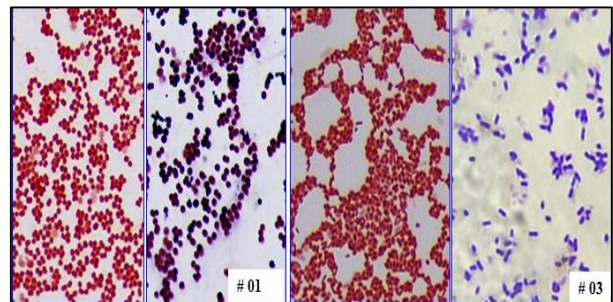


**Figure -3:** Electron micrograph of phage specific to a Gram negative free N<sub>2</sub>-fixer bacterium (# 01) isolated from maize-rhizosphere soil in Taif region.



**Figure -4:** Electron micrograph of phage specific to a Gram negative free N<sub>2</sub>-fixer bacterium (# 03) isolated from maize-rhizosphere soil in Taif region.

The selected bacterial isolates (# 01 and # 03) were morphologically identified as Gram negative bacteria and showed pleomorphic as oval cells (Isolate # 01) or rod shaped with variable size (Isolate # 03) and often in pairs (Figure-5). This was in harmony with that found by Martinez-Toledo *et al.* (1985).



**Figure -5:** Gram negative free N<sub>2</sub>-fixer bacteria (# 01 and # 03) isolated from maize-rhizosphere soil. Note, blunt rods to oval cells, and often in pairs.

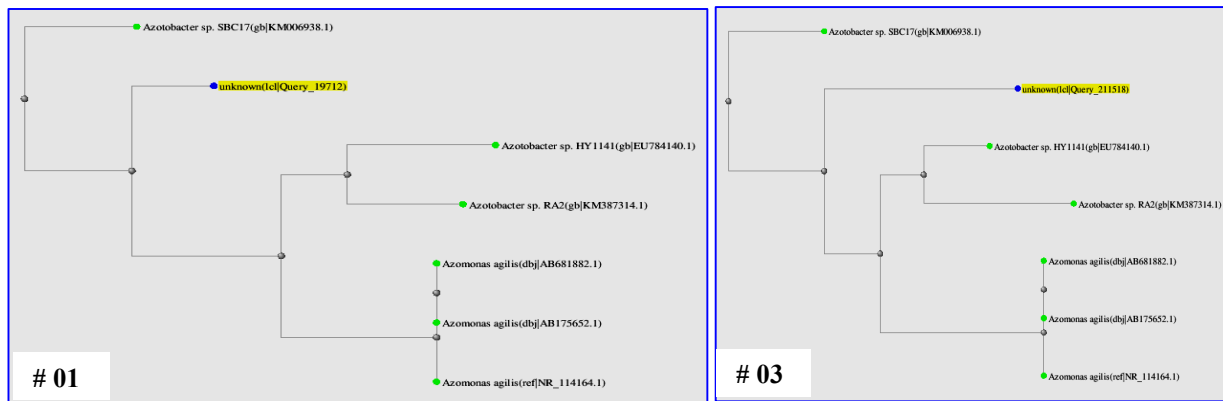
The 16S rRNA gene is used as the standard for classification and identification of microbes, because it is present in most microbes and shows proper changes. Type strains of 16S rRNA gene sequences for most bacteria and archaea are available on public databases such as NCBI (Woese, *et al.*, 1990, Palys, *et al.*, 1997, Kolbert and Persing, 1999, Harmsen and Karch, 2004, Bosshard, *et al.*, 2006, Mignard and Flandrois, 2006, Case, *et al.*, 2007 and Sadik, *et al.*, 2014). The nucleotide sequences of 16S rRNA gene of the two bacterial isolates was determined and final sequences of 927 and 873 nts for the 16S rRNA gene of two isolates of this study (# 01 and # 03), respectively, were recorded in GenBank



(ASNAz1-TKSA: LC054002.1 and ASNAz2-TKSA LC054003.1).

