Identification of Pseudomonas aeruginosa Isolated from Karish Cheese by PCR Technique

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ABSTRACT

This research aims to isolate Pseudomonas aeruginosa in karish cheese and study its related proteolytic capabilities at lower temperature, while storing when temperatures are low and contamination (neglection of hygiene) Karish cheese gets spoilage because Pseudomonas aeruginosa developed enzymes that are proteolytic. Pseudomonas aeruginosa were separated, recognized, and verified by morphological and biochemical analysis, which were followed by 16S rRNA sequencing. The isolate tested positive to oxidase, KOH, Citrate utilization, Catalase, Motility, and arginine hydrolase. While this strain was not positive for methyl red, starch hydrolysis, indole and reaction of Voges-Proskauer. based on samples of DNA fragments and PCR (polymerase chain reaction) amplification results. The National Centre for Biotechnology Information (NCBI) received these sequenced segments and added them to GenBank with the accession "OM756757". With milk agar, Pseudomonas aeruginosa proteolytic abilities were tested. by the Congo Red Agar (CRA) manner, the isolates' biofilm development was investigated. The present research provides details about the ability of Pseudomonas aeruginosa to spoil karish cheese. and focuses on the value of hygiene precautions through preparing karish cheeses.

Keywords: Karish cheese; Pseudomonas aeruginosa; Gen Bank; Biofilm formation.

INTRODUCTION

Pseudo and Monas are the two halves of the Greek word pseudomonas. Pseudo means "false," while Monas is Greek for "single unit.". “aeruginosa” refers to Greek word “aerugo” that reflects "rusty copper" or "bluish -green" (Diggle and Whiteley, 2020).

Pseudomonas aeruginosa is aerobic, methyl red, Gram-negative, and shaped like rods, motile with a unique polar flagellum, with capable of growing at lower temperatures, negative to Voges Proskauer, indole, levan manufacturing, positive to oxidase and catalase production also positive to gelatin liquefied and citrate utilization (Bergey’s Manual, 2007).

Pseudomonas was It is one of the most significant bacterial totals in the dairy sector because it has been discovered as the primary psychrotrophic bacteria associated with dairy products (Wiedmann et al., 2000; Marchand et al., 2009).

Also, proteases, lipases and lecithinases are among the heat-stable enzymes that are extracellular produced by numerous Pseudomonas isolates. that consider the reason for milk spoilage. These enzymes stay active after heating processes that kills the bacteria producing these enzymes. Enzymatic activities degrade milk constituents reducing dairy products' life span. For example, bitterness and gelation of milk were obtained when protease digest milk casein. Milk fats are broken down by lipases, resulting in free fatty acids, which give milk its sour and bitter taste (Chen et al., 2003; Morales et al., 2005; Hantsis-Zacharov and Halpern, 2007; Zhang et al., 2015).

Pseudomonas species form a great hazard to animals and human health causing great economic losses. Pseudomonas species may spread through dairy products because of unsanitary handling also manufacturing (Quintieri et al., 2019). The infection translates also through cheeses because they are ready for eating (Vrdoljak et al., 2016).

Pseudomonas aeruginosa biofilm was thought to be the cause of many chronic infectious illnesses and make treating bacterial infections more difficult. Antibiotics cannot inhibit bacterial growth because biofilms' inner surface prevents drug molecules from penetrating it (Yuan et al., 2019).

Therefore, the purpose of this work was to identify and characterize Pseudomonas aeruginosa isolated from karish cheese and its ability to produce biofilms.

MATERIALS AND METHODS

Samples:

Samples (n=18) of Karish cheese collected from different markets and dairy shops in El-Beheira governorate, Egypt and were immediately placed on ice for transportation.

