Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 91-98

Detection of Periplasmic Nitrate Reductase Enzyme in Proteolytic *Pseudomonas aeruginosa*

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Received: June 1, 2015 / **Accepted**: September 2, 2015

Abstract: A total of 50 *P. aeruginosa* isolates are leading reasons of hospital acquired infections (burns) were collected from AL-Kindy Hospital. This study focused on the quick synthesis of silver nanoparticles by this bacteria as assay for periplasmic nitrate reductase enzyme. The silver nanoparticles were described by using Atomic Force Microscopy (AFM) and found the average size was 93.55nm. From the screening profile of 50 isolates enlisted in this study the percent of *napA* gene positive encoding periplasmic nitrate reductase was 100%. Results revealed that nitrogen bases sequencing for PCR product of one isolate correspond reaching up to 88% as compared with nitrogen base sequence of the *napA* gene of *P. aeruginosa* strain of National Center for Biotechnology Information (NCBI).

Key words: periplasmic nitrate reductase , *nap A* gene , *Pseudomonas aeruginosa***,** silver nanoparticles.

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Introduction

Pseudomonas aeruginosa is a rod gram negative aerobic bacteria. It is a member of the gamma proteobacteria group (1), and a combined bacteria that can cause disease in humans and animals .It is an opportunistic pathogen causing severe, chronic and acute nosocomial infections in catheterized, immunocompromised, or burn patients (2).

Different types of virulent factors have been appointed in *P. aeruginosa*, which indicates their contribution to the pathogenesis of the disease (3). The Protease is very important virulence factor which destroy host tissues and nip up with host antibacterial defense mechanisms. (4). Proteolytic bacteria are of major importance in nitrogen mineralization, and play a significant role in proteolytic processes in soil, sewage and water (1).

It is well known that many of microbes, both multicellular and unicellular, inorganic materials produced either intra or extracellular (5). The microorganisms such as, fungi, yeast and bacteria , play a significant role in the handling of toxic metals through reduction of metal ions and employment as interesting nanofactories (6). These microbes are excessively good candidates in the synthesis of gold, cadmium, and silver nanoparticles (Ag –NPs) (7). Bacteria being prokaryotes have survived the test of time in enriching ions synthesizing magnetite nanoparticles, reducing Ag into metal particles, forming nanoparticles , and in generation of cerements (8). The aim of this study was to detect the presence of periplasmic nitrate reductase enzyme in

P. aeruginosa by synthesis of silver nanoparticles.

Materials and Methods

Bacterial Isolates

A total of 50 isolates of *Pseudomonas aeruginosa* were collected from burns from patients who were admitted to AL-Kindy Hospital in Baghdad. These isolates were identified by Conventional biochemical reaction according to the criteria established by Forbes *et al*., (9).The isolates were inoculated on pseudomonas agar plate. The results were read after 24 and 48h of incubation at 42°C.

Detection of Protease Production Isolates

The ability of the isolates to produce protease enzyme were tested by culturing in skimmed milk agar (10) then incubate at 37 °C for 24h.

Synthesis of Silver Nanoparticles by *Pseudomonas aeruginosa*

Nutrient broth was prepared , sterilized and inoculated with a fresh growth of test strain *P. aeruginosa* . The culture flasks were incubated at 37^0C for 72 hrs. in an orbital shaker at 150 rpm after the incubation period, the culture was centrifuged at 10,000 rpm for 5 min and the supernatant was used for the synthesis of silver nanoparticles (AgNPs). The supernatant of *P. aeruginosa* culture was separately added to the reaction vessels containing silver nitrate at a concentration of 0.1g/L. The reaction between the supernatant and silver ions was carried out in bright conditions for 72hrs. (5).

Characterization of Silver Nanoparticles by Atomic Force Microscopy

Atomic Force Microscopy image was taken using Park system AFM XE 100. A thin film of the sample was prepared on a glass slide by dropping 100 µl of the sample on the slide, and was allowed to dry for 5 min. The slides were then scanned with the AFM (11).

