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# Chromosomal *nif* Genes Transfer by Conjugation in Nitrogen Fixing *Azotobacter chroococcum* to *Lactobacillus plantarium*

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**Abstract:** To determine the possibility of transferring chromosomal nitrogen fixation genes (*nif* genes) from *Azotobacter chroococcum* to *Lactobacillus planetarium*, a total of 72 *Azotobacter chroococcum* isolated from Erbil governorate, Iraq were culturally, morphologically and biochemically characterized. Genes for atmospheric nitrogen fixation, located on the chromosome of *Azotobacter chroococcum* isolates were transferred by conjugation process to a recipient *Lactobacillus plantarium* isolated from Erbil city soils. The chromosomal genes transferred were verified by analysis of the genomes of donor, recipient and putative transconjugants, by polymorphism of DNA bands obtained through amplification of *nif*H1, *nif*H2, *nif*H3, *nif*U and *nif*V genes by PCR technique. The transconjugant cells promote an efficient fixation of nitrogen in liquid cultures fixed 0.2% nitrogen, and in the soil as inoculums of wheat plants, fixed 0.31% nitrogen and solublized 11.71 ppm phosphorus, beside all advantages of Lactic acid bacteria, and probably to be used as inoculums for both nitrogen fixation and solublizing insoluble phosphorus components, and used as biofertilizers

**Key words:** *Azotobacter chroococcum*, conjugation and gene transfer, *Lactobacillus plantarium*, *nif* genes, nitrogen fixation

## INTRODUCTION

Azotobacter is an aerobic, free-living $N_2$-fixing soil bacterium. Studies of the genetics of nitrogen fixation in this genus should contribute to an understanding of their *nif* genes for fixing nitrogen (Doris *et al*., 2008). The nitrogenase are two compounds enzyme complex required 3 genes *nif*H, *nif*D and *nif*K encode respectively, the Fe-protein, and the alpha and beta-subunits of Mo-Fe protein both component of nitrogenase require additional genes products for activity. *nif*M is require to give an active Fe-protein (Raina *et al*., 1993). *nif*E, *nif*N and *nif*B are required for the synthesis or insertion of the Fe and Mo containing cofactor (FeMo-Co). The *nif*V gene product subtly modifies the co-factor, altering the substrate specify of the resulting nitrogenase.

Transfer of chromosomal genes among strains of *A. chroococcum* has been reported by (Rowan, 2003), also conjugation transfer of chromosomal DNA in *Bacillus subtilis* was reported by (Lotareva and Prosorov, 2006). Jones *et al*. (2009) transferred copper resistance genes located on the chromosomal of *A. chroococcum* to *Xanthomonas citri*.

In previous studies of nitrogen fixation in *A. chroococcum* Davis *et al*. (2000), they transferred *nif*V, *nif*S, *nif*M, *nif*A, *nif*N, *nif*B and *nif*Q genes from *Azotobacter chroococcum* to *Klebsiella pneumoniae*, and they observed the expression of these genes in transconjugant cells.

The purpose of this study was to investigate if chromosomal genes transfer by conjugation occurs among *A. chroococcum* and *L. plantarum*, it was speculated that the *nif* genes located on the chromosome and thus might mobilize the chromosome to *L. plantarum* by conjugation could be an important source of nitrogen fixation and solublizing phosphorus in the soil, and evolutionary significance for biofertilizers and plant production.

## MATERIALS AND METHODS

**Bacterial strains:**

**The bacterial strains were *Azotobacter chroococcum* and *Lactobacillus plantarum* were isolated from the soil:** *A. chroococcum* was isolated from the soil of Erbil city, Iraq during 14th December 2008 to 18th February 2009, using N-free Jensen 's medium by cultural, morphological, and biochemical tests (catalase, oxidase, Indol, gelatenase, carbohydrate assimilation, according to (Atlas *et al*., 1995; Johnstone *et al*., 2000; Forbes *et al*., 2002).

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1 cagacacgaa gaagccgggc ccgtgacatg cccgccatgg actgctgctc cgtcgccgca
61 cgccacttcc tgcaccagcc ggcatgaacc ccggtaccac atgggaacgg atcgccgcgg
121 cgttactacc ggtacgccgc cagcccggga cgacgcagat cgctgccgtc cgactcccga
181 cacatgccat atgcagcatg aaatatcgct gaaaacatat tactggtttt tttatccaaa
241 aaacaaacaa catatgaaat tcacatcttg atggcaccac ccttgctcca tcccctgcga
301 caccagtcaa acgccacgaa tcaatggagg ttccaagatg gcattgcgtc agtgtgcaat
361 ttacggcaag ggtggtatcg gcaagtccac caccacccag aacctggtcg ccgcgctcgc
421 cgaggccggc aagaaggtga tgatcgtcgg ttgcgacccg aaagccgact ccacccgcct
481 gatcctgcat tccaaggccc agaacaccgt catggagatg gccgcatccg ccggctcggg
541 tgaagacctc gagctggaag acgtgctgca gatcggctac ggcggcgtca agtgcgtcga
601 gtccggcggc cctgagccgg gcgtcggctg cgcgggccgt ggcgtgatca ccgcgatcaa
661 cttcctggaa gaggaaggcg cctacagcga cgacctggac ttcgtgttct acgacgtgct
721 gggcgacgtg gtgtgcggcg gcttcgccat gccgatccgc gagaacaagg cccaggaaat
781 ctacatcgtc tgctccggcg agatgatggc catgtacgcc gccaacaaca tcgccaaggg
841 catcgtgaag tacgcccact ccggcagcgt gcgtctgggc gggctgatct gcaacagccg
901 caagaccgac cgcgaagacg agctgatcat ggccctggcc gcgaagatcg gcacccagat
961 gatccacttt gtgccgcgcg acaacgtcgt gcagcacgcc gaaatccgcc gcatgaccgt
1021 gatcgaatac gatccgaaag ccaagcaggc cgacgagtac cgtgccctgg cccagaagat
1081 cctcaacaac aagctgctgg tc atcccgaa cccggcgagc atggaggacc tcgaagagct
1141 gctgatggag ttcggcatca tggaagccga agacgagtcc atcgtcggca aggccggcgc
1201 cgagggctga tcccgccggc gcagtgtttg cgg aggagcg tgcgtcgcgg gctgtccgga
1261 atggcttctc gcggccggca cgccgccctc ccttttgaat cgccccgaat tctccaacct
1321 caggagctga ccctatggcc atggccatcg acggctacga atgcaccgtc tgcggcgact
1381 gcaagccggt ctgcccgacc ggctcgatcg tcctccaggg cggtatctac gtgatcgacg
1441 ccgacagctg caacgagtgc gccgacctgg gcgagccacg ctgtctcggc gtctgccccg
1501 tggacttctg catccagccg ctcgatgact gaagactgaa cgagccgcttgccg
1561 gcgacagagc atcccgccgc tctgccaccg gaccaccaaa cggcgatcgc tttcctcagg
1621 tcgccgtttt ttctctcctg accgcacctt

