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Research Article

Bacteriocin extraction from bacterial samples and study the effect on the other types of pathogenic bacteria

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Abstract
This study investigated the antibacterial activity of a lectin-like bacteriocin extracted from *Pseudomonas putida* isolated from various soil samples in Baghdad. Thirteen new isolates of *P. putida* were identified using morphological, physiological, and molecular methods. Real-time PCR identified three isolates carrying the bacteriocin gene. Antibiotic sensitivity was tested on pathogenic bacteria, and one isolate from each species exhibiting broad-spectrum resistance was selected. The crude bacteriocin from 13 isolates showed antimicrobial activity against six pathogenic bacterial species, with five isolates exhibiting inhibition zones. Purification resulted in a bacteriocin with a total protein concentration of ~4669µg/ml and an apparent molecular mass of ≤11 kDa. HPLC confirmed the molecular mass. This study quantified the expression of *P. putida* bacteriocin genes, characterized as putadicin, with potential applications in human medicine for burn treatment in Iraq.
Keywords: Bacteriocin, antibacterial activity, Lactobacilli, bacteriocin encoding genes

Introduction
Pseudomonas putida is a fluorescent saprotrophic soil bacterium Pseudomonad, *Pseudomonas*, Gram-negative, rod-shaped. Based on an analysis of 16S rRNA, *P. putida* was taxonomically confirmed as a *Pseudomonas species* and placed in the *P. putida* group along with many other species, to which it lends its name (Palleroni, 2008). *P. putida* is an aerobic oxidase-positive bacterium, with one or more flagella *P. putida* is environment sensitive and suppresses changes in flagella rotation direction when sensing chemoattractants. This is very helpful in guiding the *P.*

putida to propel towards plant seeds that provide nutrients to the bacterial cells (Henry & Speert, 2011). A variety of *P. putida*, called Pseudomonas multi plasmid-degrading hydrocarbons, is the world's first patented organism and they are a living organism. This demonstrates a very diverse metabolism including the ability to reduce organic solvents such as toluene. (Akkaya et al., 2018). *P. putida* developed genetically to obtain a modified strain that carries enzyme-coding genes that mediate xylene, toluene, octane, and camphor degradation to CO2 and H2O. These genes are born on plasmids and have manipulated by general microbiological and genetic engineering to obtain bug strain containing all four gene sets (Belda et al., 2016). *P. putida* has demonstrated an ability to suppress a variety of plant pathogens and reduce plant disease incidence (Kilic and Yuen, 2003). This may be partly due to its inhibition of plant pathogenic microorganisms by sequestration of iron or by the production of antibiotic metabolites. The plant may also be involved in the formation of a 66- siderophore complex. *P. putida* has been found to produce bio-surfactants that disturb the Phytophthora membrane. Capsici zoospores within minutes, causing death. Phy. capsici causing black pepper (spice crop) to become dead.

The use of *P. putida* as control method for *Phytophthora capsici* will be more effective than using pure bio-surfactants created in a laboratory (Tran et al., 2008). *P. putida* strains lack a number of key virulence determinants that are necessary. There is no evidence in the *P. putida* genome that it contains genes encoded for exotoxin A, phospholipase C (Nelson et al. 2002) or pectin lyase that are frequently present in plant and animal pathogens. Furthermore, *P. putida* strains lack the type III secretion pathway in the plant pathogens P. syringae pv. Tomato (Feil et al., 2005). *P. putida* causes septicemia, pneumonia, urinary tract infections, nosocomial bacteremia, septic arthritis, or peritonitis in immunocompromised patients (Carpenter et al., 2008). The entire sequencing of genomes of significant strains, such as *P. putida* KT2440 (Nelson et al., 2012; Kuepper et al., 2015), has been carried out, serving as a fundamental resource for comprehending metabolic networks and using advanced methodologies in strain production (Martínez-García et al., 2014). *Pseudomonas putida* is considered a favorable host for the heterologous expression of GC-rich bacterial genes, such as those found in actinobacteria or myxobacteria. This is due to its comparatively high guanine-cytosine (GC) content of 61.5%. These bacterial groups are known for their abundance of secondary metabolite gene clusters..

Martial and methods

All media were prepared according to the manufacturing company's instructions; the ingredients dissolved in distilled water (DW). The pH was adjusted to 7.2 ± with 0.1N NaOH or 0.1N HCl 0.2, all constituents had to be completely dissolved in the water bath. Media sterilization was done at 121°C for 15 min at 15 pound / inch^2 by autoclaving. Then, it was distributed in sterile tubes or Petri dishes; the media was incubated overnight at 37°C to ensure sterility.

Collection of samples for isolation of bacteria

Twenty-eight samples were collected from different Iraqi cities including Baghdad and Al-Kut. The highest number of samples was collected from Baghdad. Soil samples were collected from different areas of Iraq. The samples were collected from a depth (5-10) cm from the soil surface near the roots area. Each sample was collected under aseptic conditions and then transported to the laboratory until use.

Morphological examination

The primary diagnostic tests utilized in this study involved examining the morphological characteristics of bacterial growth on various media, including MacConkey agar, nutrient agar, pseudomonas agar, and Uti agar. These tests were employed to differentiate the phenotypic attributes of bacterial isolates obtained from each implant medium. Subsequently, the isolates were cultured on MacConkey agar and subjected to diagnosis using the Vitek 2 compact system device. The plates were incubated for three days at a temperature ranging from 25 to 37 degrees Celsius. Following this incubation period, the bacterial colonies were subjected to purification by sub-culturing on a nutrient agar medium until a pure culture was achieved. Pseudomonas agar, MacConkey agar and UTi agar have been studied including the colony shape, texture, color and edges (Bailey & Scotte, 2014).

Identification and sensitive tests using VITEK 2 System

A specific number of bacterial isolates selected using the Vitek 2 system, to confirm their susceptibility to identification. The Vitek 2 system consists of an instrument, a computer, and an instrument consisting of five basic components.