DNA Preparation and PCR

A PCR reaction with specific primer
5'-CCATGGGCTTCAACC-'3
Smith *et al*., (12) was performed to identify *napA* genotype of each *Pseudomonas aeroginosa* isolate. DNA template was prepared as described by Ruppe' *et al*.(13), 25 µl of PCR amplification mixture contained 4.5 µl deionized sterile water , 12.5 µl Green Go *Taq* Master Mix pH (8) (Promega ,USA) [contained 50 unit /ml of Go *Taq* DNA polymerase, 400 Mm of each dNTPs and 3Mm of MgCl2 and 1pmol for specific primer (Alpha DNA, Canada)]. The PCR cycles for *napA* were as followed : initial denaturation at 95°C for 4min , 30 cycles of denaturation at 94°C for 30sec, annealing at 53°C for 45sec and extension at 72°C and final extension at 72°C for 4 min using Gradient PCR (TechNet-500,USA).

DNA Sequence Analysis

The DNA fragments for sequencing were obtained by PCR amplification of each chromosomal DNA as the template. The fragments of each PCR products were sequenced with the set of primers by Macrogen, USA). The program (BioEdit Pro. version: 7.0.0)

was used for bioinformatic analysis of nucleotide sequences.

Results and Discussion

Detection of Protease Production Isolates

Fifty isolates of *P. aeruginosa* were collected from Al –Kindy hospital-Baghdad, and according to classifications in Bergeys Manual of Systematic Bacteriology (14), were investigated for morphological and physiological features. All locally isolates produced protease enzyme, (Figure-1). Protease can affect abroad range of biological roles including the infection practicability which is not only a simple and fast multiplication of bacterial cells in the human body. *Pseudomonas aeruginosa* the opportunistic human pathogen has an arsenal of effectively proteases that helps establishing and maintain the infection and thereby controlling and modifying the environment according to the requirements of the bacterium within the host tissue (15).

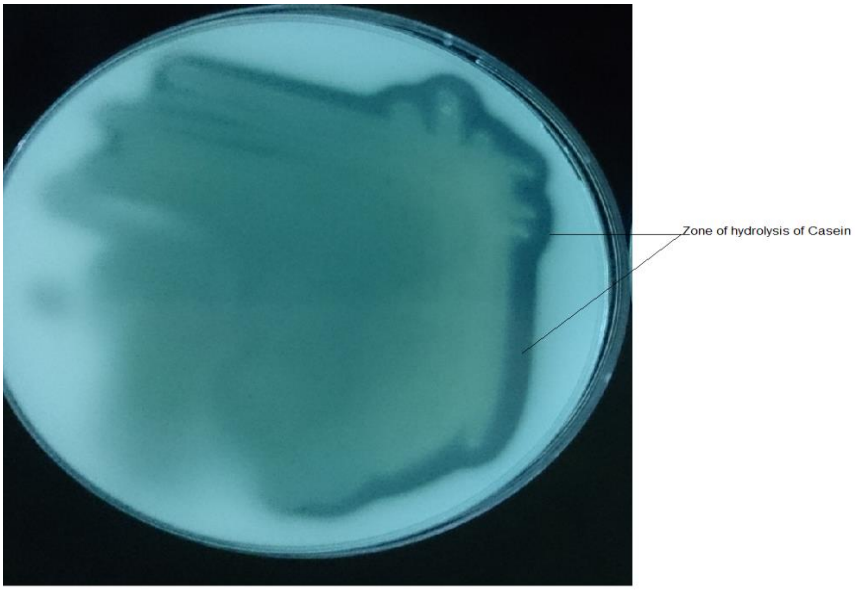

Figure (1) *P. aeruginosa* protease positive on Skimmed milk agar

Synthesis and Characterization of Silver Nanoparticles by *P. aeruginosa*

A protease positive isolates were tested for synthesis of silver nanoparticles. Extracellular biosynthesis of silver nanoparticles by the culture supernatant of *P. aeruginosa* was studied. Visual watching showed a change of colour in supernatant from yellow or green bluish (for isolate produced pyocyanin) to brown (figure-2) , while no colour change was noted in the culture supernatant without silver nitrate or in media with silver nitrate alone. The release of brown colour in silver nitrate treated culture supernatant suggested the fashioning of silver nanoparticles

(16). A similar observation was made by Duran *et al*., (17). In the biosynthesis of Ag –NPs by *P. aeruginosa* strain by extracellular process, the excitement of surface Plasmon vibration of Ag –NPs, the change colour of the medium to brown could be consequent (7). The mechanism of biosynthesis of Ag-NPs is exacting known, however, it has been assumption that silver ions desired the NADPH - dependent nitrate reductase enzyme for their reduction which was produced by the bacteria in its extracellular environment (18).