**Primers:**

*nif*H1-F-( cagacacgaagaagccgggc) (20)
*nif*H1-R-(gaccagcagcttgttgttga)(20)
*nif*H2-F-(cgccggcgcagtgtttgcgg)(20)
*nif*H2-R-(cactcgttgcagctgtcggc)(20)
*nif*H3-F-(cgatgactgaagactgaacgag)(22)
*nif*H3-R-(aaggtgcggtcaggagagaa)(20)

**Analysis of predicted*nif*H1, *nif*H2, and*nif*H3 genes products:**

| Sequence | gene | No.of amino acids |
|---|---|---|
| 1-1102 | nifH1 (1102)bp | 367 |
| 1213-1459 | *nif*H2 (246)bp | 82 |
| 1522-1550 | *nif*H3 (128)bp | 42 |

Fig. 1: Sequence of the chromosomal region contains *nif*H1 gene,*nif*H2 gene, and *nif*H3 gene in *A. chroococcum*. Sequence with red are sites for primers *nif*H1-F and *nif*H1-R, yellow are sites for primers *nif*H2-F and *nif*H2-R, and bright green are sites for primers *nif*H3-F and *nif*H3-R for amplification

*L. plantarum* was isolated from rhizospheres of fruit trees and wheat plants in Erbil city using Man-Rogosa-Sharpe agar (MRS agar) supplemented with 1% $CaCO_3$ according to (Chen *et al*., 2006), and accumulation method was used according to (Coolborn, 2005). According to cultural, morphological and biochemical tests (catalase, gelatenase, acid and gas production from glucose, production of ammonia from arginin, oxidase test, growth at 4, 15 and 45ºC, growth on MRS agar with 6.5 and 8% NaCl, milk curdle and carbohydrate fermentation test were conducted in MRS broth with 0.004% chlorophenol red but without sucrose and beef extract, and containing 1% from (glucose, manitol, maltose, rhamnose, xylose, ribose, melezitose, trehalose, collbiose, raffinose, arabinose, mannose, salicilin and starch) identification was performed according to combination criteria proposed by (Garive, 1986, Holt *et al*., 1994, Stevanova *et al*., 2002; Axelsson, 2004).

**Preservation of bacterial isolates:** The bacterial isolates were preservated at -20ºC after suspension in 20% (V/V) glycerol (Delves *et al*., 1996).

