

Cloning and Nucleotide Sequence of 16S-like Rna Coding from Lipolytic *Pseudomonas Fluorescens* Eg Strain from Animal Blood Waste

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Abstract: An lipolytic producing bacterial strain was isolated from slaughter house wastes (animal blood). In order to identify the bacterial strain microbiological, physiological and biochemical tests were performed, along with 16S-like rRNA and DNA-DNA hybridization analysis. The bacterium was found to be a strain of *Pseudomonas fluorescens* which was designated as (EG). The strain was grown in SHBM medium. *Ps. Fluorescens* EG strain secretes a thermostable lipase into the extracellular medium. To further characterize the strain phylogenetic analysis of the strain EG were performed using 16S rRNA analysis. The 1451 bp 16S rRNA gene was amplified from EG strain and sequence compared with that of other *Pseudomonas* spp. Which showed up to 88% to *Ps. Fluorescens* strains (chloro, coronas; congs. S, syrin, cari, Flo. Se, Flo.S and Flo.S). The phylogenetic tree developed from this comparative study also indicated that EG was closer to *Ps. fluorescens* with an aberration to *Ps. fluorescens* Strains EG. Nucleotide sequence of 16S-rRNA was recorded in gene bank with Accession number EU 2703406.

Key word: *Pseudomonas fluorescens*, lipase, nucleotide sequence, of 16S rRNA gene, PCR, DNA-DNA hybridization.

INTRODUCTION

Lipase is a triacylglycerol-hydrolyzing enzyme whose activity depends on the occurrence of an oil-water interface. This enzyme occurs in many microorganisms, plants and animal (Borgstrom and Brockman, 1984 and Ahn *et al.*, 1999). In particular bacterial lipases are generally more stable than animal or plant lipases. The *Pseudomonas lipases* constitute a major group; they have been reported from *Ps. aeruginosa*; *Ps. fluorescens*, *Ps. glumae* and other *Pseudomonas* species (Kumura *et al.*, 1998 and Ihara *et al.*, 1991). The production of enzymes depends on substrates. Slaughter houses such, animal blood were screened as substrate for lipase production (Sayed, 2006).

The comparison of 16S rRNA sequence is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaeobacteria and eucaryotic organisms. These sequences have been derived previously by methods including oligonucleotide cataloging (Weisburg *et al.*, 1991; Fox *et al.*, 1980; Ipper *et al.*, 2005), Sequencing of clones, direct sequencing of RNA by using reverse transcriptase (Lane *et al.*, 1985) and sequencing of material amplified by polymerase chain reaction (PCR) (Edwards *et al.*, 1989). This investigation was primarily concerned with the identification of the *Ps. Fluorescens* isolated from animal blood waste, with the use of morphological, biochemical, productivity and molecular methods. We have further described the lipolytic productivity by *Ps. fluorescens* E.G. isolate using animal blood waste as substrate.

MATERIALS AND METHODS

Isolation of Bacteria :

Animal blood wastes were obtained from slaughter house at Benha, Qalubia Governorate, Egypt. The blood were homogenized using demineralized water and the homogenate was serially diluted and plated into Mannitol-Glutamate- yeast (MGY) agar media (Keane *et al.*, 1970), followed by incubated for overnight at 28°C. Different morphological colonies were recorded on the dilution plates. One colony was inoculated nutrient broth medium containing 20% glycerol, by freeze-drying in 10% skimmed milk for its long-term preservation as described by Perry (1995).

Identification of the Bacterial Isolate:

The bacterial isolate was identified by examination of the morphological physiological, biochemical and molecular analysis according to Schallmey *et al.* (2004), Collee *et al.*, (1996), Parker (1984) and Ipper *et al.* (2005), other *Ps. fluorescens* (ATCC 1033) and *Bacillus subtilis* identified were obtained from Cairo Microbiological Resources, Center (MIRCEN).

Tributylin Clearing Zone Technique (TCZ):

The used medium for assay of lipase enzyme productivity according to TCZ technique (Elwan *et al.*, 1977).