DNA extraction from bacteria

Genomic DNA was extracted using the extraction kit G-spin DNA. DNA extracted from the fresh cultures of the selected bacterial isolate as described, according to the protocol stated by the kit manufacturer. Taking 3 µl of the processor loading buffer (Intron / Korea) was mixed with 5 µl of the supposed DNA being electrophoresis (loading dye), the loading process is now in the holes of

the gel after the mixing process. The tincture was subjected to an electrical current of 7 V/cm² for of 1-2 hours, resulting in its migration to the other side of the gel. The gel was subjected to analysis using a 336 nm ultraviolet (UV) source after its immersion in a pool consisting of a 30µl solution of red safe nucleic acid stain and a 500 ml solution of distilled water.

The primers used in the interaction

The primers were investigated by Integrated DNA Technologies Company (IDT) in Canada. The primers were lyophilized and subsequently dissolved in deionized water (ddH2O) to achieve a final concentration of 100 picomoles per microliter (pmol/µl) as a stock solution. The stock solution was stored at -20 degrees Celsius. To prepare the working primer suspension with a concentration of 10 pmol/µl, 10 microliters of the stock solution were mixed with 90 microliters of ddH2O, resulting in a final volume of 100 microliters. The iNtRON Maxime PCR Pre-Mix Kit offers a diverse range of experience-based Pre-Mix Kits, in addition to a 2X Master Mix solution. The Maxime PCR Pre Mix Kit I is a product that combines all necessary components for a single reaction polymerase chain reaction (PCR) into a single tube. All the compounds mixed in table (3). In a PCR tube containing the lyophilized premix, the PCR mixture was set up in total volume, and the remaining volume was completed with sterile DW. Then vortexed to 25µl. In the thermocycler PCR instrument, PCR reaction tubes were placed where DNA was amplified as shown in Tables (1, 2).

Table 1. The optimum condition for detection of the universal gene of *Pseudomonas putida* by 16SrRNA

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min.	1 cycle
2-	Denaturation -2	95°C	45 sec	35 cycle
3-	Annealing	58°C	45 sec	
4-	Extension-1	72°C	45sec	
5-	Extension -2	72°C	7 min.	1 cycle

Table 2. The optimum condition for detection the specific gene of *Pseudomonas putida* by 16SrRNA

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min.	1 cycle
2-	Denaturation -2	95°C	45sec	40 cycle
3-	Annealing	56°C	45sec	
4-	Extension-1	72°C	45sec	
5-	Extension -2	72°C	7 min.	1 cycle

Real time PCR (qPCR)

The reaction mixture was made under low-temperature conditions using an ice bath. To account for potential pipetting losses, a slightly higher quantity of master mix was prepared than the amount necessary. The RNA sample was administered after the dispensation of aliquots of this combination into the micro-tubes.

Table 3. Compound mixture of Rreal-time PCR

Reagents	Volumes
5 × Prime Script TM mix	2 µl 1
Total RNA	
RNase Free dH2O	up to 10 µ l
	Total 10 µ l *2

Measurement of the activity of bacteriocin

Bacteriocin activity was measured using the standard well-diffusion assay described by Kang and Lee (2005).

Sample and Electrophoresis Loading (Hames, 1985)

The procedure for loading liquid samples onto both native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is outlined as follows. During the process of electrophoresis, the gel matrices are securely positioned, and the reservoir buffer is carefully added until it reaches the uppermost region of the gel. The sample is meticulously loaded onto the gel surface using a micro-syringe or micropipette. The experimental setup included the use of electrophoresis equipment, which was coupled to a power pack. The gel, which was under investigation, was exposed to a voltage of 100 volts for 2 hours.

Gel Staining

The solution used in this study is Coomassie Brilliant Blue. G-250 was synthesized by the dissolution of 100 mg of Coomassie blue in a 50 mL combination of water, methanol, and glacial acetic acid in a 5:5:2 volume ratio. The solution was stirred and then brought to a final volume of 1 L using distilled water. Subsequently, it was filtered through a Whatman No.1 filter paper and kept in an opaque container at a temperature of 4°C until it was ready for use. The approach described by Chang et al. (1991) was used to obtain protein staining in the native gel. The protein bands were seen in SDS-PAGE by the immersion of the gel in a solution containing Coomassie blue dye. The reaction was terminated after three hours by the process of washing the stain solution with a solution containing acetic acid at a concentration of %.

Results and Discussion

DNA extraction from bacteria DNA was extracted using the G- spin kit; the concentration ranged from 50 to 300 µg / µl while the purity was approximately 1.8-2.0 with a ratio reaching 1.8. Considering the DNA pure generally is acceptable. Unless the ratio was lower than previously reported, certain pollutants with an absorbency of 260/280 nm such as protein or phenol could be present (William, 1997). In addition, the technique of gel electrophoresis explained the presence of a single band; thus, it can be concluded that the DNA was completely pure (Figure 1).