3721 ACCCGCTTCA TCAGCAGCAC TGGCAACGCC GGTCGCCGGT TTGCGCATCC GGTTGAGGGT
3781 GGCGGCTGCT CCGGTCTGAA ATACAGCCTG AAGCTGGAGG AGGCCGGTGC CGAAGGCGAT
3841 CAGCTGATCG ACTGCGACGG CATCACCCTG CTGGTCGACG ATGCCAGCGC CCCTCTGCTC
3901 AATGGCGTGA CCATGGACTT TGTGGAAAGC ATGGAAGGTA GCGGTTTCAC CTTCGTCAAT
3961 CCGAATGCCA GCAACAGCTG TGGTTGTGGC AAGTCTTTTG CTTGCTGATT AGGCAACCCT
4021 GAGGTCGCCG GCTGGGGCCC CAAAGACTCA CTGGGAGATG AAGCCGACAT GTGGGATTAT
4081 TCGGAAAAAG TCAAAGAGCA TTTTTACAAC CCCAAGAATG CCGGAGCCGT GGAAGGCGCC
4141 AACGCCATCG GCGACGTCGG ATCGCTGAGC TGCGGTGACC GGCTGCGTCT GACCCTGAAG
4201 GTGGATCCGG AAACCGACGT GATCCTGGAT GCCGGCTTCC AGACCTTCGG CTGTGGCTCC
4261 GCCATCGCTT CCTCCTCGGC ACTGACCGAG ATGGTCAAGG GGGCTGACCT GGACGATGCG
4321 CTGAAGATCA GCAACAGGGA CATCGCCCAT TTCCTCGGAC GGCTGCCGCG GGAGAAGATG
4381 CACTGCTCGG TGATGGGCCG CGAACGCCTG CAGGCCGCCG TGGCCAACTA CCGTG6CGAA
4441 GAGCTCAGGA CCGACCACGA GAAGCCGCAG CTGATCTGCA AGTCGTTCGC CATCGACGAA
4501 GTGATGGTCC GCGACACCAT TCGCGCCAAC AAGCTGTCCA CCGTCGAGGA CGTGACCAAA
4561 CACACCAAGC GCGGCGGCGG CTGCTCGGCC TGTCATGAGG GCATCGAGCG CGTGCTGAGC
4621 GAGGAGCTGG CGCCCGTGGC GAGGTCTTCG TCGTGCGCCG ACCAAGGCCA AGAAGAGGTC
4681 AAGGTGCTCG CCCCGAGCCG GCTCCGCTCG TGGCCGAGGA GACTCCGCGC CACGCCGAAG
4741 CTGAGCAACC TGCAGCGCAT CCGCCGCATC GAAACCGTGC TGGCGGCGAT CCGTCCGACC
4801 CTGCAGCGCG ACAAGGGTGA TGTCGAGCTG ATCGATGTCG ACGGCAAGAA CATCTACGTC
4861 AAGCTAACCG GCGCCTGCAC CGGCTGCCAG ATGGCCTCCA TGACCCTTGG CGGCATCCAG
4921 CAGCGCCTGA TCGAGGAACT CGGCGAATTC GTCAAGGTGA TCCCGGTCAG CGCCGGCCCA
4981 CGGCAGATGG AGGTCTGACA TGGCTGACGT CTATCTCGAC AACAACGCCA CCACCCGGGT
5041 GGACGACGAA ATCGTCGAGG CCATGCTGCC GTTCTTCACC GABCAGTTCG GCAACCCCTC
5101 GTCGCTGCAC AGCTTCGGCA ACCAGGTCGG CCTGGCGCTG AAGAGGGCGC GGCAGCAGCG
5161 TGCAGGCGTG CTCGGCGAGC ATGATTCGGA AATCATCTTC ACTTCCTGCG GCACCGAGTC
5221 GGATCACGCG ATCCTCTCGG CGCTCAGCCC AGCCCGAGCG CAAGACCTGA TCACCACCGT
5281 GGTCGAGCAC CCGGCAGTGC TGAGCCTGTG CGACTACCTC GCCAGCGAGG GCTACACCGT
5341 GCACAAGCTG CCGGTGGACA AGAAGGGCCG CCTGBATCTG GACCATTACG CCAGCCTGCT
5401 GAACGACGAC GTCGCCGTGG TGTCGGTGAT GTGGGCCAAC AACGAGACCG GCACCCTGTT
5461 CCCGGTCGAG GAAATGGCAC GCATGBCCGA CGAGGCCGGC ATCATGTTCC ACACCGACGC
5521 CGTGCAGGCC GTGCGCAAGC TGCCGATCGA CCTGAAGAAC TCGTCGATCC ACATGCTCTC
5581 GCTGTCGGGC CACAAGCTGC ATCGCAAGGG CGTCGGCGTG CTCTACCTGC GCCGCGGCAC
5641 CCGCTTCCGT CGCTGCTGCC GCGGCCACCA GGAGCGGCCC GCGGGCGGTA CCGAGAACGC
5701 TGCCTCGATC ATCGCCATGG GCTGGGCCGC CGAGCGCGCG CTGGCCTTCA TGGAGCACGA
5761 GAACACCGAG GTCAAGCGCC TGCGCGACAA GCTGGAGGCC GGCATCCTCG CCGTCGTGCC
5821 GCACGCCTTC GTCACCGGCG ACCCGGACAA CCGCCTGCCC AACACCGCCA ACATCGCGTT
5881 CGAGTACATC GAGGGCGAGG CCATCCTGCT GCTGCTGAAC AAGGTCGGCA TCGCCGCCTC
5941 CAGCGGCTCG GCCTGCACCT CCGGCTCCCT GGAGCCCTCC CACGTGATGC GCGCCATGGA
6001 CATTCCCTAC ACCGCCGCCC ACGGCACCGT GCGTTTCTCC CTGTCGCGCT ACACCACCGA
6061 GGAGGAGATC GACCGGGTGA TCCGCGAGGT GCCGCCGATT GTGGCCCAGC TGCGCAACGT
6121 GTCGCCCTAC TGGAGCGGCA ACGGTCCGGT GGAACATCCG GGCAAGGCCT TCGCGCCGGT
6181 CTACGGCTGA GCCGCCGCCT GCGGGAGCGC ATCCCGCAGG AAACCGCCTC GGGGAGCCCC
6241 GCCCGAGTTG TTGGAGAAAG CCATGGCTAG CGTGATCATC GACGACACCA CCCTGCGTGA
6301 CGGCGAGCAG AGTGCCGGGG TCGCCTTCAA TGCCGACGAG AAGATCGCCA TCCGGCGTGC
6361 GCTCGCCGAG CTGGGCGTAC CGGAGCTGGA GATCGGCATT CCCAGCATGG GCGAGGAGGA
6421 GCGCGAGGTG ATGCGCGCCA TTGCCGGCCT CGGCCTGTCG TCGCGCCTGC TGGCCTGGTG
6481 CCGGCTGTGC GACTTCGACC TCTCGGCCGC GCGCTCCACC GGGGTGACCA TGGTCGACCT
6541 GTCACTGCCG ATCTCCGACC TGATGCTGCG CCACAAGCTC AATCGTGATC GCGACTGGGC
6601 ACTGGGCGAG 6TCGCCCGGC TGGTCAGCGA GGCGCGCATG GCCGGGCTTG AGGTGTGCCT
6661 GGGCTGCGAG GACGCCTCGC GGGCGGATCA GGACTTCATC GTGCGGGTGG GGGCGGTGOC
6721 GCAGGCCGCG CGCCCGCCGC CTGCGTTCGC CGATACCGTC GGGGTGATGG AGCCGTTCGG
6781 CATGCTCGAC CGCTTCCGTT TCCTCCGCCA GCGCCTGGAC GTGGAGCTGG AGGTGCACGC
6841 CCACGACGAC TTCGGGCTGG CCACCGCCAA CACCCTGGCG GCGGTGATGG GCGGGGCGAC
6901 CCACATCAAT ACCACGGTCA ACGGGCTCGG CGAGCGCGCC GCCAACGCCG CGCTGGAAGA
6961 GTGCGTGCTG GCGCTCAAGA ACCTCCACGG CATCGACACC GGCATCGACA CCCGCGGCAT
7021 CCCGGCCATC TCGGCGCTGG TCGAGCGGGC CTCGGGGCGT CAGTGGCCTG GCAGAAGAGC
7081 GTGGTTGGCG CCGGTGTTCA CCCACGAGGC CGGCATCCAC GTCGACGGGC TGCTCAAGCA
7141 CCGGCGCAAC TACGAGGGAC TGAATCCCGA CGAGCTCGGC CGCAGCCACA GCCTGGTGCT
7201 GGGCAAGCAT TCCGGCGCGC ACATGGTGCG CAACAGCTAC CGCGAGCTGG GCATCGAGCT
7261 GGCCGACTGG CAGAGCCAGG CACTGCTCGG CCGCATCCGC GCCTTCTCCA CCCGCACCAA
7321 GCGCAGCCCG CAGGCTGCCG AGCTGGAGGA CTTCTATCGC CAGCTGTGCG AGCAGGGGAC

7381 TGCCGAACTG GCGGCAGGAG GAATGGCATG AGCCTGCTTG CGCAATGGCG TGAAGACATC
7441 CGCTGCGTGT TCGAGCGCGA TCCGGCGGCA CGCACCACCT TCGAGGTGCT GACCACCTAT
7501 CCGGGCTGCA CGCGATCATG CTCTACCGGC TCGC4CCATC GTCTGTGGCG GCCGAATGCG
7561 TTACCTCGCC CGGCTGCTGT CGTTCGCGCG CGCCTGGTGA GCAACGTCGA CATCCATCCC
7621 GGGGCCGTCA TCGGTGCGCG CTTCTTCATC GACCACGGCG CCTGCGTGGT GATCGGCGAG
7681 ACCGCCGAGA TCGGCCGGGA CGTGACTCTC TACCACGGCG TCACCCTGGG CGGCACCACT

**Primers:**

1-nifU-F-(ATGTGGGATTATTCGGAAAAA) (21)
2-nifU-R-(TCAGACCTCCATCTGCCGTGGG) (22)
3-nifV-F-(CATGGCTAGCGTGATCATCGACGA) (24)
4-nifV-R-GCCATTCCTCCTGCCGCCAGTTCG) (24)

**Analysis of predicted*nif*U and*nif*V genes products:**

| Sequence | gene | No.of aminoa cids |
|---|---|---|
| 4068-4998 | *nif*U (930)bp | 310 |
| 6261-7407 | *nif*V (1146)bp | 382 |
| | nifH3 (128)bp | |

Fig. 2: Sequence of the chromosomal region contains *nif*U gene and *nif*V gene in *A. chroococcum*. Sequence with pink are sites for primers *nif*U-F and *nif*U-R, blue are sites for primers *nif*V-F and *nif*V-R for amplification

**Antibiotic resistance test:** Antibiotics were used at the following concentrations (µg/mL) Ampicillin (50), Rifampicin (5), Cefotaxim (30), Erythromcin (10), Trimethoprim (20), Strptomycin (25), Chloramphenicol (30) and Tetracycllin (15). *A. chroococcum* isolates were grown under air at 30ºC on Jensen's medium and MRS medium for *L. plantarum* with antibiotics added when required.