It contained, basal medium which was prepared according to Vincent (1970), it was modified to include the following constituents (g/L) 6.0 NaCl, 1.0 (NH₄)₂ SO₄; 0.5 KH₂PO₄; 0.1 CaCl₂, 1.0 yeast extract and distilled water up to one litre and supplemented with 0.2% (v/v) of emulsified tributyrin. Tributyrin emulsion was prepared by grinding 20 ml tributyrin and 20 gm arabic gum (v/w) were 1:1 thoroughly in a mortar which was finally powdered. Warm distilled water being added in small aliquots throughout grinding with obtain smooth permanent emulsion of a total volume of 250 ml (8% emulsion) as a stock emulsion and was kept at (4°C). From this stock 25 ml was added to 1 L of medium plus agar for solidification, pH was adjusted at 9.0 using Tris-buffer (0.2M).

Isolation of DNA:

Genomic DNAs were prepared by using a lysozyme dodecyl sulfate lysis procedure (Owen *et al.*, 1987). Bacterial cells were cultured in MGY broth at 28°C, with shaking at 200 rpm, and harvested by centrifugation. The cells were lysed by the addition of 10% SDS solution followed by incubation with 100 µl of proteinase K solution (10 mg/ml) was added and the mixture incubated for 1 h at 37°C. The DNAs was then extracted using the phenol extraction method. The DNAs in aqueous phase was precipitated with 93% ethanol, followed by washing with 70% ethanol. The DNA pellet was allowed to dry, then dissolved in TE buffer (pH 8.0) and stored at -20°C for further work.

PCRAmplification :

PCR analysis was performed with a DNA thermal cycler (COY temp Cycler 11 Model 110S). The 16S, RNA gene was amplified using FDI (CCGAATTCGTCGACAACAGAGTTTGATC-CTGGCTCAG) and rP2 (CCC GGATCCAAGCTTACGGCTACCTTGT-TACGACTT) primers (Weisburg *et al.*, 1991). Amplification was performed in a total volume of 50 µl, containing 20 pmol of each primer, 200 mM of mixture from dATP, dCTP, dGTP, dTTP (Promega Co., USA), IX buffer, template DNA (20 ng) and 2.5 units of Taq polymerase (Promega Co., USA) under the following reaction conditions; initial denaturation at 94°C for 4 min, then 35 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 3 min, followed by a final elongation step at 72°C for 10 min.

Cloning and sequencing:

The PCR product of the 16S rRNA was analyzed by electrophoretic separation in 1% (w/w) QA-agarose containing 0.5 µg/L ethidium bromide. The resultant PCR product was excised from the gel and purified using a QIA quick gel extraction kit (Qiagen Inc., Germany). Purified DNAs were ligated into the pGEM-T assay vector (Promega Co., USA). Plasmids containing the 16S rRNA region was then directly sequenced using an Alfred autocycle sequencing kit, with forward and reverse primers. DNA homology searches were carried out with the NCBI data bases, using the BLAST network service (Altschut *et al.*, 1990).

Phylogenetic Analysis :

The Mega Align Software package (Window 3.88, Dnastar, Inc., USA) was used for alignment of the nucleotides of different *Pseudomonas* species. The relationship between the bacterial strains was further analyzed by a phylogenetic tree, using the MEGA Program (Kumura *et al.*, 1998; MEGA: molecular evolutionary genetic analysis version 1.0. The Pennsylvania State University).

Dot blot:

The eubacterial primer FDI (Weisburg *et al.*, 1991) was used as universal oligonucleotides probe. 100 µl of TE buffer, 4 µl of 10M NaOH and 35 µl of 20 x SSC were added to 100 µl of unlabeled total DNA (1 µg/µl) and denatured by boiling at 50°C for 10 min. Denatured DNA was applied to Hybond-N nylon membrane, using dot blot apparatus. Total DNA of *Ps. Fluorescens* KACC 10327 was used for probe. Labeling Native DNA was labeled with DIG11-dUTP using Dig-High Prime (Roche Molecular Biochemicals, Germany), prehybridized at 49°C for 3h, and then hybridized for 16 h at the same temperature. Hybridization signals were detected using a DIG Luminescent Detection Kit and quantified using a Densitometer (Bio-Rad, USA).

RESULTS AND DISCUSSION

Qualitative Screening for Selection of Potent Lipase Producer:

Ten bacterial isolates were isolated from animal blood waste sample on MGY agar media. These isolates were purified and subjected to a screening program in order to evaluate their lipolytic activities by measuring (observing) the tributyrin around the bacterial colonies. Out of ten there was found that 4 bacterial isolates gave lipolytic activities while only one of these isolates considered to give a good lipolytic activities.