Synthesis of silver nanoparticle under anaerobic conditions using this enzyme has previously been demonstrated. Nitrate reductase is known to shuttle electron from nitrate to the metal group. These results cited the role of nitrate reductase enzyme in the biosynthesis of silver nanoparticles (19). It is reported that reduction of Ag^{+} to Ag^{o} occurs through nitrate reductase enzyme, these enzymes secreted in the solution can reduce the silver nitrate to silver nanoparticles through proteins as capping agents (20). By atomic force microscopy in this study converted particle size was analyzed. AFM was used to show the nanoparticle both in surface and three dimensional view (figure-3) and found the average size of particles 93.55 nm. The image gave the clear shape and size of the AgNPs synthesized by *P. aeruginosa*, while Jeevan *et al.*, (5) found that the particles size ranges from 20-100 nm.

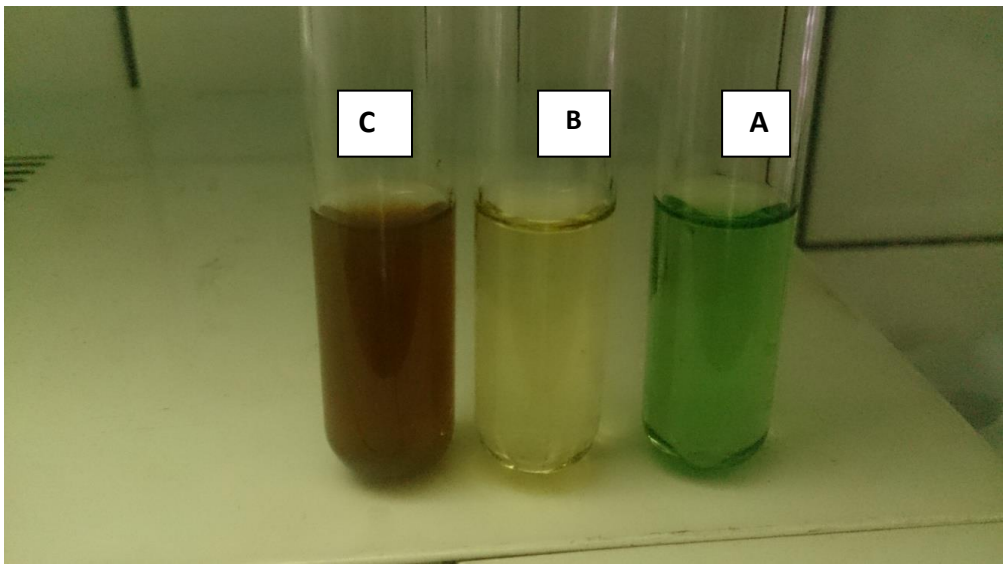

Figure (2) supernatant of *P.aeruginosa* : (A) Supernatant without AgNO3, (B) Nutrient broth before culturing and (C) Supernatant With AgNO3