**Isolation of genomic:** Genomic DNA was extracted and purified from bacterial cells using the QIAamp DNA Mini Kit (Jayashree *et al*., 2007).

**PCR amplification of nitrogenase genes:** The *nif*H1, *nif*H2, *nif*H3, *nif*V, and *nif*U genes sequence were obtained from NCBI site, and were designed in OPERON diagnostic ltd, Germany. The primer length and melting temperature were designed with coordination between forward and reverse primers. The melting and annealing temperature were calculated following (Womble, 2000). Primers Amplification was completed using the protocol and reagents followed of (Rajeswari and Kasthuri, 2009). The programmed temperature sequence was 96ºC followed by 55ºC for one minute, and 72ºC for 1 min, the temperature sequence was run for 30 cycles, the final product extension was conducted at 72ºC for 6 min followed by 4ºC temperature hold.

**Agarose gel electrophoresis:** Agarose gel electrophoresis protocol followed (Helmut *et al*., 2004).

**Determination of nitrogen:** Total nitrogen was determined in the soil and broth cultures according to (Saribay, 2003).

**Determination of phosphorus:** A solublized Phosphorus was determined in the soil followed (Ryan *et al*., 2003).

## RESULTS

A total of 27Azotobacter were isolated from the soil samples from Erbil city, Iraq. Approximately all of the isolates 100% obtained in this study were very closely related to well study *A. chroococcum*. Conjugation at frequency of $7.2x10^{-5}$ of transconjugant. The transconjugant colonies were subjected to microscopic examination, cultural characteristics and biochemical test, the results showed that these colonies were rod shapes, occurred singly or in chain, gram positive, non spore former, non motile, without capsule, small colonies with insoluble brown pigmentation grow poorly in air but better under reduced oxygen tension, curled milk, negative to oxidase test, positive to catalase test, microaerophilic, did not lequdify gelatine, grow at 4 and 15ºC but not at 45ºC, did not produce gas from glucose, did not grow on nutrient agar, with 18% NaCl, without producing ammonia from arginine, and they utilized glucose, manitol, maltose, lactose, melebiose, ruffinose, arabinose, ribose, trehalose. Cellobiose, sorbitol, mannose, salicin, starch, but they differed in their ability of xylose, inositole and melezitose fermentation, while they were not utilized rhaminose, resistant to ampicillin, rifampicin and erythromycin. Complementation was considered when growth was comparable to that of original bacteria *A. chroococcum*, a *nif* strain of lactic acid bacteria. The mentioned results confirmed that the putative transconjugant colonies were derived from the recipient *Lactobacillus plantarum* isolates. The complementation was also determined by measuring

Table 1: Percent total nitrogen in plant, soil, total phosphorus in the plant and available phosphorus in the soil inoculated with transconjugant cells.

| Treatments | Total nitrogen in the plant (%) | Total phosphorus in the plant (%) | Total nitrogen in the soil (%) | Available phosphorus in the soil (ppm) |
|---|---|---|---|---|
| Control 1 | 2.01 | 0.18 | 0.11 | 2.08 |
| Control 2 | 2.00 | 0.18 | 0.11 | 2.08 |
| *L. plantarum* | 2.01 | 0.28 | 0.10 | 7.80 |
| *A. chroococcum* | 4.10 | 0.32 | 0.24 | 5.3 |
| Transconjugant *L. plantarum* | 6.94 | 0.59 | 0.31 | 11.71 |
| Transconjugant *L.plantarum*+*A. chroococcum* | 8.50 | 0.62 | 0.41 | 12.81 |

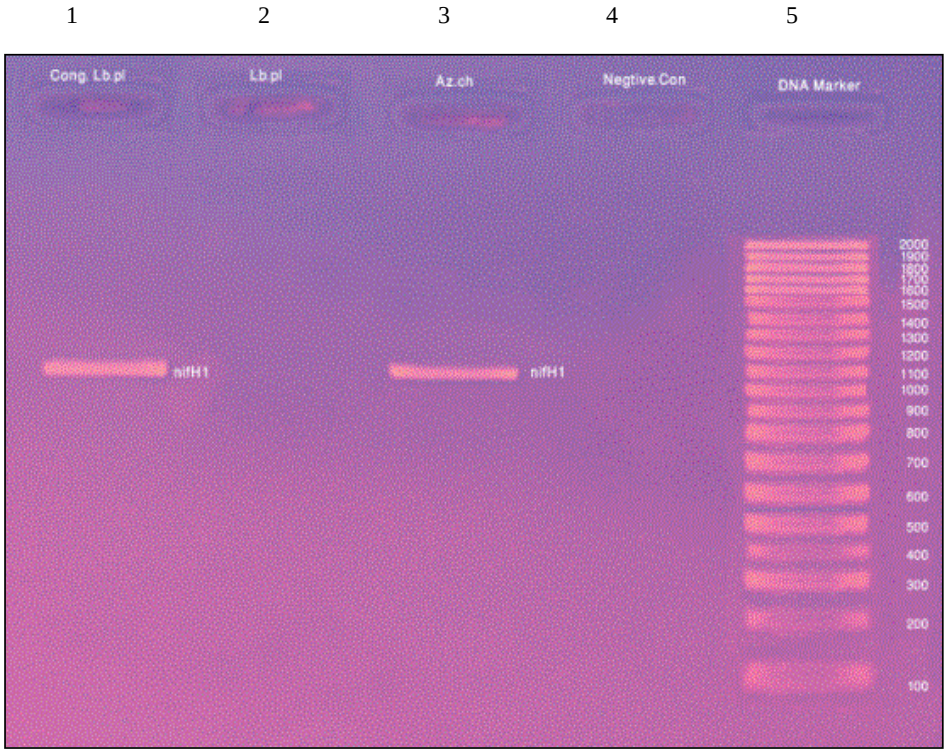


Fig. 3: Agaros gel electrophoresis showing the PCR amplified products of the*nif*H1 gene (1102bp). Lane 5: DNA marker 2kb, lane 4: negative control (-ve PCR product), lane 3: *A. chroococcum* (+ve PCR product), lane 2: *L. plantarum* (-ve PCR product), lane 1: transconjugant *L. plantarum* (+ve PCR product)