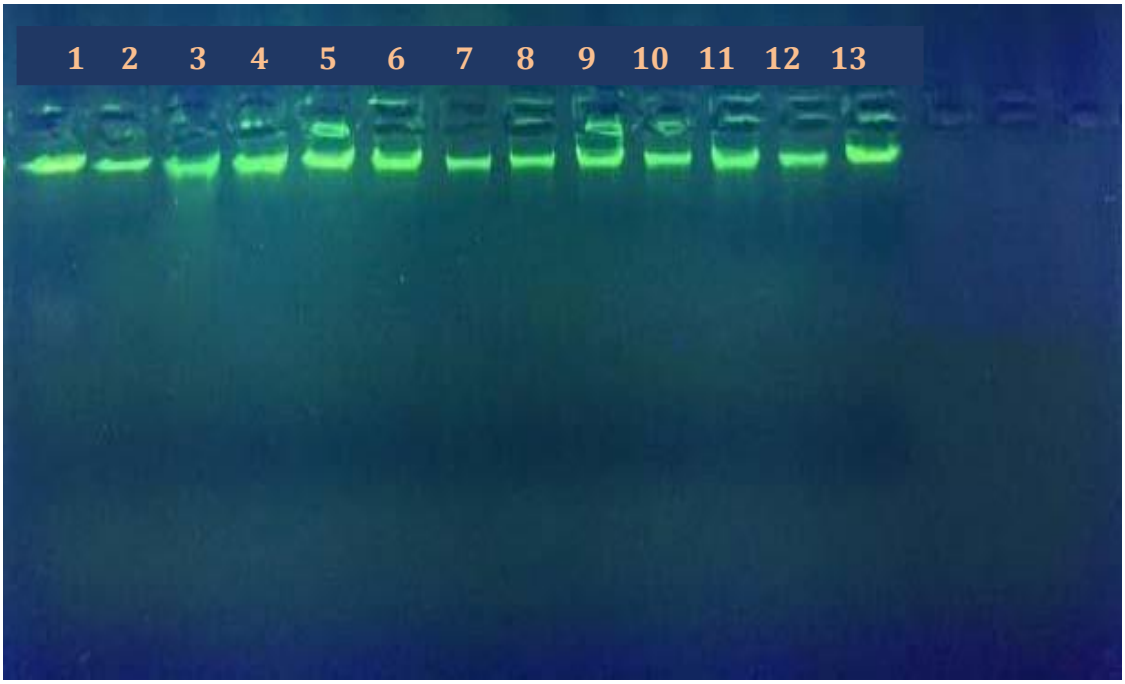

Figure 1. Gel electrophoresis of genomic DNA extraction from bacteria, 1% agarose gel at 5 vol. /cm for 1hr.

Identification of *P. putida* by16s rRNA and Sequencing

To validate the identification of *P. putida* isolates, a 16s rRNA gene fragment was amplified using a set of primers specifically designed for *P. putida* species specificity. The findings indicated that all isolates, which were previously classified as *Pseudomonas putida* using the Vitak 2 technique, had consistent results. The molecular methodology was used to confirm the identification of *P. putida.* This was achieved by using the universal gene of 16S rRNA primer and afterward subjecting the samples to sequencing. The PCR method was employed to amplify all 13 isolates, resulting in successful identification as *P. putida* (Fig. 2).

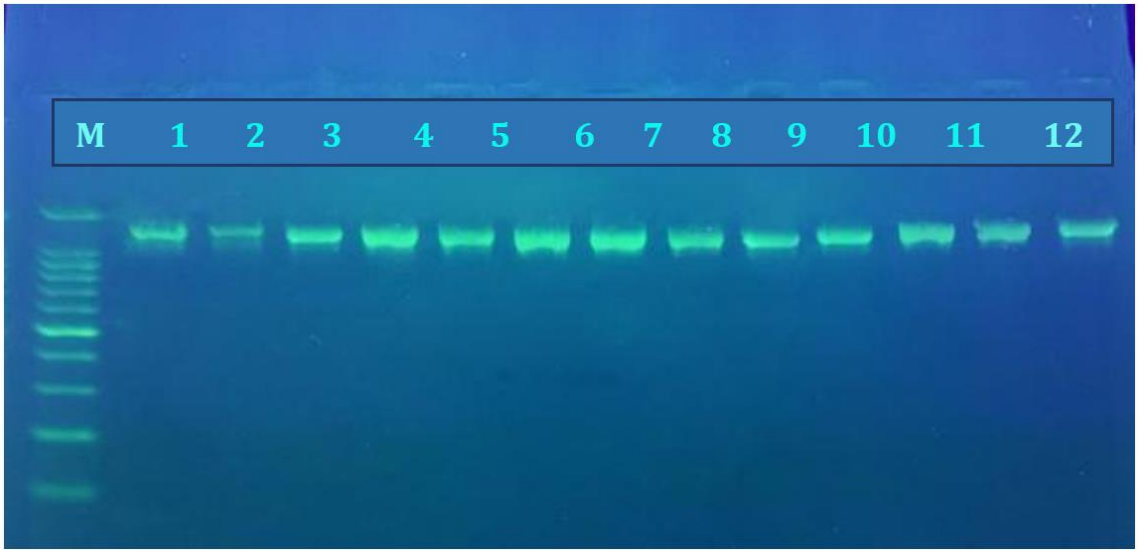

Figure 2. Agarose gel electrophoresis for 16 sRNA gene (1250 bp.) amplicon of *P. putida*. Bands were fractionated by electrophoresis on a 2% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100) and visualized under U.V. light after staining with red stain.

Then used the specific gene for detection of the isolates that carried the Putadicin gene, which showed 3 isolates only from 13 carried this gene (Figure 3). Using the appropriate gene probes; any bacterial pathogen can be detected and identified even at the species; this technique also allows for a quick and accurate identification of bacteria or other microbes regardless of their growth rates or metabolic activity. This is especially helpful in detecting metabolically inactive and dormant bacteria, as the number of ribosomes in such organisms is not significantly affected (Almanza et al., 2017).

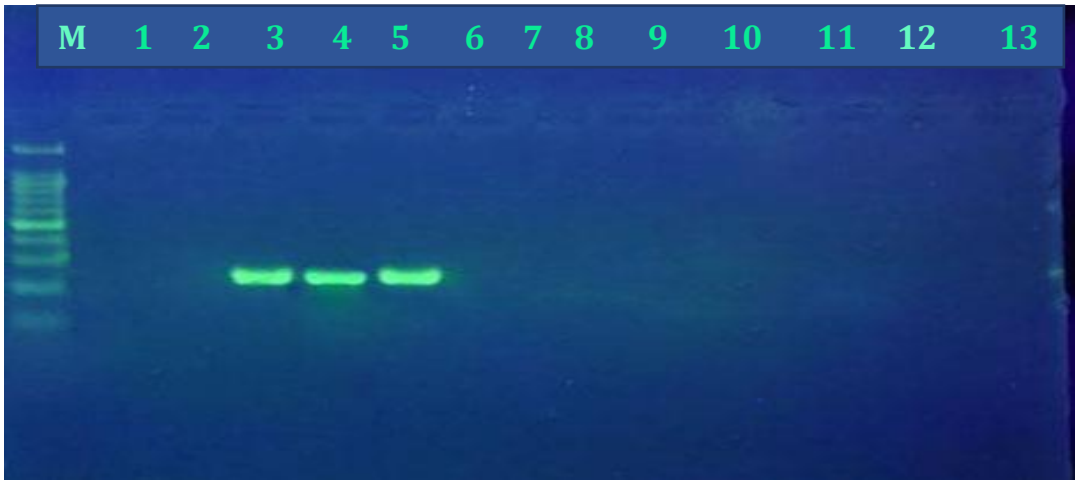

Figure 3. Agarose gel electrophoresis for Puticin gene. Bands were fractionated by electrophoresis on a 2% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100) and visualized under U.V. light after staining with red stain.