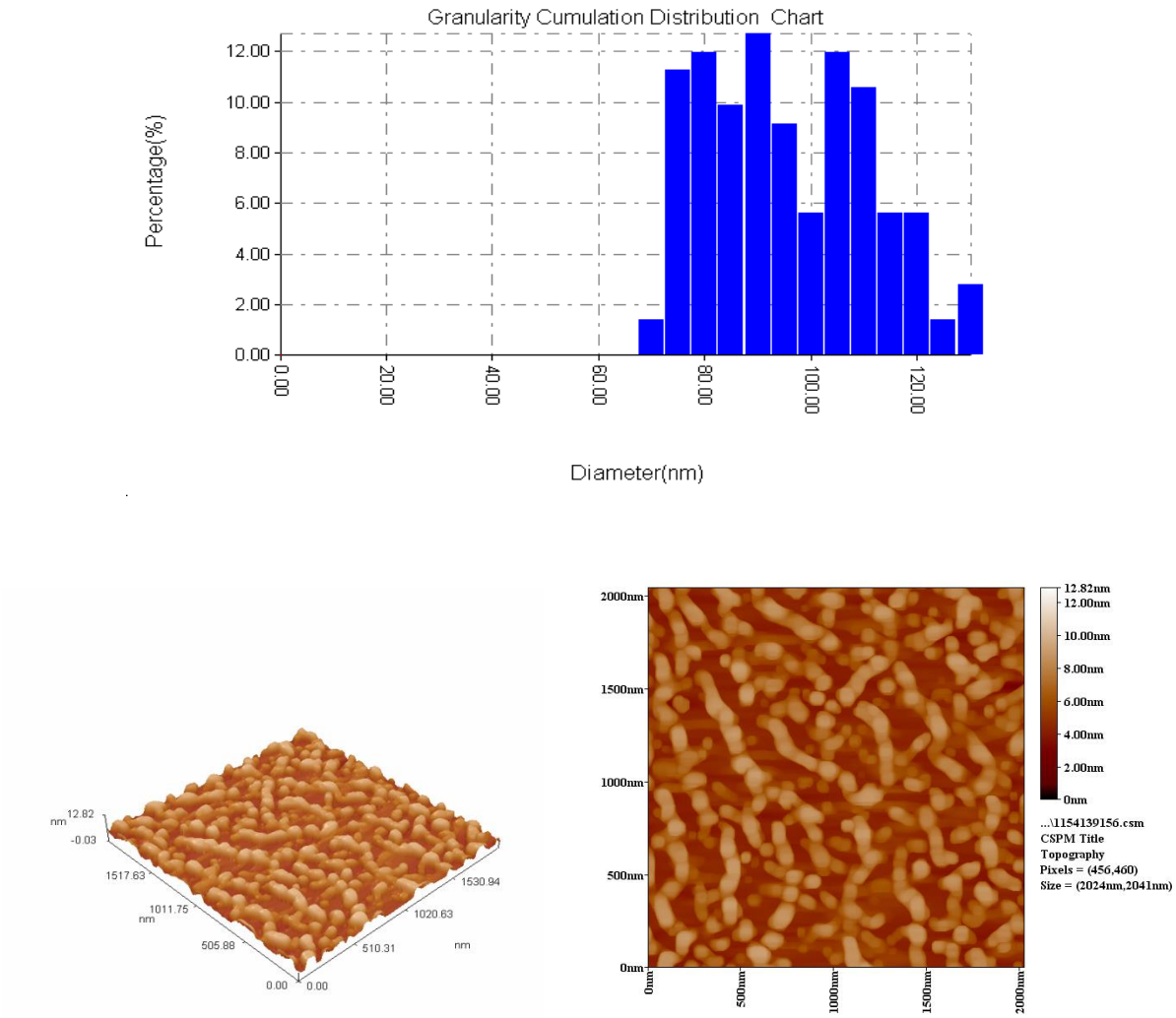

Figure (3) Atomic Force Microscopy image of silver nanoparticles synthesized by *P.aeruginosa*

Polymerase Chain Reaction (PCR)

Technique was performed by using specific primer targeting the specific sequences of the *nap A* gene. The results showed that *nap A* gene found 100% of tested isolates (Figure-4).