when the transconjugant colonies grow well on N-free Jensen's agar medium, and showed high efficiency in $N_2$-fixation when grow in N-free Jensen's broth fixed 0.2% nitrogen after 20 days of incubation, followed the usual pattern as *A. chroococcum* and same $N_2$-fixing genes were detected by PCR amplification technique same size of DNA fragments were detected (Fig. 3, 4, 5, 6, 7 and 8). These results suggested that all of these transconjugant are utilized nitrogenase, the $N_2$-fixation are consistent with genetic characterization of these bacteria and grow on N- free medium (it is possible that these cells were used dinitrogen when grow under N-deficient condition). More over the transconjugant cells were applied to the soil cultivated with wheat plants in pot experiment as a biofertilizer, the results are cleared in Table 1, and appeared that total nitrogen in the plants, total nitrogen in the soil, total phosphorus in the plants and available phosphorus in the soil of inoculated treatments with transconjugant cells were higher than the control, or treatments were inoculated with *A. chroococcum* or *L. plantarum* only, on the other hand treatment with mixture of transconjugant, *A. chroococcum* and *L. plantarum* the studied data were higher than other treatments.

## DISCUSSION

*A. chroococcum* resisted ampicillin and erythromycin and was sensitive to rifampicin, and lactic acid bacteria was sensitive to ampicillin and erythromycine and resisted rifampicin. Transconjugant colonies obtained on MRS agar plate containing erythromycin, ampicillin and rifampicin, success of obtaining transconjugant colonies maybe due to the presence of one of conjugation requirements of the donor cells (mob gene, bom sequence and formation of conjugation bridge) (Snyder and

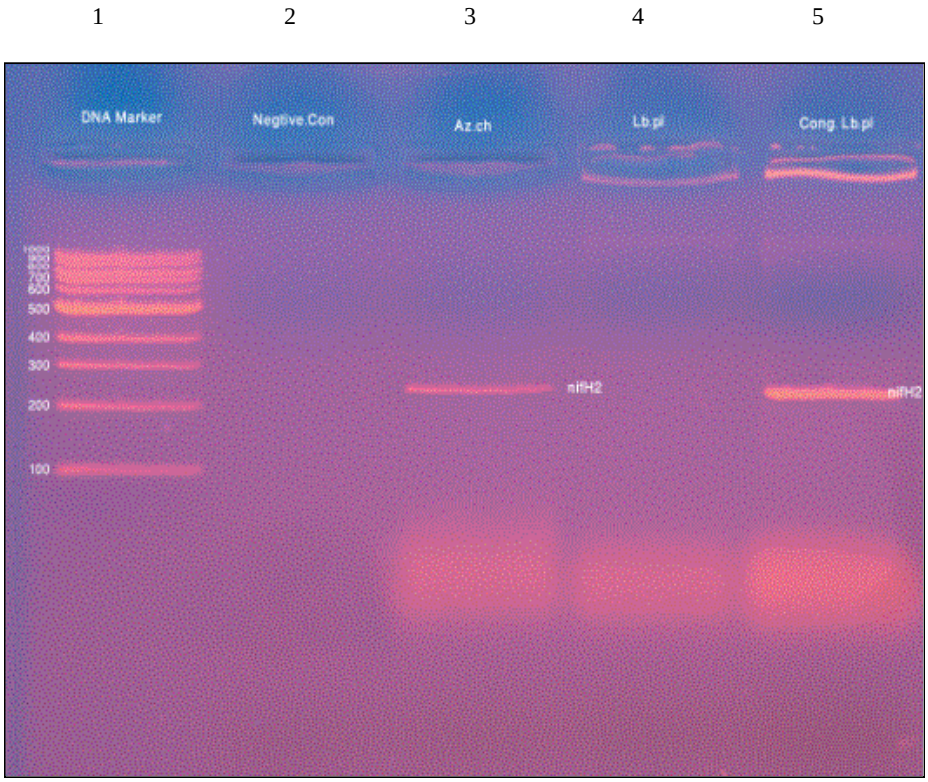


Fig. 4: Agaros gel electrophoresis showing the PCR amplified products of the *nif*H2 gene (246bp). Lane 1: DNA marker 1kb, lane 2: negative control (-ve PCR product), lane 3: *A. chroococcum* (+ve PCR product), lane 4: *L. plantarum* (-ve PCR product), lane 5: transconjugant *L. plantarum* (+ ve PCR product)

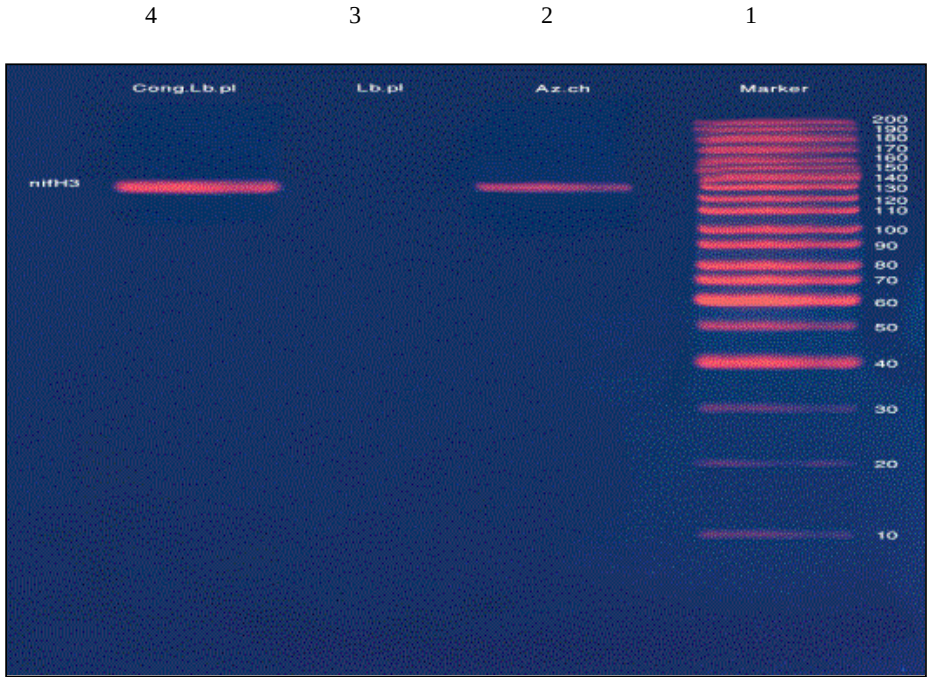


Fig. 5: Agaros gel electrophoresis showing the PCR amplified products of the *nif*H3 gene (128bp). Lane 1: DNA marker 200bp, lane 2: *Azotobacter chroococcum* (+ve PCR product), lane 3: *Lactobacillus plantarum* (-ve PCR product), lane 4: transconjugant *Lactobacillus plantarum* (+ve PCR product)