Sequencing of *P. putida*

The PCR products were sequenced and compared to the standard 16s rRNA sequence with accession numbers of the 16s rRNA gene fragments for the nucleotide sequences. In addition; the results of this study revealed a high similarity level (98-100 %) which further confirms the identification of these isolates as *P. putida.* (Al-Sudani et al., 2020) The process of sequencing the 16S rRNA was conducted at Macrogen Company. The resulting findings were compared with the data collected from the ExPASY program, which is published by the Gene Bank and may be accessed online at the National Center for Biotechnology Information (NCBI). The investigation of 16S rRNA genes often involves the examination of nine hypervariable areas, which exhibit significant sequence variation across distinct bacterial species and may serve as a means of species identification (Montazeri et al., 2015). After sequencing, both DNA sequences were also analyzed and compared to the partial and complete 16S rRNA gene with the Basic Local Alignment Search Tool (BLAST)as shown (tables 4, 5) in the National Center for Biotechnology Information (NCBI) for classification (Liu et al.,2012; Li et al.,2012). All thirteen samples of *Pseudomonas putida* were sequenced for the near complete length of the 16S rRNA gene. Two samples were specifically chosen based on their proximity to the desired length. These selected samples were then registered with the National Center for Biotechnology Information (NCBI) and successfully passed the validation process, resulting in the establishment of reference sequence entries. The present compilation will be used in continuing scholarly investigations when further type strains become

accessible for retrieval from the National Center for Biotechnology Information (NCBI) website, located at https://www.ncbi.nlm.nih.gov. According to the study conducted by Al_Sudani et al. (2020),

Specific target regions in the *P. putida* genome were amplified using the amplification method such as PCR. The 16S rRNA gene's broad range of PCR targets, which is the most common housekeeping genetic marker since the ribosomal RNA was defined as the small subunit that is universally present in all bacteria and consists of two regions– conserved and variable (Janda et al., 2007; Vetrovsky et a*l*., 2013). The method of PCR amplification of conserved sections from the bacterial genome, particularly the 16S rRNA gene, followed by sequence analysis, has been widely used for the identification of *P. putida* strains (Relman et al., 1999). The 16SrRNA gene sequence is widely recognized as the optimal standard for determining the phylogenetic connection of microorganisms (Nayak et al., 2011).Universal primers are used to target the conserved sections in order to ascertain the presence of a microorganism, whereas species-specific primers are employed to target the variable regions. The amplified target regions undergo sequencing to identify the genus and species using universal primers (Mignard et al., 2006).

Table 4. Represents polymorphic types of parital 16S rRNA gene isolated from *P. putida*

No.	Type of substitution	Location	Nucleotide	Sequence ID	Score	Expect	Identities	Source
1	Transition	861	A>G	ID: MG554688.1	1803	0.0	99%	***P. putida* 16S ribosomal RNA gene**
	Transition	867	A>G					
	Transvertion	940	G>C					
2	----------			ID: MG554688.1	1896	0.0	100%	***P. putida* 16S ribosomal RNA gene**
3	Transvertion	133	T>G	ID: MG554688.1	1651	0.0	99%	***P. putida* 16S ribosomal RNA gene**
	Transition	641	G>A					
	Transition	644	G>A					
	Transvertion	675	G>C					
	Transvertion	727	G>T					
	Transvertion	738	G>C					
	Transvertion	840	G>T					

	Transition	861	A>G					
	Transition	867	A>G					
	Transvertion	881	G>T					
	Transvertion	884	C>A					
	Transvertion	940	G>C					
4	----------			ID: MG554688.1	1840	0.0	100%	***P. putida* 16S ribosomal RNA gene**
5	----------			ID: MG554688.1	1920	0.0	100%	***P. putida* 16S ribosomal RNA gene**
6	----------			ID: MG554688.1	1840	0.0	100%	***P. putida* 16S ribosomal RNA gene**
7	----------			ID: MG554688.1	1896	0.0	100%	***P.putida* 16S ribosomal RNA gene**
8	----------			ID: MG554688.1	2039	0.0	100%	***P. putida* 16S ribosomal RNA gene**
9	Transvertion	743	A>C	ID: MG554688.1	1621	0.0	99%	***P. putida* 16S ribosomal RNA gene**
	Transvertion	777	A>C					
	Transvertion	803	T>A					
	Transition	860	A>G					
	Transition	867	A>G					
	Transvertion	881	G>C					
	Transition	912	A>G					
	Transvertion	916	G>T					
	Transition	926	G>A					
10	----------			ID: MG554688.1	2026	0.0	100%	***P. putida* 16S ribosomal RNA gene**

Table 5. Represents polymorphic types of complete 16S rRNA gene isolated from *P. putida*.

No. Of sample	Type of substitution	Location	Nucleotide	Sequence ID	Score	Identities	Source
1	Transition	3193654	A>G	ID: CP009974.1	712	98%	***Pseudomonas putida***
	Transvertion	3193808	G>C				
	Transition	3193840	A>G				
	Transition	3193888	A>G				
	Transvertion	3193891	C>G				
	Transition	3193939	G>A				
	Transition	3193953	G>A				
	Transition	3193957	T>C				
2	Transvertion	3193792	G>C	ID: CP009974.1	655	99%	***Pseudomonas putida***
	Transition	3193809	C>T				
	Transvertion	3193810	T>G				
	Transition	3194056	G>A				
3	Transition	3193654	A>G	ID: CP009974.1	800	99%	***Pseudomonas putida***
	Transition	**3194016**	**C>T**				

The phylogenetic tree analysis

The analysis of phylogenetic tree by the software Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 shows the phylogenetic trees of these species in Figures (4) which clarified the Neighbour-joining tree for phylogenetic analysis (Table 6). These alignments appeared to be the *P. putida* in 16S ribosomal gene for translating specific region between Iraq and other global strains by partial sequence similarity. Hierarchical cluster analysis determines the following clusters including *P. putida* Iraq isolates that are identical in partial sequence between 99-100 %, close to Iran (ID: MG554688.1), whereas in complete sequence it is close to the Netherlands between 98-99 % (ID: CP009974.1) The phylogenetic analysis showed that the compatibility values for Proximity and the genetic dimension between themselves and the world exceeded 98 %. The study detected the high genetic similarities between isolates from Iraqi *P. putida* and other global strains. The Neighbor-Joining approach was used to infer evolutionary history. The

evolutionary distances were calculated using the Jukes-Cantor model to determine the phylogenetic distance provided for in the Gene6 program.