Preparation of cheese samples:

A volume of 225 ml of trisodium citrate was added to the stomacher bags, each of them contains 25 g of cheese sample, and the samples were homogenized for 3 min by stomacher (Wehr and Frank, 2004). Tenfold serial dilutions were plated onto Pseudomonas agar base (Oxoid) supplemented by supplemented with penicillin (100,000 IU/L, Sigma Aldrich, St. Louis, MO), pimaricin (0.01 g/L, Sigma) Following 24 hrs incubation at 25°C, three morphologically different colonies from plates were inoculated on to nutrient agar. Presumptive identification of Pseudomonas spp. was made based on colony morphology, Gram staining, oxidase production, motility, gelatin hydrolysis, pigment production, ability to grow at 42°C, indole production, utilization of carbohydrates (glucose and fructose) (Garrity et al., 2005).

Morphological and biochemical tests of the isolates:

Isolated typical colonies from Pseudomonas agar base were tested for Gram-staining (Brock et al., 1994), KOH (Suslow et al., 1982), Catalase activity (Herrero et al., 1996), Gelatin Liquefaction (Collins et al., 2004). All isolates were also examined for additional tests such as motility, catalase, Utilization of citrate, acid production from glucose, Starch Hydrolysis, Utilization of Citrate and Voges-Proskauer reaction (APHA, 1992).

Proteolytic Activity:

Pseudomonas single colonies were streaked onto Pseudomonas agar (Oxoid Ltd.) supplemented with penicillin (100,000 IU/L, Dr. Ehrenstorfer GmbH, Augsburg, Germany), pimaricin (0.01 g/L, Dr. Ehrenstorfer GmbH), and skim milk (10%) as described by Scatamburlo et al., (2015). The plates were incubated at 2, 4, 7, 10 and 25°C for 5 days. The temperature of 25°C was set as a control. The plates were monitored daily. Proteolytic halos in the inoculated areas were indicative of proteolytic activity (Scatamburlo et al., 2015).

Screening of extracellular lipase enzyme:

To determine production of lipases, Pseudomonas isolates were plated on tributyrin agar (Merck). Tributyrin agar plates were incubated at 30°C for 48 h. Presence of a zone of hydrolysis around the bacterial colonies indicated lipase activity (Meghwanshi et al., 2006).

Determination of biofilm production:

Biofilm formation of the isolates was investigated in vitro using Congo Red Agar (CRA) method. CRA method was carried out with the method reported (Atshan et al., 2012).

Molecular Identification:

DNA Extraction:

DNA was extracted from the bacteria and actinomycetes according to Cheng and Jiang, (2006), this described method is an improved method of the standard phenol/chloroform method described by Neumann et al., (1992).

16S analysis:

***PCR Reactions*:**

The PCR amplification was performed in a total volume of 50 μl, containing 1X reaction buffer, 1.5 mM MgCl2, 1U Taq DNA polymerase (Promega), 2.5mM dNTPs, 30 picomole (pmol; 1 picomole = 10-12 mole) of each primer and 30 ng genomic DNA.

Thermo-cycling PCR program:

PCR amplification was performed in a Perkin-Elmer/Gene Amp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 30 sec., an annealing step at 54ºC for 30 sec. and an elongation step at 72ºC for 1 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

Detection of the PCR Products:

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 ug/ml) in 1X TBE buffer at 95 volts. A 100 bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000).

Purification of PCR Products:

Amplified products for all PCR were purified using EZ-10 spin column PCR products purification PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes was added of binding buffer 1 after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 min after that centrifuge, 750 μl of wash solution was added to the column

and centrifuge at 10.000 rpm for two min, repeated washing, 10.000 rpm was spine for an additional minute to remove any residual wash solution. The column was transferred into a clean 1.5 ml microfuge tube and adds 50 µl of elution buffer, incubated at room temperature for 2 min and when store purified DNA at -20°C.

16S sequencing analysis:

The sequencing of the product PCR was carried through in an automatic sequencer ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle Sequencing Kits following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using Rbcl Forward primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Microgen Company).

Computational analysis (BLASTn):

The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) Sequences were aligned using Align Sequences Nucleotide BLAST.