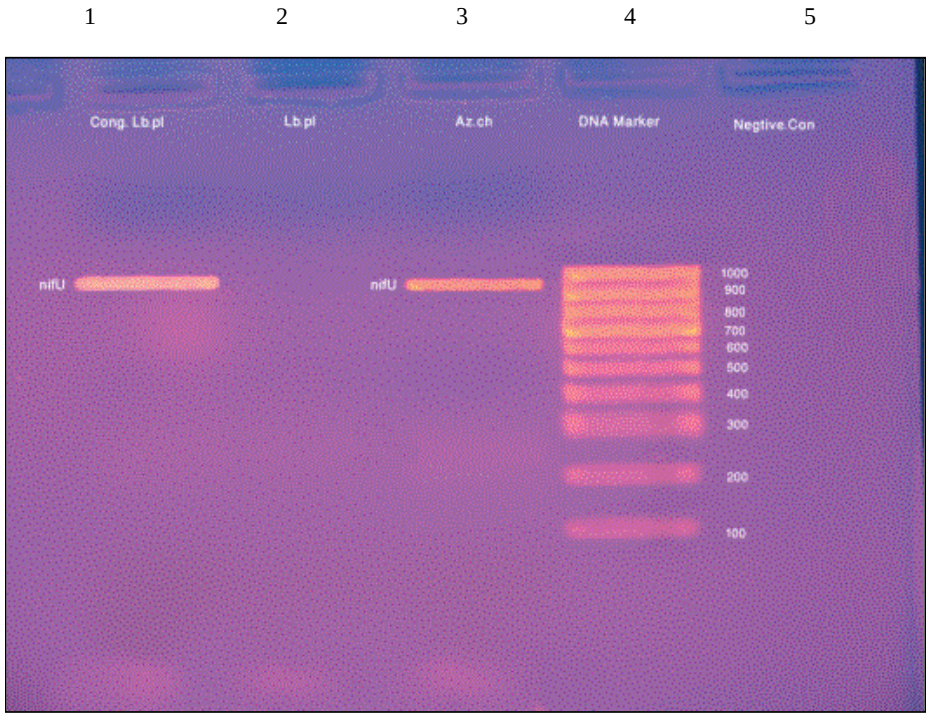


Fig. 6: Agaros gel electrophoresis showing the PCR amplified products of the *nif*U gene (930bp). Lane 1: negative control (-ve PCR product), lane 2: DNA marker 1kb, lane 3: *Azotobacter chroococcum* (+ve PCR product), lane 4: *Lactobacillus plantarum* (-ve PCR product), lane 5: transconjugant *Lactobacillus plantarum* (+ve PCR product)

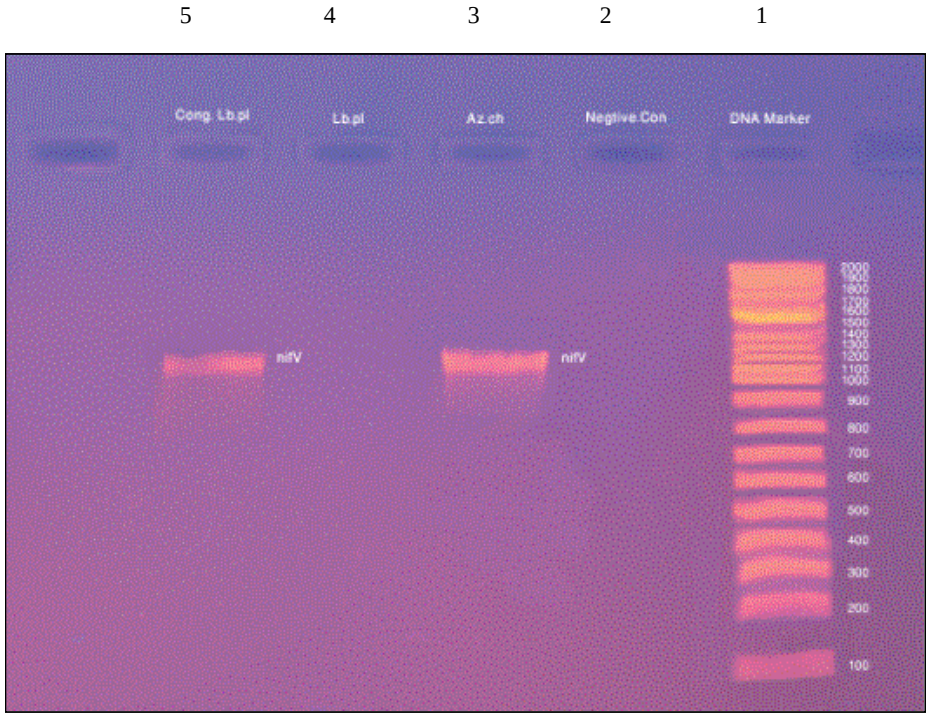


Fig. 7: Agaros gel electrophoresis showing the PCR amplified products of the *nif*V gene (1146bp). Lane 1: DNA Marker 2kb, lane 2: negative control (-ve PCR product), lane 3: *Azotobacter chroococcum* (+ve PCR product), lane 4: *Lactobacillus plantarum* (-ve PCR product), lane 5: transconjugant *Lactobacillus plantarum* (+ve PCR product)