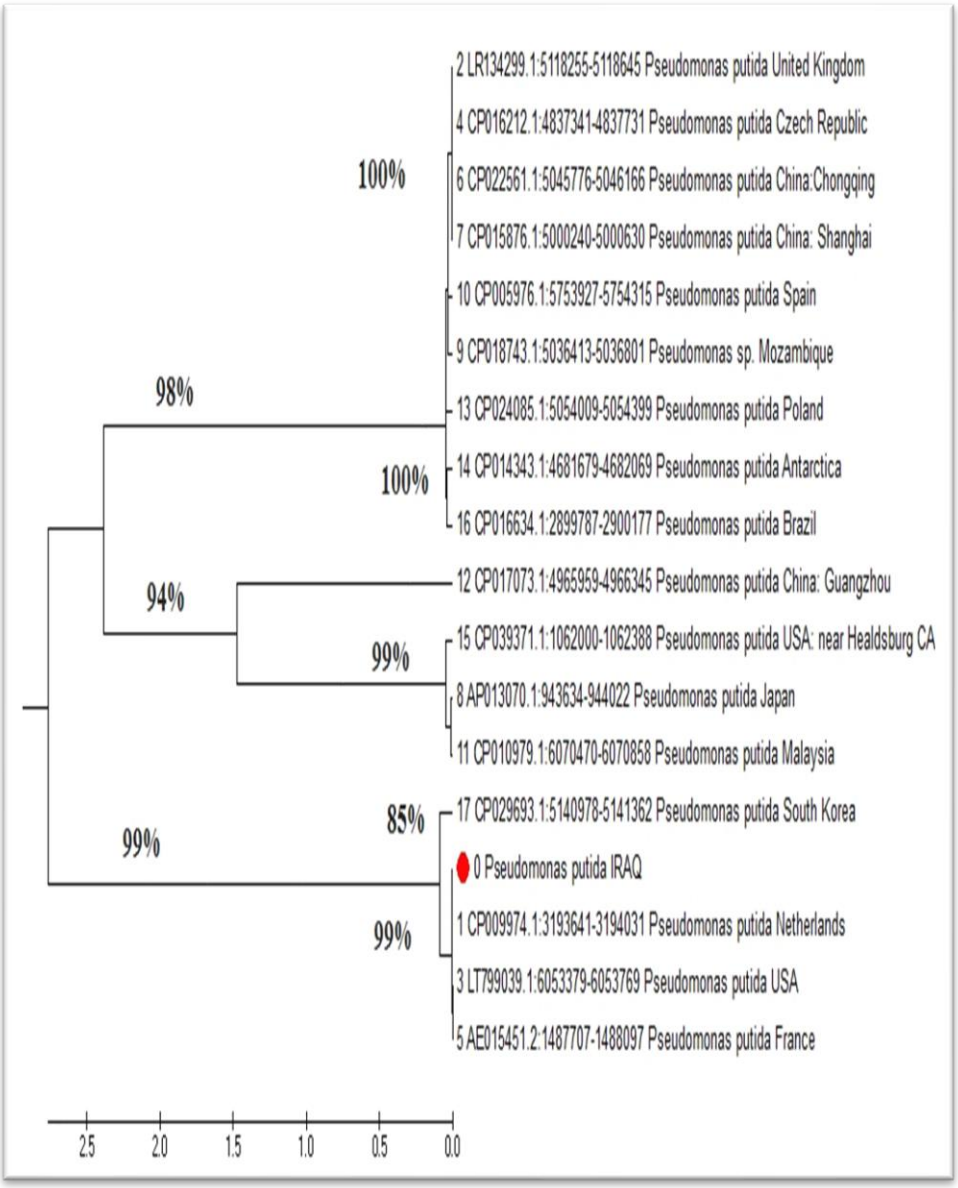

Figure 4. The genogram of the promising Iraqi *P. putida* phylogenetic relationship has closely related type and genetic distance to the global strains recovered from the NCBI Gen Bank. Bootstrap values have shown similarity and homology of the adjacent full-length 16S rRNA gene sequences

Table 6. Represents comparison of *P. putida* between different countries registered in the National Center Biotechnology Information (NCBI) but with different sources of isolation.

No.	Accession	Country	Source	Compatibility
1.	ID:MG554688.1	Iran	*P. putida*	**100%**
2.	ID: CP009974.1	Netherlands	*P. putida*	**99%**
3.	ID: LR134299.1	United Kingdom	*P. putida*	**99%**
4.	ID: LT799039.1	USA	*P. putida*	**99%**
5.	ID: CP016212.1	Czech Republic	*P. putida*	**99%**
6.	ID: AE015451.2	France	*P. putida*	**99%**
7.	ID: CP022561.1	China: Chongqing	*P. putida*	**98%**
8.	ID: CP015876.1	China: Shanghai	*P. putida*	**98%**
9.	ID: AP013070.1	Japan	*P. putida*	**94%**
10.	ID: CP018743.1	Mozambique	*P. putida*	**94%**
11.	ID: CP005976.1	Spain	*P. putida*	**94%**
12.	ID: CP010979.1	Malaysia	*P. putida*	**94%**
13.	ID: CP017073.1	China: Guangzhou	*P. putida*	**94%**
14.	ID: CP024085.1	Poland	*P. putida*	**91%**
15.	ID: CP014343.1	Antarctica	*P. putida*	**91%**
16.	ID: CP039371.1	USA: near Healdsburg, CA	*P. putida*	**91%**
17.	ID: CP016634.1	Brazil	*P. putida*	**91%**
18.	ID: CP029693.1	South Korea	*P. putida*	**85%**

Submission of local Iraq isolates in NCBI

PCR amplification method has been used to amplify specific target genetic regions within the isolated *P. putida* genome from Iraq's soil. The use of broad-range polymerase chain reaction (PCR) is focused on amplifying the 16S rRNA gene, which serves as a prevalent genetic marker in housekeeping due to its universal presence in all bacteria (Taghavi et al., 2010; Shin et al.,

2012). The sequence analysis test detected 13 isolates similar to data from the *P. putida* Genome sequence was deposited with NCBI, Gene Bank, as shown in figure (5).