Identification by 16S rRNA:

All bacteria contain 16S ribosomal RNA (rRNA) genes of approximately 1500bp in length. RNA genes contain regions of variable DNA sequence that are unique to the species carrying the gene. The species identity of an unknown bacterium may therefore be deduced from its unique rRNA gene sequence rRNA genes are first amplified using PCR technology. After amplification, PCR cycle sequencing is performed, and the rRNA sequence determined using a capillary sequence analyzer. The resulting sequence is then matched to known rRNA sequences in Gen-Bank® and validated using a rigorous review process.

RESULTS AND DISCUSSION

Employing morphological and biochemical testing to identify and characterize bacterial isolates:

Thirty-eight bacterial isolates were obtained from 18 karish cheese samples. All isolated (38 isolates) grown upon pseudomonas agar media as indicated, gender distinctions were determined at the level of several according to Garrity, (2007), conventional techniques include gram stain, gelatin liquefaction, oxidase, motility, catalase, citrate using, acidity production by glucose, starch hydrolysis, and the Voges-Proskauer interaction.

Generally, Table (2) exhibits the features of isolated strains. According to the information in this table, out of the 38 isolates, 16 (42.1%) were gram-negative, shaped like a rod, pigment producing likewise, motile. however, the remainder 22 (57.9%) strains that produced outcomes in opposition to those of the prior results were disregarded. Additionally, Table (2) data showed that 16 (100%) of 16 isolates successfully produced catalase and pigment production, were positive for glucose fermentation, KOH, oxidase and utilization of citrate, while these strains gave negative results with voges proskauer, methyl red, indole, starch hydrolysis and levan formation. In addition, from these strains, 14 (87.5%), 12 (75 %), 10 (62.5%) and 12 (75.4%) gave positive results with arginine hydrolysis, gelatin liquefied, development at 4°C and development at 41°C, respectively.

Proteolytic Function:

At all temperatures investigated, we measured the isolates' proteolytic function. The formation of a halo in the infected area served as a marker for extracellular enzyme activity on milk agar. After a 5 days of milk agar incubation, proteolytic function was found in 87.5% (14/16) of the isolates at 25ºC, 75 % (12/16) of the isolates at 10ºC and 56.25% (9/16) at 7ºC and 37.5 % (6/16) at 4ºC and 25 % (4/16) at 2ºC.

According to Ralyea et al., (1998) and Hayes et al., (2002), Pseudomonas spp. are typically a sign of contamination after pasteurization in food. The thermal setups used by the dairy operations can quickly kill Pseudomonas species, however, most of the enzymes protease as well as lipase abilities resist pasteurization at extreme temperatures for a short period of time and even ultra-high temperatures treatments and continue to be present in the foods that are created from them (Zhang Chen et al., 2003; Hantsis-Zacharov and Halpern, 2007) According to Rajmohan et al., (2002); Datta and Deeth (2003), enzymes protease as well lipase can lead to a variety of troubles and deterioration, such as rancidness, bitterness and proteolysis.

Screening of extracellular lipase produced Pseudomonas spp:

The isolated strains showed the ability to produce lipase enzyme when strains plated

on tributyrin agar (Merck). Plates of tributyrin agar were left to incubate for 48 hrs at 30°C. there was a space from 16 isolates indicated lipase activity.

Synthesis of biofilms:

Isolated Pseudomonas aeruginosa (n=16) that could form biofilms was performed using the CRA technique, 10 isolates (62.5%) were determined to have biofilm activities. The medium had been incubated at 37°C at conditions of aerobic growth lasting a period of 24 to 48 hrs. When the period of incubation is over, Colonies began to show color variations. Biofilm generation was assessed as being present in isolates that formed black-gray colonies on CRA, and colonies with red-pink color considered negative.