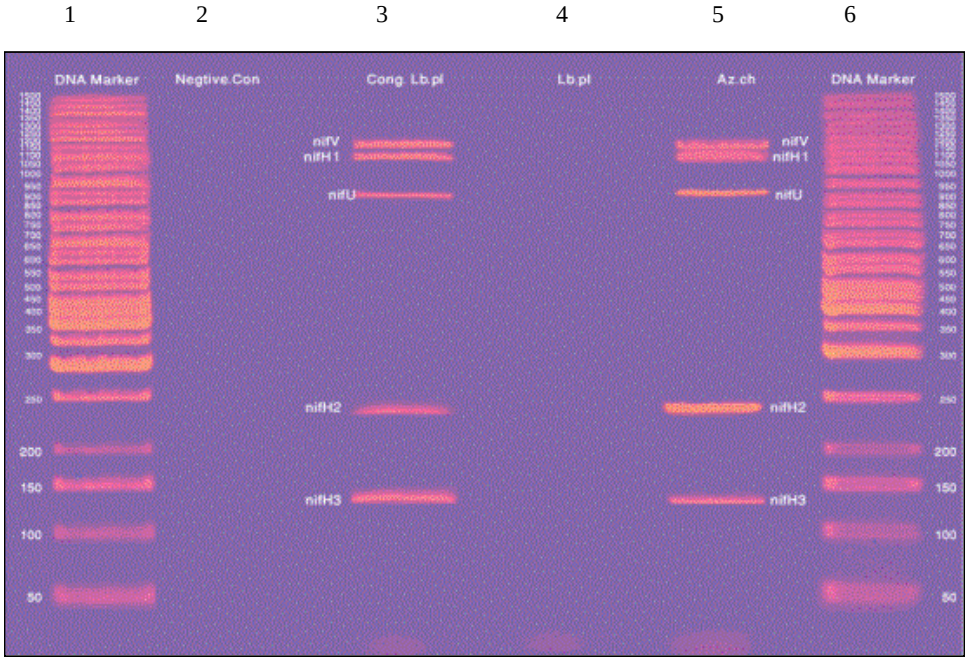


Fig. 8: Agaros gel electrophoresis showing the PCR amplified products of the *nif*H1, *nif*H2, *nif*H3, *nif*U, and *nif*V genes (1146bp). Lane 1,6: DNA Marker 1500bp, Lane 2: negative control (-ve PCR product), Lane 3: transconjugant *Lactobacillus plantarum* (+ve PCR product), Lane 4: *Lactobacillus plantarum* (-ve PCR product), Lane 5: *Azotobacter chroococcum* (+ve PCR product)

Champness, 1997). Chromosomal mobilization of *nif* markers has been previously reported by (Blanco *et al*., 1991 and Davis *et al*., 2000), and transfer of chromosomal DNA among Azotobacter (Rowan, 2003), or in *Bacillus subtilis* (Lotareva and Prosovov, 2006) or copper resistance genes located on the chromosomal of *A. chroococcum* to xanthomonase (Jones *et al*., 2009). Transconjugant shows resistant to ampicillin and erythromycin may be due to the transfer of antibiotic resistance genes which located on R-plasmid (Stall *et al*., 1999) for donor *A. chroococcum*, on the other hand the transconjugant were grown on N-free Jensen's agar medium, and able to fix atmospheric nitrogen, and produced brown pigment and catalase enzyme similar to the donor cells, this may be due to transferring of *nif* genes, pigmentation genes, and catalase genes, which were chromosomally located (Evans *et al*., 2005), moreover to verify the transfer of N2-fixation genes *nif* genes (*nif*H, *nif*U, *nif*V, *nif*K, *nif*D, *nif*M, *nif*A, *nif*N, *nif*B, *nif*Q, *nif*Z, *nif*T, *nif*P, *nif*F, *nif*S, *nif*W, *nif*L and *nif*Y) from *A. chroococcum* (the donor) to nun-nitrogen fixed *L. plantarum* (the recipient cells) by conjugation of *nif*H, *nif*U and *nif*V were selected. *nif*H genes (nifH1, *nif*H2 and *nif*H3) which encoded for biosynthesis of Fe-protein (component 2) and β-subunits of MoFe-protein (component 1) were clustered in region of *A. chroococcum* chromosome spanning about 1650bp. The *nif*H1 gene was 1102bp, while *nif*H2 gene was 2461bp; these two genes were separated by flank of DNA of 111bp. The *nif* H3 gene 128bp was separated from *nif* H2 gene by 63bp.The genes organization and their sequence are show in Fig. 1.

The *nif*U and *nif*V gene is clustered in a region of *A. chroococcum* genome spanning about 3339bp (Fig. 2). The gene *nif*U which involved in maturation of Fe for Fe-S cluster synthesis and repair was 930bp long, while *nif*V gene, which involved maturation of FeMo-complex, was 1146bp, these two genes were separated by 1263bp, and *nif*S is located between them. The region of chromosome which contain *nif*K, *nif*D, *nif*M, *nif*A, *nif*N, *nif*B, *nif*Q, *nif*Z, *nif*P, *nif*F, *nif*W, *nif*B, *nif*L, and *nif*Y genes are located between the fragment of chromosome which contain*nif*H1 ,*nif*H2, *nif*H3, and the fragment contain *nif*V, *nif*S, and *nif*U. Because important of region of *nif*H1, *nif*H2 and *nif*H3, *nif*V and *nif*U genes on chromosome they were selected for this study, detected by PCR technique in each of *A. chroococcum* and *Lactobacillu plantarum* of transconjugant cells (Fig. 3), which indicated that *A. chroococcum* and transconjugant cells contain *nif* H1-F and *nif*H1-R primers show positive PCR product on the gel lane 3 and lane 1, with 1102bp, while *L. plantarum* show a negative control on PCR product lane 2. Figure 4 and 5 show positive amplification PCR product of *nif*H2 (246bp), *nif*H3 (128bp) using *nif*H2-F plus *nif*H2-R and *nif* H3-F plus *nif* H3-R primers respectively. Figure 6 and 7 show positive amplification PCR product of *nif*U (930bp) and *nif*V (1146bp).Clear bands (Fig. 8) of *nif*H1, *nif*H2, *nif*H3, *nif*V and *nif*U

which present on chromosome of diazotrophic *A. chroococcum*, were transferred to nun nitrogen fixer *L. plantarum* by conjugation. The conjugant cells were tested for expression ability of the foreign genes (*nif* genes) and examine variation in expression associated with different cells in vitro, in order to confirm all genes expression (Hogg, 2005). The results revealed that a transconjugant *L. plantarum* show high efficiency in $N_2$-fixation, and fixed 2950 ppm of $10^5$ cell/mL during 30 days of incubation at 28ºC, same amount as *A. chroococcum* at same conditions. These results was incontrast with (Davis *et al*., 2000) who transfer*nif*V, *nif*S, *nif*M, *nif*A, *nif*N, *nif*B, *nif*Q genes from *A. chroococcum* to *Klebsilla pneumonia* with expression of these genes in transconjugant.

The stimulating effect observed in this study due to inoculation with selected effective microorganisms on wheat plants and microbial communities and activity in the rhizospher can be explained by the capacity of such microorganisms to produce growth promoting substances, phosphorus solubilization and $N_2$-fixation which improve the plant growth (Kumar *et al*., 2001; Milani and Anthofer, 2008). Previous investigations revealed that inoculation with *A. chroococcum* enhanced wheat growth promoting ability of biofertilizer agents isolated from rhizospher has been reported (Milani and Anthofer, 2008; El-Shanshoury, 2008; Abd-El-Gawad and El-Sayed, 2009; Abd El-Ghany *et al*., 2010).

Transconjugants which promotes an efficient fixation of nitrogen in the liquid cultures and in the soil beside all advantages of lactic acid bacteria could probably be used to introduce $N_2$-fixation and used as inoculums for both $N_2$fixation and solublizing unsolublizing phosphorus components or organic phosphorus in the soil. Finally we can concluded that transconjugant bacteria are useful to be used as biofertilizer for improving the nitrogen and phosphorus in the agriculture farms.