Quantitative Screening for Selection of Potent Lipase Producer Bacterial Isolate:

This experiment was carried out to determine the most potent lipase producer bacterial isolates on the basis of mean diameters of clearing zones (mm) by testing the potency of the best bacterial isolates producers selected from the previous test to attack animal blood wastes.

Tributyryn clearing zone technique (TCZ) showed the ability of bacterial isolates selected from the previous experiment to attack animal blood wastes for lipase (s) productivities. It was found that bacterial isolate No. 3 and 5 gave the highest lipolytic activities where it reached up to 23.5 and 25.5 mm respectively compared with other isolates.

Identification of Bacterial Isolates:

Physiological and biochemical tests showed levan production, oxidase and arginine dehydrolase activity and utilizations of 2-ketogluconate, mannitol and D. arabinose as most *Ps. fluorescens* species. However, no grolatin liquifaction was observed which is usually shown by *Ps. Fluorescens*, such a the ATCC 1003 strain. The pectolytic activity, HR in tobacco and growth of higher temperatures tests were negative. On the basis of 33 other test results, as shown in Table 1, indicates that the isolates is closely placed with the other *Ps. Fluorescens* Egyptian strain.

Table 1: Morphological, physiological and biochemical characteristics of bacterial isolates.

Characteristic	<i>Bacillus</i> sp.		<i>Pseudomonas</i> sp.	
	<i>B. subtilis</i>	<i>B. isolate</i>	<i>Ps. Fluorescens</i>	<i>Ps. isolate</i>
Cell shape	Bacilli	Bacilli	Bacill	Bacilli
Gram reaction	+	+	-	-
Cell diameter (µm)	3 x 1.2	3 x 1.1	0.9 x 1.2	0.8 x 1.2
Pigments	-	-	0.9	V
Oxidase	+	+	+	+
Amylolytic activity	+	+	-	-
Pectolytic activity	-	-	+	+
Cellulolytic activity	-	+	-	-
Lipolytic activity	-	V ⁺	+	+
Protolytic activity	+	-	-	V
Urease	-	V	-	-
Utilization of:				
Benzoate	-	V	+	V
Mannitol	+	+	+	+
Cellubiose	-	-	-	-
Sorbitol	-	+	-	V
Sucrose	-	+	-	-
D-arabinase	-	+	-	V
L-rhamnose	-	-	-	V
D-aspartate	-	-	-	-
Xylose	+	+	+	+
Glucose	+	+	+	+
Lactose	-	-	-	-
Maltose	-	-	-	-
Ribose	+	+	+	+
Glycerol	-	+	+	+
Fructose	+	V	-	-
Raffinose	-	-	-	-
Galactose	+	+	+	+
Ethanol	-	+	-	-
Butyrate	-	-	+	+
Arginine test	-	+	-	-
Malonate test	-	-	+	+
VP test	+	+	+	+
O/F test	+	+	+	+
Anaerobic growth	-	-	-	+
Indole	-	-	-	-
Growth at 55°C	-	-	-	-
41°C	-	-	-	-
37°C	-	-	+	+
4°C	+	+	+	+

* C.F. Cairo Microbiological Resources Center (MIRCEN).

O/F : Oxidation/fermentation

VP : Voges-Proskauer

V: Variable

+ Positive result

- Negative result

+⁺ 75% or more isolate positive

- 80% or more isolate negative

PCR amplification of 16S RNA gene:

Genomic DNAs were isolated from bacterial isolates *Ps. Fluorescens* (ATCC 1003) and *Ps. Fluorescens*, EG) using a Lysozyme dodecyl sulfate lysis procedure with high quality and substantially free RNA contamination. The DNA was then used as a template for PCR to amplify the 16S RNA gene via the QIAGEN PCR system by use of an oligo (dt), Fdl and rP2 primer sets, nearly full length 16S rRNA gene could be synthesized. The amplified 16S rRNA gene was used as a template using the internal primer combination (Fdl and rP2) in PCR for confirmation its specificity to the *Ps. Fluorescens* 16S-rRNA gene as a PCR product with a size of about 1451 bp DNA was amplified (Fig. 1).