One of the main causes of failing to treat Pseudomonas aeruginosa is the growth of biofilms. The presence of exopolysaccharide (EPS), which is in the structure of the biofilm is regarded to be essential for the survival of the bacteria., and for situations where EPS is removed out of biofilms during test conditions, according to Watnick and Kolter (2000), the bacteria grow increasingly dependent on the way of treating against microbes. It is believed that a variety of factors contribute to biofilms' resistance. Not each of the layers in the biofilm are penetrated by the antibacterial substance. Polymeric components in the matrix of biofilm are known to make antibiotics diffusion more difficult. that indicates they will never accumulate enough antibiotic.

Biofilms contain at minimum some nutrient-starved cells, forcing them to transition into a slow development stage. Several antimicrobial substances do not affect bacteria that are growing slow or fails to develop, and a lot of such cells remain alive. Inside the structure of biofilm, bacteria move resistant gene sequences with one another (cIftçi et al., 2005).

Molecular characterization for examined isolates:

A single species had been selected from a 16 isolates after being first recognized using biochemical as well morphological testing as it suited the description of Pseudomonas aeruginosa in Systematic Bacteriology in Bergey’s Manual for (2007); Garrity, (2007). After that, a molecular study of this strain was performed.

One Pseudomonas aeruginosa strain was investigated, the findings of the 16 S ribosomal RNA (rRNA) gene with Electrophoresis fragments and fragment amplification using PCR in a 1.5% agarose gel are shown in Fig (1). The 16 S ribosomal RNA (rRNA) gene's partial sequencing was used to identify the species. These findings in Fig. 1 reveal that the 1380 bp amplified segment from the karish cheese obtained in lane (1) was Pseudomonas aeruginosa.

Relationship between the phylogeny of the genus's and species Pseudomonas:

Fig (2) provides a description of the use of 16S ribosomal RNA (rRNA) gene sequences to identify relations among closely linked species. According to phylogenetic research, isolates from the same species are linked to one another deeper than strains from other species. We have demonstrated how different subspecies of exactly the same kinds can be distinguished based on the degree of differentiation of their 16S ribosomal RNA (rRNA) gene sequences. Consequently, the phylogenetic tree was produced using the BLAST tree construct in https://www.ncbi.nlm.nih.gov/blast/treeview according to Fast Minimum Evolution, which used database sequences, length polymorphism of the PCR-amplified, and 16S ribosomal RNA sequence comparisons.

Submission to database and accession number:

The data shown in Fig (3) demonstrates a new overview of the discovery of a novel Pseudomonas aeruginosa strain by the molecular isolation from the karish cheese in Egypt. The results of the genome sequence analysis were then overviewed by GenBank in the NCBI database. Under the new accession number "OM756757" and the designation Pseudomonas aeruginosa G4, it has been officially certified to have been added to GenBank and made accessible to the general public.

CONCLUSION

The research above provides proof that Pseudomonas aeruginosa can easily spoiled karish cheese when it is impacted by temperature changes in the refrigerator. That emphasizes how important it is to prevent these species from contaminating cheese within manufacturing.

Finally, the Pseudomonas aeruginosa G4 strain of karish cheese was isolated, recognized by biochemical and morphological analysis then later verified by 16S rRNA sequence. Fragment of the DNA

sample were recovered during polymerase chain reaction (PCR) amplification. With the accession number "OM756757," the fragments have been sequenced and uploaded to GenBank (NCBI). it was determined that the examined Pseudomonas aeruginosa has the ability for biofilm formation. This study suggests the intensive need to improve hygienic practices during karish cheese preparing.