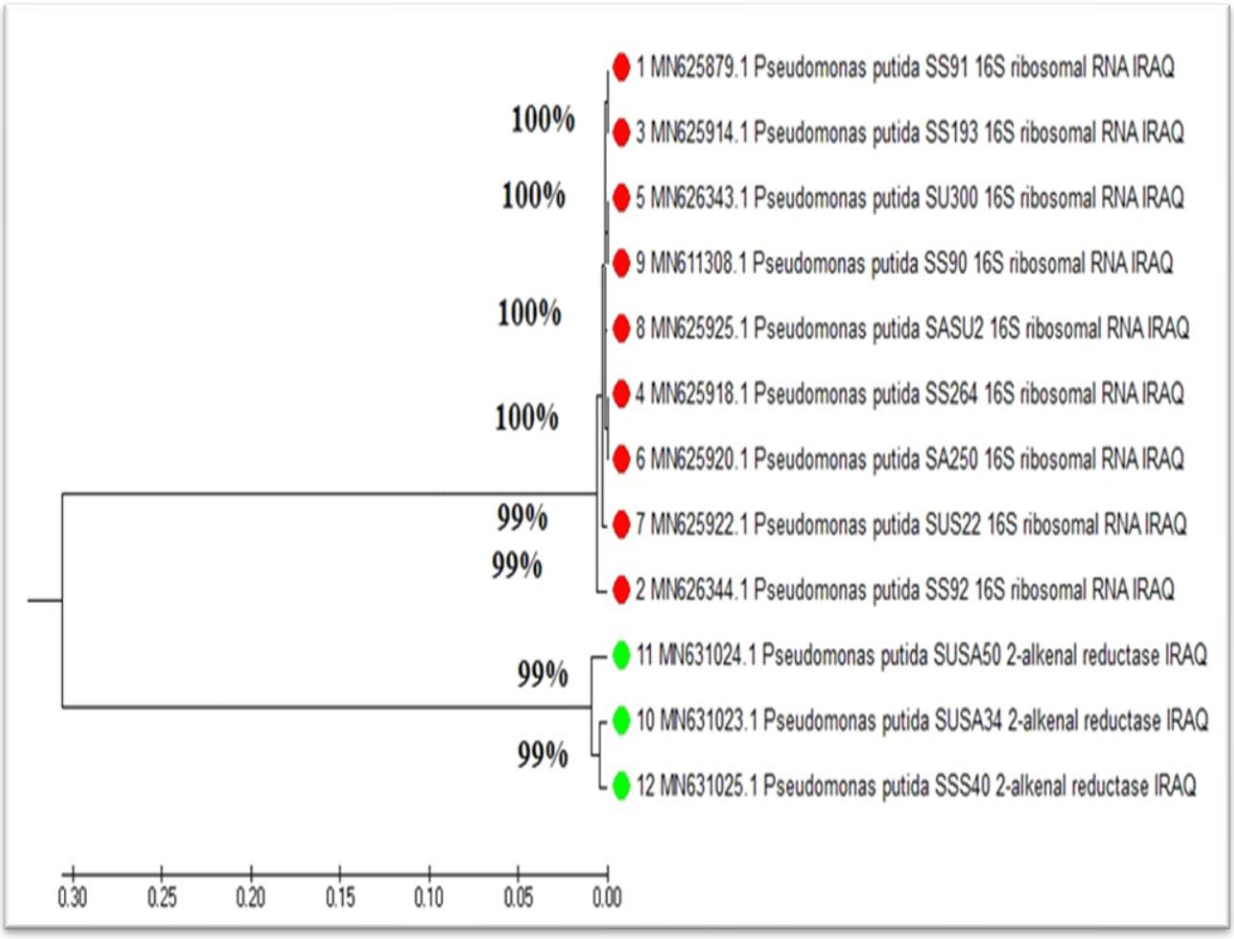

Figure 5. Showing phylogenetic relationships of promising Iraqi *P. putida* strains and recovered genetic distance from NCBI Gen Bank

The results of strains were coordinated at 98 -100% and have different length efficient in Database framework for comparative genome analyses when using BLAST score; which due to new recorded strains get ID: (MN611308.1, MN625879.1, MN625914.1, MN625918.1, MN625920.1, MN625922.1, MN625925.1, MN626343.1, MN626344.1, MN631023.1, MN631025.1, MN631024.1) in NCBI compared to NCBI obtaining *P. putida*; there appears to be a nucleotide difference for the translated specific region after comparison with the online database and the bacterial strains belonging to *P. putida* and most closely related to *P. putida* with ID: MG554688.1 and ID: CP009974.1(NCBI, gene bank data base). In this work, a phylogenetic analysis was conducted to assess the genetic distances across different geographic locations. These distances

were then used as a search criterion in the NCBI database to identify orthologues using the BLASTP/BLASTX algorithms. A stringent E-value cut-off of 0e was applied to identify specific genes of interest, particularly focusing on a portion length of the 16S rRNA gene in *Pseudomonas putida*. The value that is very close to zero means that these sequences were the same and the bit score: statistical measurement of the moral similarity and the higher value indicates the high degree of similarity, the sense that there is no mention of similarities. The gene hit sequences were acquired, followed by the construction of numerous DNA sequence alignments. Subsequently, a Neighbor-joining tree replicate bootstrap was generated to facilitate phylogenetic analysis. The genetic divergence of *Pseudomonas putida* strains from Iraq and those from other regions was assessed by alignments of partial and full sequences of the 16S ribosomal RNA gene, specifically focusing on a particular area. The average genetic distance between these groups was among the largest found in this population, determined for the larger branch (father group in genetic tree) spatial coordination. Neighborhood graduation came from the comparison recorded in the National Center for Biotechnology Information (NCBI) between *P. putida* isolates to appear to be in sequence respectively with source of *P. putida*. To facilitate the comparison of phylogenetic trees, we used sequences derived from the 16S rRNA gene sequences that were accessible in the GenBank / NCBI databases (Fig. 4). To complete this study, the sequences of the 16S rRNA gene of 13 *P. putida* samples were determined (Figure 5). These Iraqi strains had full sequence length similarity for this, it obtained the submission of the NCBI. For each strain, the distance analysis was used to determine the strains of *P. putida* from geographic genetic distances compared to other world strains (Baharak et al., 2018). Nevertheless, it is possible to distinguish the phenotypically similar *P. putida* species based on genotypic differences, particularly by analyzing housekeeping genes like 16S rRNA genes. The use of 16S rRNA gene distances was effective in facilitating the unambiguous differentiation of several species. Initially, a comparative analysis was conducted between Iraqi *P. putida* and other NCBI *P. putida* strains, focusing on particular nucleotide variations within the translated region. Additional research has corroborated the suitability of using sequence analysis of 16S rRNA genes as a viable approach for distinguishing taxonomic categories at more advanced levels. For instance, it has been shown that variations in sequences within 16S rRNA operons may impact the accuracy of phylogenetic analysis at the species level (Romilio et al., 2018).