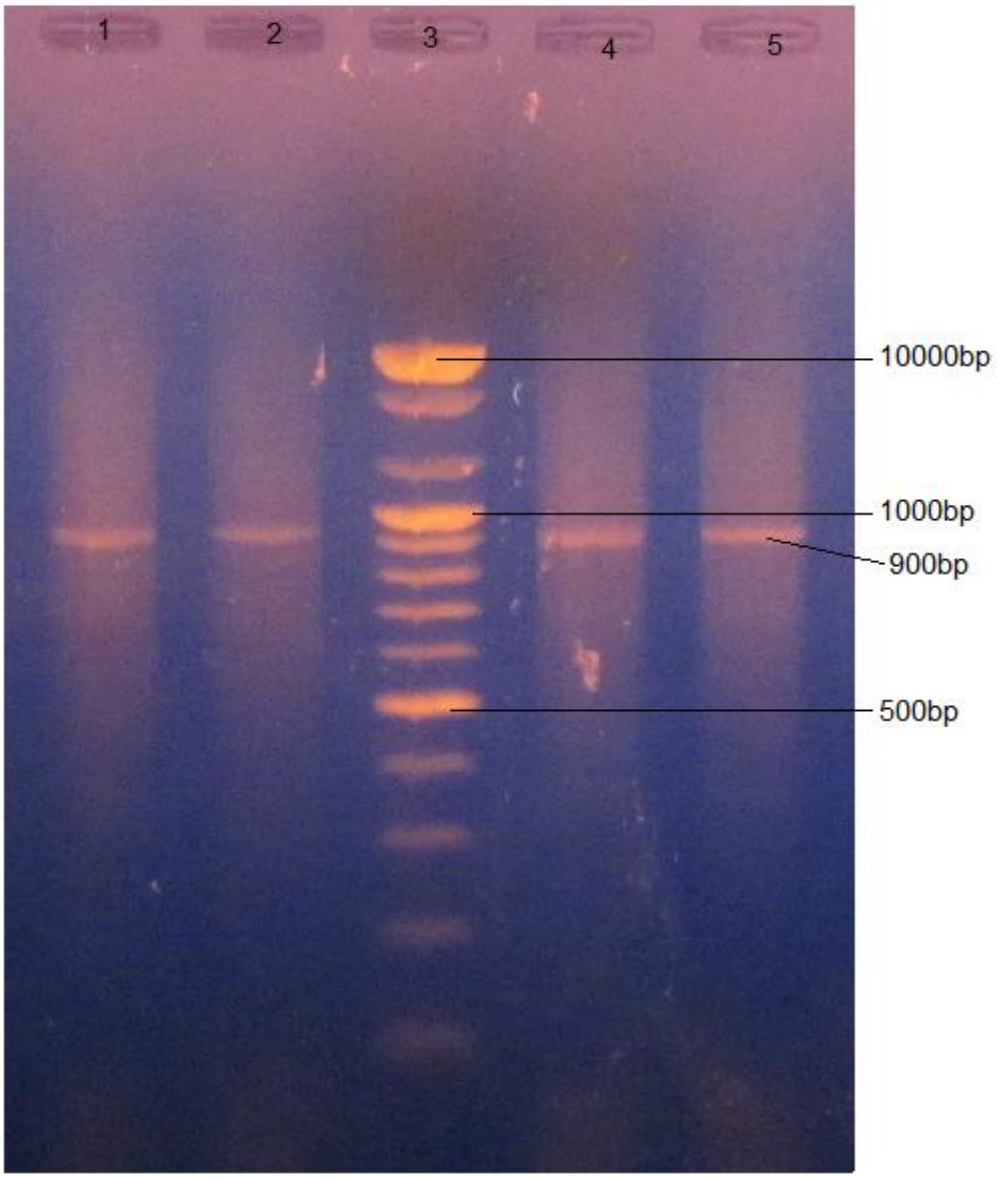

Figure (4) Gel electrophoresis (1% agarose, 7 v/cm) of *napA* gene (900 bp)

Lane 1,2,4,5 DNA of *Pseudomonas aeruginosa* isolates, Lane 3 DNA ladder (10000bp)

Results of the Nitrogen bases sequencing for PCR product of one isolate revealed consistency reaching up to 88% as compared with Nitrogen bases sequence of the *napA* gene present in the *Pseudomonas aeruginosa* strain in NCBI (Figure-5). Possessing *napA* gene will reflect a release of periplasmic nitrate reductase enzyme (21).