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Table 1: The standard 16S rRNA gene primers:

Primer Code	Sequence	Product Size
27F	5′- AGAGTTTGATCCTGGCTAG -3′	1500bp
1492R	5′- GGTTACCTTGTTACGACTT -3′	

Table 2: Morphological and Biochemical tests used for the identification of isolates:

Source of samples	No. of samples	No. of isolates	Morphological tests by used Microscope							
			Gram staining		Morphology		Pigment		Motility	
			+	-	Rods	Cocci	+	-	+	-
Karish cheese	18	38	22	16	24	14	16	8	16	8

Biochemical tests used for identification of isolates

No. of isolates	KOH		Catalase production		Starch Hydrolysis		Oxidase production		Arginine Hydrolysis		Voges Proskauer		Gelatin liquefied		Levan formation	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
16	16	-	16	-	-	16	16	-	14	2	-	16	12	4	-	16

Biochemical tests used for identification of isolates

No. of isolates	Utilization of Citrate		Methyl red		Acid from glucose		Indole		Growth at 4 °C		Growth at 41 °C	
	+	-	+	-	+	-	+	-	+	-	+	-
16	16	-	-	16	16	-	-	16	10	4	12	4

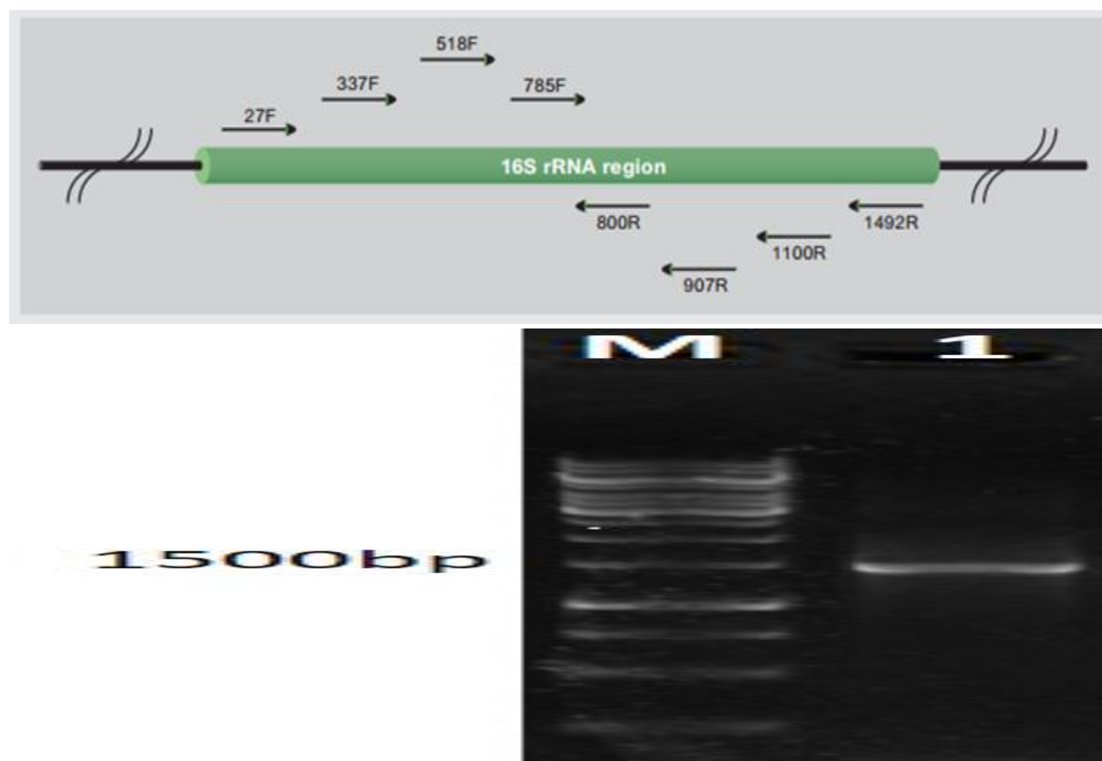

Figure 1: PCR amplified fragments and electrophoresis analysis using 16S ribosomal RNA (rRNA) gene in a 1.5 % agarose gel, for (1) *Pseudomonas aeruginosa* G4 (Lane M: DNA ladder), amplified fragment 1380 bp from karish cheese.