RNA extraction from *P. putida*

Purified RNA was approximately 1.6- 1.8, which was quantified using Nano-Drop ND-1000 spectrophotometer at 260 nm. The RNA sample integrity number values were always above 8.5, which indicate that the RNA was not degraded; therefore, it was appropriated for the real-time reverse transcription-PCR analysis. The confirmation of the absence of contaminating DNA in RNA samples was achieved by observing the lack of amplification in samples that did not undergo reverse transcription. On the other hand, the confirmation of the absence of reverse transcription inhibitors and amplification reactions in the samples was accomplished by comparing the standard curves obtained from serial sample dilutions before and after reverse transcription. These curves exhibited identical slopes and intercepts. Furthermore, it is worth noting that a solitary melting temperature peak was detected for each reaction in the real-time PCR results, as seen in Figures 6 and 7. This observation strongly implies the absence of non-specific amplification, including primer dimers, DNA contamination, and other forms of non-specific binding.

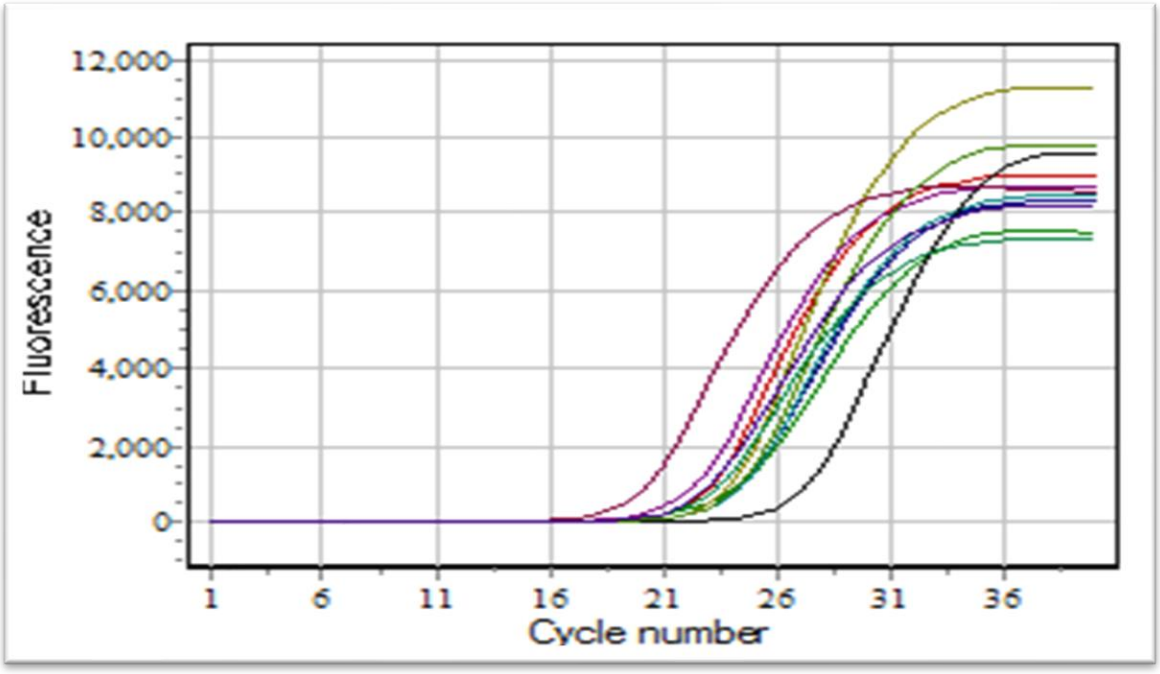

Figure 6. Amplification curve plot of reference gene in all 13 samples.

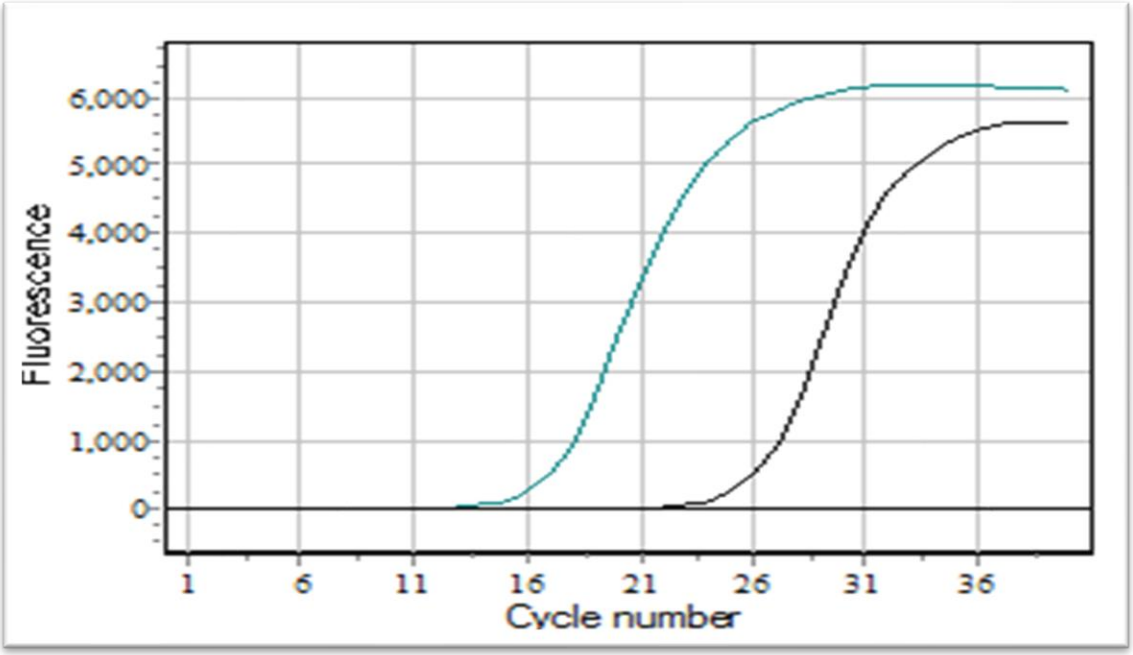

Figure 7. Amplification curve plot of the target in bacteriocin gene.