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## REFERENCES

Abd-El-Gawad, A.M. and Z.T. El-Sayed, 2009. Evaluation the response of wheat to bio-organic agriculture under Siwa Oasis conditions. Ind. J. Agric. Sci., 49(6): 34-43.

Abd-El-Ghany, B.F., R.A. Arafa, T.A. El-Rahmany and M.M. El-Shazly, 2010. Effect of some soil microorganisms on soil properties and wheat production under north Sinai conditions. J. Appl. Sci. Res., 4(5): 559-579.

Atlas, R.M., L.C. Parks and A.E. Brown, 1995. Laboratory Manual of Experimental Microbiology. Mosby-Year Book, Inc., USA.

Axelsson, L.T., 2004. Lactic Acid Bacteria: Classification and Physiology. In: Salminen, S., A. VonWright and A. Ouwehand (Eds.), Lactic Acid Bacteria: Microbiology and Functional Aspects. Marcel Dekker Inc., NewYork.

Blanco, G., F. Ramos, J.R. Medina, J.C. Gutierrez and M. Torolero, 1991. Conjugal retrotransfer of chromosomal markers in *Azotobacter* spp. Curr. Microbiol., 22: 241-246.

Chen, Y.S., F. Yanagida and T. Shinohara, 2006. Searching for bacteriocin-producing lactic acid bacteria in soil. J. Gen. Appl. Microbiol., 52: 21-28.

Coolborn, A.F., 2005. Antibacterial Quantification from Lactic acid bacteria isolated from food sources and soil. J. Food Technol., 3(4): 568-571.

Davis, N., L. Cannon, A. Wilson and T. Williams, 2000. Expression of *Azotobacter* nitrogen fixation genes (*nif*) in *Klebsiella*. J. Gen. Microbiol., 105(4): 321-330.

Delves, B., P. Black-burn, R.J. Evans and Z. Hugenhott, 1996. Application of bacteria. Anatonic Van Leeuwen Hock, 69: 193-202.

Doris, A.B., M.L. Telisa, W.B. James and E.B. Paul, 2008. Characterizationof diazotrophs containing Mo-Independent nitrogenase, isolated from Divers natural environments. Appl. Environ. Microbiol., 47: 11.

El-Shanshoury, A.R., 2008. Interactions of Azotobacter chroococcum, *Azospirillium brasillense* and *Streptomyces mutabilis*, in relation to their effect on wheat development. J. Agron. Crop Sci., 175(2): 119-127.

Evans, L., A. Daniel and S. Pearson, 2005. Nucleotide sequence and genetic analysis of the *Azotobacter chroococcum nifUSHVWZMP* genes. J. Mol. Gen. Genet., 467: 345-352.

Forbes, B.A., D.F. Sahm and A.S. Weissfeld, 2002. Diagnostic Microbiology. 11th Edn., Mosby, Inc., USA.

Garive, E.I., 1986. Genus *Lactobacillus*. In: Sneath, P.H.A., N.S. Mair, M.E. Sharpe and J.G. Holt, (Eds.), Bergy's Manual of Systematic Bacteriology. Vol. 2, Williams and Wilkans Co., Baltimore. London.

Helmut, B.F., W. Widmer, V. Sigler and J. Zeyer, 2004. New molecular screening tools for analysis of free-living diazotrophs in soil. Appl. Environ. Microbiol., 70: 240-247.

Hogg, S., 2005. Essential Microbiology. John Wiley & Sons Ltd., The Atrium, Southern Gate, Chichester, England.

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley and S.T. Williams, 1994. Bergey's Manual of Determinative Bacteriology. 9th Edn., Williams and Wilkins, Baltimore.

Jayashree, V.S., A. Karthick and V. Kalaigandhi, 2007. Molecular study on *Azotobacter nif* H Gene by PCR. J. Gen. Microbiol., 152: 1871-1884.

Johnstone, G.F., H.L. Marynko and B. Jones, 2000. Classification and Identification of *Azotobacter* ssp. In: Jensen, H.L. (Ed.), The Azotobacteriaceae. Lange Agricul. Publication, Los Altos, California Bact. Rev., 18: 195-214.

Jones, H., J. Graham, R. Stall and T. Leite, 2009. Chromosomal gene transfer by conjugation in the *Azotobacter chroococcum* to *Xanthomonas citri.* Biotechniques, 51: 1221-1229.

Kumar, V., R.K. Behl and N. Narula, 2001. Effect of phosphate-solubilizing strains of *Azotobacter chroococcum* on yield traits and their survival in the rhizoshere of wheat genotypes under field conditions. Acta Agron. Hung., 95(7): 141-149.

Lotareva, O.V. and A.A. Prosorov, 2006. Conjugation transfer of chromosomal and plasmid genes in *Bacillus subtilis*. Doklady Biol. Sci., 408: 226-228.

Milani, P.M. and J. Anthofer, 2008. Effect of *Azotobacter* and *Azospirillium* on the yield of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) in Kermanshah and Lorestan, Iran. Arch. Agron. Soil Sc., 52(8): 588-604.

Raina, R., U.K. Bageshwar and H.K. Das, 1993. The ORFencoding a putative ferredoxin-like protein downstream of the vnf H gene in *Azotobacter vinelandii* is involved in the vanadium-dependent alternative pathway of nitrogen fixation. Mol. Gen. Genet., 236: 459-462.

Rajeswari, K. and M. Kasthuri, 2009. Molecular characterization of *Azotobacter* spp. nifH gene isolated from marine source. Afr. J. Biotechnol., 8(24): 6850-6855.

Rowan, S.L., 2003. Kinetics of chromosome transfer in *Azotobacter chroococcum* conjugation. Mol. Plant-Microbe Interact., 18: 256-260.

Ryan, J., J. Astufan and A. Al-Rashid, 2003. Laboratory Manual of Soil and Plant Analysis. ICARDA, Aleppo, Syria.

Saribay, G.F., 2003. Growth and nitrogen fixation dynamics of Azotobacter chroococcum nitrogen-free and OMW containing medium. M.Sc. Thesis, The Graduate School of Natural and applied Sciences, Middle East Technical University, Turkey.

Snyder, L. and W. Champness, 1997. Molecular Genetic of Bacteria. American Society for Microbiology. ASM Press, Washington D.C.

Stall, H., A. Ludwig, K. Lamar and F. Cullen, 1999. Nature and role of plasmids in *Azotobacter chroococcum*. Biotechnol. Lett., 22: 643-649.

Stevanova, S., L. Tserovaska and T. Yordanova, 2002. Identification of lactic acid bacteria isolated from katyk, goats milk, cheese, and soils. J. Cult. Collect., 5: 48-52.

Womble, D.D., 2000. GCG: The Wisconsin package of sequence analysis programs. Methods Mol. Biol., 132: 3-22.