Data of the phylogenetic trees show that the two

bacterial isolates (# 01 and # 03) could be strains of *Azotobacter* sp. (Figure-6).

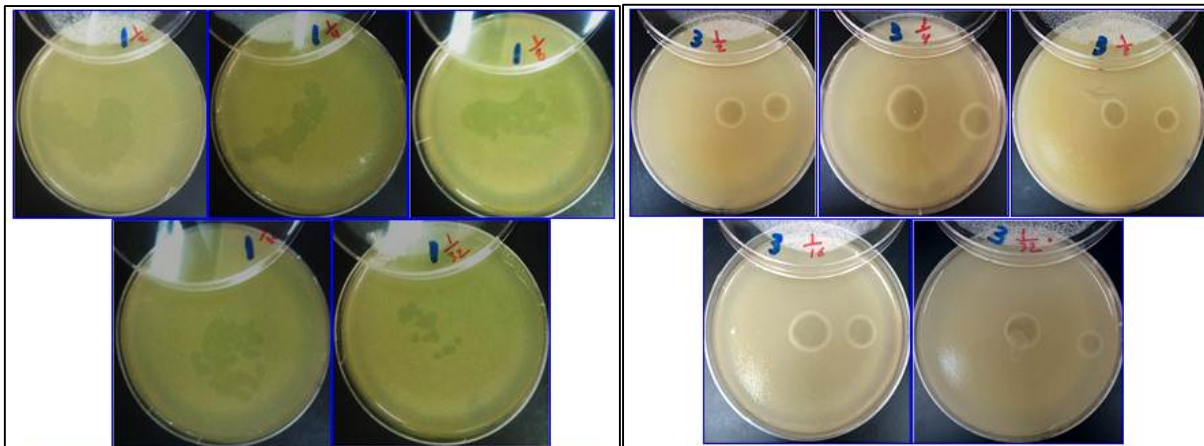


**Figure -6:** Phylogenetic tree shows the taxonomy of partial nucleotide sequence of 16S rRNA gene a Gram negative free N2- fixer bacteria (# 01 and # 03) isolated from maize-rhizosphere soil of Taif region and 6 nitrogen-fixer bacterial strains recorded in GenBank.

Using both of DEP and longevity at 4°C the phage stability was studied. Results proved the presence of phage specific to both of *Azotobacter* sp. strain ASNAz1-TKSA (isolate # 01) and *Azotobacter* sp. strain ASNAz2-TKSA (isolate # 03) in the two fold dilutions, *i.e.*, 1/2, 1/4, 1/8, 1/16 and 1/32 of their enriched phage solutions (Figure-7). The phages of *Azotobacter* sp. strain

ASNAz1-TKSA (isolate # 01) and *Azotobacter* sp. strain ASNAz2-TKSA (isolate # 03) were detected 0, 2, 4, 6, 8, 10, 12, 14 and 16 weeks post storage at 4°C, this was obvious from the clear zones of spot tests for each period. These results are in agreement with that obtained by Marie (2013) and Zain and Al-Othman (2013).

Dilutions	1/2	1/4	1/8	1/16	1/32
Spot test	+	+	+	+	+



**Figure-7:** Dilution end point of phage suspension specific to a Gram negative free N2-fixer bacteria (# 01 and # 03) isolated from maize-rhizosphere soil of Taif region.

**CONCLUSIONS:** Bacteriophage(s) specific to using a number of ten free N2-fixer bacterial isolates maintained on Waksman base No.77 Medium and used as hosts via spot test and confirmed by

turbidity test in the phage suspension. Based on cultural, morphological and molecular properties, the two selected bacterial isolates (# 01 and # 03) were identified as strains of *Azotobacter* sp. and

submitted in GenBank with accession numbers of LC054002.1 and LC054003.1, respectively.

**ACKNOWLEDGMENT:** The author would like to thank Dr. Mohammed S. Al-Harbi, Head of Biology Department, Faculty of Science, Taif University for his continuous encouragements during carrying out this thesis.

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