Partial sequence of *B. fluorescens* – 16S rRNA gene :

Cloning into pGEM-T easy vector :

The PCR product of 16S rRNA gene was extracted from the agarose gel using the Agarose Gel DNA Extraction Kit and cloned into the pGEM-T easy vector as mentioned in the materials and methods. The plasmid was then introduced into *E. coli* strain DH5 and the recombinant colonies were detected by blue/white colony screening. White and ampicillin resistant colonies were selected for further analysis. The white colonies were subcultured on LB ampicillin containing broth. The plasmid was then isolated and purified using the Wizard® Plasmid Miniprepation System.

PCR Confirmation:

To confirm the presence of the insert (1451 bp), the purified plasmid was used as a template for PCR detection of the recombinant colonies and a nested-PCR product with a size of about 1451 bp (Fig. 2) was amplified.

Digestion Analysis:

As an addition confirmatory test, the new plasmid was digested with *Eco RI* restriction enzyme and a band with a size of about 1451 bp was released to prove the successful cloning of the 16 rRNA gene under investigation.

DNA Sequence:

The created plasmid was sequenced as mentioned before. Results in Fig. (3) showed that the nucleotide sequences of the PCR fragment which appeared to be containing 1451 nucleotides.

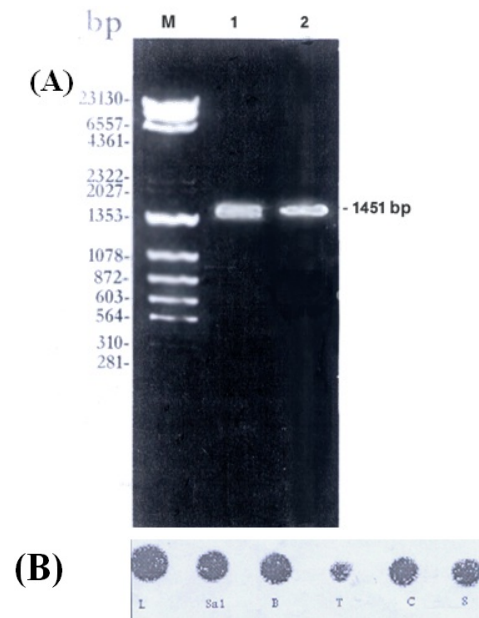


Fig. 1: Molecular detection of 16S- rRNA of *P. fluorescens* EG in total DNA extractin.

- A. Agarose gel, 1% stained with ethidium bromide showing PCR amplification of 16S rRNA gene using primers. Lane M; DNA marker, Lane1. *Pseudomonas fluorescens* (ATCC 1003) and Lane 2, *P. fluorescens* EG strain.
- B. Dot blot hybridization of DNA prepared from *Ps. fluorescens* EG., *Pseudomonas* spp. isolated from animal blood as well as *Ps. fluorescens* (ATCC 1003) (a, b, c, d, e and f) respectively with general eubacterial probe.

Sequencing Analysis:

On the level of DNA sequencing, the similarity between the present sequence (16 rRNA) and that of the overseas strains belonging to different groups could be summarized as follows: In the first groups in case a RAJ 308301-chloro, Z7660-corona-s, AJ492828-congs, AY574913-syrin and PSEATCC09-cari strains the similarity was formed 100% on the level of nucleotide sequence. The phylogenetic analysis of the present nucleotide sequence (Fig. 4) showed that the 16S rRNA *P. fluorescens* EG strain was classified in a separated cluster. Regarding the second group, that contains AY951875-Flase; EF694708- Flo.said FJ 470030-Flo.s strains, the identity was 100% on the level of nucleotide sequence. Considering the third group, that contains the first group and the second group, the homology was 99% on the level of nucleotide sequence. The phylogenetic analysis of the present nucleotide sequence (Fig. 4) showed that the 16S rRNA *Ps. fluorescens* EG strain, was classified in a separated cluster whereas the homology was 88% with the first and second groups.