Pseudomonas aeruginosa strain PSE305, genome
Sequence ID: emb|HG974234.1|Length: 6762448Number of Matches: 1
Related Information
Range 1: 5975211 to 5975837GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
745 bits(403)	0.0	554/628(88%)	6/628(0%)	Plus/Minus

Features:
hypothetical protein PLES 00921hypothetical protein PLES 00931

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Query  166      GGACGACGCCGAAGGACGAGGGACTGTTCACCAATGCTGCCCCCACGGATGACTTCTGGT  225
                |||||||  | |||||  |||||   |||   ||  ||   |||||  ||  ||  ||||||||||||||
Sbjct  5975837  GGACGACCCAGAAGGTCGAGGTCCTGCGCAGCACCGCTGCGCCGACCGATGACTTCTGGT  5975778

Query  226      TGCTCAAGGGACA-TGGTGATCCCGTCAAAGGCGATCCACTTCCACGGTTCTCCCACTAC  284
                 |  | ||||||||  |   |  ||||||  ||||||||    |  |   |  ||  |||||||  ||  |
Sbjct  5975777  CGGTAAAGGGACAGTCAT-ATCCCGCCAAAGGCGGCGCGCAACAACTGTTCTCCAACGAG  5975719

Query  285      AAATGCCCCTTCAGTCTGCGTCCCCGGGAGGGATCATGACACCCATCGAATACATCGAGC  344
                |||  ||  |||||  ||||||  |||||||||||||||||||||||||||||||||||||||||  |
Sbjct  5975718  AAAGGCTCCTTCGGTCTGCTTCCCCGGGAGGGATCATGACACCCATCGAATACATCGACC  5975659

Query  345      GCGCTCTGGCGCTGGTCCTGCACCGGGTGGACCGCTATCCGGGATACGAAGTCCTGCTGT  404
                ||||||||||||||||  |   |||||  |||  ||||||||||||||||||  ||||||||||
Sbjct  5975658  GCGCTCTGGCGCTGGTCGTCGACCGGCTGGCCCGCTATCCGGGATACGAGGTCCTGCTGT  5975599

Query  405      CCGCGAAAAAGAAATTGCTGTACATCAGGTCCGTCCTGCTCGACCGCAGCCTGAATCGTT  464
                |||||  |||||  ||||||  ||||||||||||||||||||||||||||||||||  ||||||
Sbjct  5975598  CCGCGGAAAAGCAATTGCAGTACATCAGGTCCGTCCTGCTCGACCGCAGCCTGGATCGTT  5975539

Query  465      CCGCACTGCACCGGTTGACCCTCGGCAGCATCGCCGTGAAGGAATTCGACGAAACCGACC  524
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5975538  CCGCACTGCACCGGTTGACCCTCGGCAGCATCGCCGTGAAGGAATTCGACGAAACCGACC  5975479

Query  525      CGGAACTCTCCAGGGCCCTCAAGGACGCCTACTACGTCGGCATACGCACTGGCCGCGGCC  584
                ||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||
Sbjct  5975478  CGGAACTCTCCAGGGCCCTCAAGGACGCCTACTACGTCGGTATACGCACTGGCCGCGGCC  5975419

Query  585      TGAAAGTCGATCTGCCCTGAGCGCAACACCATCGGTCTTGAGCGACCGCCGCTTCTGCCT  644
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5975418  TGAAAGTCGATCTGCCCTGAGCGCAACACCATCGGTCTTGAGCGACCGCCGCTTCTGCCT  5975359

Query  645      GGCGAGGACATGCGCGAATCGTCAAGGCTCCCGCGGCGTCCCGCCCAATGGCGTTCAGTC  704
                ||||||||||||||||||||||||||||||||||||||||||||||||||||  ||||  |||  |
Sbjct  5975358  GGCGAGGACATGCGCGAATCGTCAAGGCTCCCGCGGCGTCCCGCCCAAGGGCGGGCAGCC  5975299

Query  705      ACGATGCTGCCGTCTCATCC-TTCGCCGGCTAAG-AAACTTTGTCTGTATCTGGCCTACG  762
                |  |||||  ||||  ||||  ||  |||||||||    |  ||||  |  |  |||||  ||||||  ||
Sbjct  5975298  AGGATGCCGCCGCCTCAGCCCTTCGCCGGCGGCGGAAACATCGCCTGTACCTGGCCGTCG  5975239

Query  763      ATGTTGCC-TGCTCTCCTTT-CCTTTGG  788
                |||  ||||  |||  |  ||  ||  ||  ||||
Sbjct  5975238  ATGGTGCCCTGCACGCCCTTGCCCTTGG  5975211
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(Figure-5) : Nitrogen bases sequencing of *napA* gene in clinical isolate of *Pseudomonas aeruginosa*

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