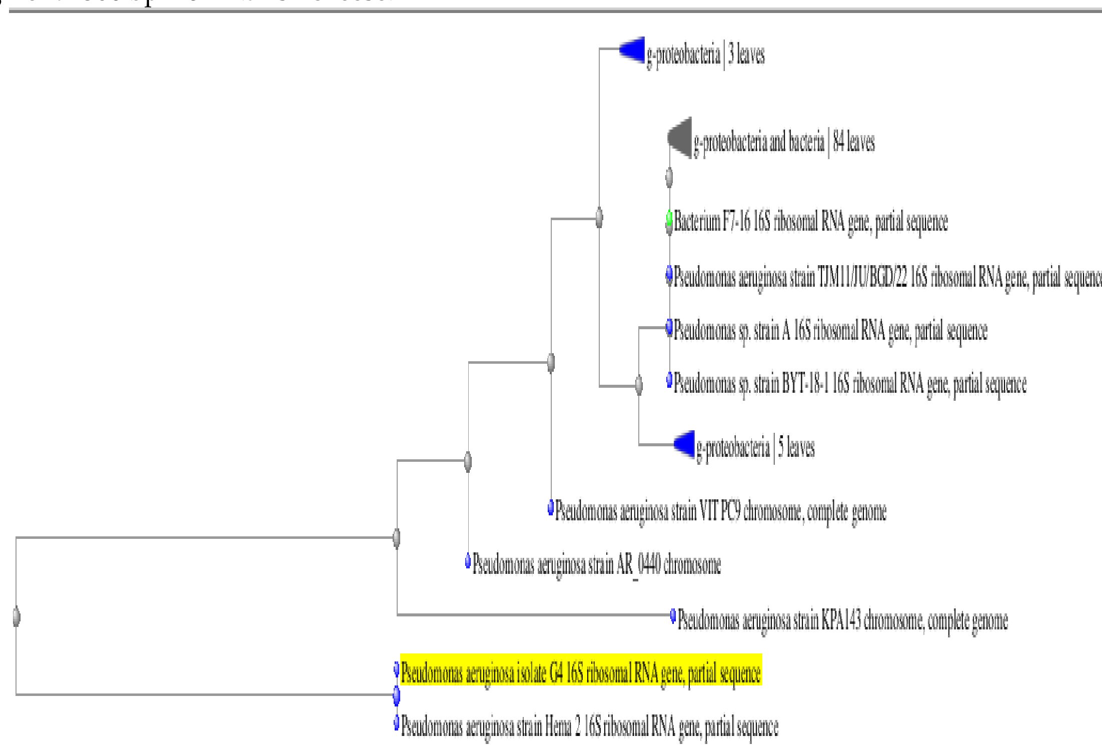

Figure 2: The relationships between individuals belonging to the *Pseudomonas aeruginosa* G4 is presented in a phylogenetic tree based on 16S ribosomal RNA sequences.

Pseudomonas aeruginosa isolate G4 16S ribosomal RNA gene, partial sequence

GenBank: OM756757.1

FASTA Graphics

Go to:

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LOCUS       OM756757                1112 bp    DNA     linear   BCT 18-APR-2022
DEFINITION  Pseudomonas aeruginosa isolate G4 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   OM756757
VERSION     OM756757.1
KEYWORDS    .
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Pseudomonadota; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1112)
  AUTHORS   Nour,M.A., Soliman,S.A., Abdella,S.A. and Elgharabawy,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-FEB-2022) Dairy Department, Faculty of Agric.
            Al-Azhar Uni, Cairo, Egypt
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /mol_type="genomic DNA"
                     /isolate="G4"
                     /isolation_source="Karish cheese"
                     /db_xref="taxon:287"
                     /country="Egypt"
     rRNA            <1..>1112
                     /product="16S ribosomal RNA"
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       61 cttaagttgg gaggaaggca gtaaggttaa taacctttgc tgtttttgac gttagcaaca
      121 agaaataaag caccggctaa acttcgtgcc agcagcgcgg taatacgaaa gggtgcaagc
      181 gttaatcgga attactgggg cgtaaagcgc gcgtaggtgg ttcagcaagt tggatgtgaa
      241 atccccgggg ctcaaacctg ggaactgcat cccaaaacta ctgagctaga gtacggtaga
      301 gggtggtgga atttcctgtg tagcggtgaa atgcgtagat ataggaagga acaccagtgg
      361 cgaaggcgac cacctggact gatactgaca ctgaggtgcg aaagcgtggg gagcaaacag
      421 gattagatac cctggtagtc cacgccgtaa acgatgtcga ctagccgttg ggatccttga
      481 gatcttagtg gcgcagctaa cgcgataagt cgaccgcctg gggagtacgg ccgcaaggtt
      541 aaaactcaaa tgaattgacg ggggcccgca caagcggtgg agcatgtggt ttaattcgaa
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      661 accttcggga actcagacac aggtgctgca tggctgtcgt cagctcgtgt cgtgagatgt
      721 tgggttaagt cccgtaacga gcgcaaccct tgtccttagt taccagcacc tcgggtgggc
      781 actctaagga gactgccggt gacaaaccgg aggaaggtgg ggatgacgtc aagtcatcat
      841 ggcccttacg gccagggcta cacacgtgct acaatggtcg gtacaaaggg ttgccaagcc
      901 gcgaggtgga gctaatccca taaaaccgat cgtagtccgg atcgcagtct gcaactcgac
      961 tgcgtgaagt cggaatcgct agtaatcgtg aatcagaatg tcacggtgaa tacgttcccg
     1021 ggccttgtac acaccgccct cacaccatgg gagtgggttg ctctcagaag tagctagtct
     1081 aaccgcaagg ggacgtactg tcaacgatgt ga
//
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Figure 3: NCBI Flat file for Egyptian *Pseudomonas aeruginosa* G4 16S ribosomal RNA gene, partial sequences.

تعريف بكتيريا البسيدومونس أرجونزا المعزولة من الجبن القريش بواسطة تقنية تفاعل البلمرة المتسلسل

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الملخص العربي:

هدفت هذه الدراسة إلى عزل بكتيريا Pseudomonas aeruginosa من الجبن القريش ودراسة خصائصها المحللة للبروتين عند درجات الحرارة المنخفضة أثناء التخزين والتلوث بسبب وجود الإنزيمات المحللة للبروتين التي تنتجها بكتيريا Pseudomonas aeruginosa. تم عزل Pseudomonas aeruginosa وتعريفها بالإختبارات المورفولوجية والكيميائية الحيوية ، كما تم تأكيد تعريفها بواسطة تسلسل 16S rRNA ، حيث كانت السلالة المعزولة موجبة لتفاعلات الأوكسيديز ، الكتاليز ، الحركة ، KOH ، إستهلاك السترات وتحليل الأرجينين . بينما كانت هذه السلالة سالبة لإختبار الإندول ، أحمر الميثيل ، تحلل النشا وتفاعل Voges-Proskauer. وطبقاً لتفاعل البلمرة المتسلسل (PCR) قد تم تعريف السلالة وتسجيلها عن طريق تقديمها إلى GenBank في المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI) وتحريرها للجمهور تحت رقم الإنضمام "OM756757" ، كما تم دراسة الخصائص المحللة للبروتين لسلالة Pseudomonas aeruginosa بإستخدام الوسط الغذائي Milk agar ، وكذلك تم دراسة تكوينها Biofilm بإستخدام الوسط الغذائي Congo red agar (CRA) . هذه الدراسة تقدم معلومات حول مدي تواجد Pseudomonas aeruginosa في الجبن القريش بسبب قلة تدابير النظافة والمعاملة الحرارية خلال تصنيع الجبن القريش .

الكلمات الاسترشادية : جبن قريش، البسيدومنس ارجونزا، الجين بنك، تكوين البيوفيلم .