By using Dot blot technique (DNA-DNA hybridization) with the universal oligonucleotides eubacterial probe against denatured DNA immobilized on Hy band N nylon membrane (Fig. 1-B), performed to analyze the genetic relatedness of *Ps. fluorescens* EG isolate with other *Pseudomonas* spp. The EG-isolate showed 74% relative binding ratio (RBR) with *Ps. fluorescens* (ACC 1003). A very low hybridized signal was observed with other *Pseudomonas* spp. Based on this evidence along with the biochemical and physiological analysis and the 16S rRNA, it can be inferred that the EG strain is *Ps. fluorescens*.

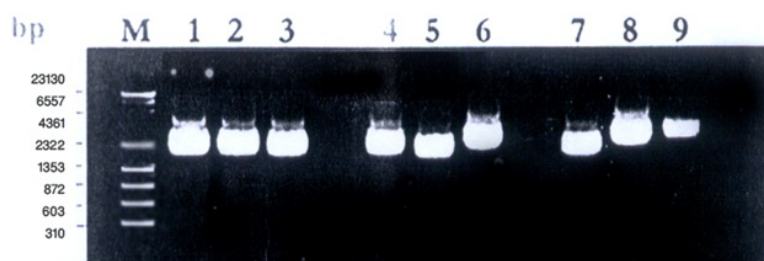


Fig. 2: Agarose gel, 1% stained with ethidium bromide showing plasmid minipreparation of white clones containing the 16S rRNA-fragment. M. 1 DNA marker.

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LOCUS 16SrRNAPseudomonas 1451 bp DNA linear UNA 03-jan-2009
DEFINITION
FEATURES Location/Qualifiers
ORIGIN
1 gcctaacaca gcaagtcgag cggtagagag gtgcttgcac ctcttgagag cggcggacgg
61 gtgagtaatg cctaggcaat ctgcctggta gtgggggata acgctcggaa acggacgcta
121 ataccgcata cgtcctacgg gagaaagcag gggaccctcg gcccttgcgc taccagatga
181 gcctaggtcg gattagctag ttggtggggg aatggctcac caaggcgacg atccgtaact
241 ggtctgagag gatgatcagt cacactggaa ctgagacacg gtccagactc ctacgggagg
301 cagcagtggt gaattattgg gtctgagagg atgatcagtc aactggaac tgagacacgg
361 tccagactcc tacgggaggc agcagtgggg aatattggac aatgggcgaa agcctgatcc
421 agccatgccg cgtgtgtgaa gaaggtcttc ggattgtaa gcaacttaag ttgggaggaa
481 gggcagttac ctaatacgtg attgttttga cgttacgcac agaataagca cgggctaact
541 ctgtgccagc agccgcggta atacagaggg tgcaagcgtt aatcggattt actgggcgta
601 aagcgcgcgt aggtggtttg ttaagttgga tgtgaaagcc ccgggctcaa cctgggaact
661 gcatccaaaa ctggcaagct agagataggt agagggtggt ggaatttcct gtgtagcggg
721 gaaatgcgta gatataggaa ggaacaccag tggcgaagcc gaccacctgg actgatactg
781 aactgaggtg gcaaaagcgt ggggagcaaa caggattaga taccctggta gtccacgccg
841 taaacgatgt caactagccg ttgggagcct tgagctctta gtggcgacg taacgcatta
901 agttgaccgc ctggggagta cggccgcaag gttaaaactc aatgaattg acggggccc
961 gcacaagcgg tggagcatgt ggtttaattc gaagcaacgc gaagaacctt accaggcctt
1021 gacatccaat gaactttcta gagatagatt ggtgccttcg ggaacattga gacagggtgt
1081 gcatggctgt cgtcagctcg tctcgtgaga tgttgggtta agtcccgtaa cgagcgcaac
1141 ccttgcctt agttaccagc acgttatggt gggcactcta aggagactgc cggtgacaaa
1201 ccggaggaat ggattgtgtc cttcgggaac attgagacag gtgctgcatg gctgtcgtca
1261 gctcgtgtcg tgagatgttg ggttaagtcc cgtaacgagc gcaacccttg tccttagtta
1321 ccagcacggt atggtgggca ctctaaggag actgccggtg acaaacccga ggaaggtggg
1381 gatgacgtca agtcatcatg gcccttacgg cctgggctac acacgtgcta caatggtcgg
1441 tacagagggg t
    
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Fig. 3: Nucleotide sequence of the 1451 bp fragment containing the *Ps. fluorescens* EG 16S rRNA structural gene.

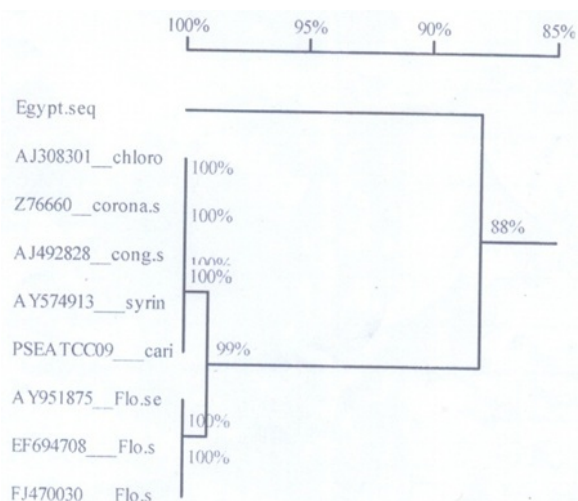


Fig. 4: Phylogenetic tree based on the 16S rRNA gene sequences of *Ps. fluorescens* EG strain and *Ps. fluorescens* spp. The branching pattern was generated by the neighbor-joining method. The number at the nodes indicate the levels of boot strap support based on a neighbor-joining analysis of 100 resampled data sets.

Discussion:

The main object of the present work an investigation of screening, purification and identification of lipolytic producing *Pseudomonas fluorescens* from slaughter house wastes (animal blood).

In this regard ten bacterial isolates were isolated from animal blood samples collected from slaughter house wastes. These bacterial isolate were grown at 28°C and 37°C and pH 9.0 to be able to produce a thermostable lipolytic enzymes. A screening test of lipolytic productivity of all bacterial isolates resulted in the fact that, only one bacterial isolate was found to be the best lipolytic enzymes producer.

For development of an lipase production, it is better to replace the usual protein source in microbiological media (peptone, leaf extract, yeast extract ... etc.) with natural proteins as cheaper sources of nitrogen for the fermentation (Sodhi *et al.*, 2005). The nature of solid substrate is the most sold state fermentation is receiving a renewed surge of interest, primarily because increased productivity and prospect of using a wide agroindustrial residues as substrates (Park *et al.*, 2002). In order to production of low cost of lipase, these bacterial isolates under study were allowed to grow on natural substrate such as animal blood waste under submerged fermentation conditions. However, the selection of the previously mentioned substrates for the process of lipase biosynthesis was based on the following factors Viz (1). It represent the most cheapest agro-industrial wastes in Egypt; (2) it is available at any time of the year; (3) it storage represents no problem in comparison with other substrates and (4) it resist any drastic effect due to the exposure to other environmental conditions e.g temperature variation in the weather from season to season and or from day to night. This trial resulted in the fact that the bacterial isolate *Ps. fluorescens* was considered to be the best for lipase production by growing on animal blood. The environmental conditions in solid state frmentation conditons can stimulate the microbe to produce the extracellular enzymes with different properties other than these of enzymes produced by the same organism under the condition performed in submerged fermitation (Pandey *et al.*, 2000).

Since the bacterial EG-lysate was the most potent producer of hydrolytic lipase, it was selected purposely for their identification as well as lipase productivity. Identification trials indicated that, it is related to the genus *Pseudomonas*. Therefore it was identified as *Pseudomonas fluorescens* EG-isolate.

Physiologicl and biochemical test showed, oxidative and arginine dihydrolase activity, and utilization of 2-ketogluconate, mannitol and D-arabinose, as most *Ps. fluorescens* species. The pectolytic activity and growth at higher temperatures tests were negative. These results were agreement with (Ipper *et al.*, 2005). To further characterize the EG-isolate, phylogenetic analysis of EG-isolate were performed using 16S rRNA. The 1451 bp 16S rRNA gene was amplified from EG-isolate and its sequence compared with that of other *Pseudomonas* spp. The phylogenetic tree developed from this comparative study revealed the isolate EG to be closer to *Ps. Fluorescens*. DNA-DNA hybridization was performed to analyze the genetic relatedness of the isolate EG with other *Pseudomonas* spp. (Ipper *et al.*, 2005; Weisburg *et al.*, 1991 and Fox *et al.*, 1980).

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