Real-time Polymerase Chain Reaction Validation (qPCR)

The assessment of gene expression related to putadicin production has been conducted on all 13 isolates using real-time reverse transcription-PCR. The consequences of the amplification response of the bacteriocin gene and all the isolates were reported in Tables 7. The bacteriocin gene was expressed using particular primers developed for real-time PCR analysis. The amplification reaction of the bacteriocin gene is dependent on the DNA copying process, namely following the conversion of mRNA to cDNA in the first stage.

Table 7. Bacteriocin gene expression was analyzed using semi-quantitative PCR and target and reference gene real-time PCR.

	Ct. target gene		Ct ref. gene	Δ Ct.	Gene expression
A1		B1	25.7		
A2		B2	24.4		
A3	**22**	B3	24.4	-2.4	0.189
A4		B4	23.3		
A5	13	B5	24.4	-11.4	0.0003
A6		B6	24.4		
A7		B7	24.4		
A8		B8	24.5		
A9		B9	24.5		
A10		B10	24.5		
A11		B11	24.5		
A12		B12	24.4		
A13		B13	24.5		
P-value	---	---	---	0.0001 **	
**** (P≤0.01).**					

The role of bacteriocins in different environments has not fully been explained, primarily due to the difficulties associated with detecting their production. Putadicin, a *P. putida* produced antimicrobial peptide, has a long history of safe use in food products, and has been studied from many aspects of genetics, biosynthesis, immunity, regulation, and mode of action. Even, some aspects of putadicin gene expressions dynamics remain unclear.

The primary aim of this work was to quantitatively assess the expression of bacteriocin genes from *P. putida* that were obtained from soils in Iraq and are known to be putadicin. This work is the first comprehensive quantification of the evolutionary trajectory of *P. putida* transcripts bacteriocin gene in the context of burn therapy in Iraq. Real-time PCR amplification efficiency was usually at least 94 % and not more than 100%. Non-template control Cq values were always above 38 cycles and over 10 cycles above the Cq values of the samples with the lowest target concentration. The genus-specific killing can be shown with lectin-like bacteriocins, which is explained by the

occurrence of certain types of sequencestypes in different *Pseudomonas* species (Ghequire et al., 2018). The armamentarium of bacteriocin possessed by *Pseudomonads Spp*. varies according to strain (Sharp *et al*., 2017). An evolutionary advantage for the bacteria that secrete these compounds has been shown for various bacteriocin groups (Dorosky et al., 2017; Príncipe et al., 2018). The occurrence of LLP in *Pseudomonad*s connected with plants and residing in the soil seems to be more prevalent, maybe indicating an evolutionary connection with lectins recovered from monocot plants. According to Ghequire and De Mot (2014), it has been shown that *Pseudomonas spp*. typically possess a single L-type bacteriocin gene in their genomes, with a few outliers. Bacteriocin sequences from strains encoding two LlpAs are usually dissimilar (30–52 per cent pairwise AA Id), arguing against a duplication event, while the latter strongly contrasts with modular S-type bacteriocins, which usually appear as multiple representatives, although present in a single *Pseudomonas* genome with different receptor binding/toxin domain combinations (Sharp et al., 2017; Beaton et al., 2018).

Isolation and purification of bacteriocin

One isolate was selected from the five isolates that exhibit the inhibition zone after 3 days for bacteriocin isolation and purification. Then the same above method was used to measure and compare the antibacterial activity against pathogenic bacteria in each purification step, which observed that the pure bacteriocin gave the largest and most efficient inhibition zone against all species of pathogenic bacteria as shown in Table (8).

Table 8. Results of all bacteriocin purification steps against different species of pathogenic bacteria.

Bacterial species	Crude	Ammonium sulfate	Dialysis	Pure bacteriocin
Staphylococcus aureus	19mm	19mm	10mm	21mm
Streptococcus mitis	18mm	18mm	11mm	22mm
Proteus mirabilis	18mm	15mm	9mm	15mm
E-coli	0mm	11mm	0mm	11mm
Pseudomonas aeruginosa	10mm	4mm	5mm	10mm
Kelbsiella pneumoniae	0mm	0mm	0mm	12 mm

Ahmad et al. (2013) documented the synthesis of bacteriocin-like substances (BLS) by a strain of Pseudomonas putida that was obtained from Sharkskin. In a separate study, Parret et al. (2005) conducted a characterization of a bacteriocin-like compound that exhibited notable similarity to

mannose-binding lectins present in monocotyledonous plants. This chemical was designated as lectin-like putidacin. The lectin-like bacteriocin generated by *P. putida* is likely to have a distinct mechanism of action compared to previously documented bacteriocins from other Pseudomonas species (Yoshino et al., 2011; Molina et al., 2014). However, it has been shown that these strains exhibit a lower level of pathogenicity in comparison to Pseudomonas aeruginosa. In the course of the inquiry about bacterium soils, a total of 15 strains of Gram-negative bacteria were recovered (Ghrairi et al., 2013). The current investigation observed that bacteriocin synthesis by the chosen isolate started during the late exponential phase, reaching its peak output towards the middle of the stationary phase. These findings align with previous studies that have shown similar patterns of bacteriocin production in other bacterial species (Migaw et al., 2013).

The bacteriocins of *P. putida* have the antimicrobial activity towards a broad range of Gram-negative and Gram-positive bacterial species for these characteristics suggested a potential use in medical applications (Saeidi et al., 2011), While Turgis et al. (2016) found a synergistic antimicrobial activity of two types of bacteriocins (nisin and pediocin) against foodborne and food spoilage bacteria. For these reasons, further experiments are needed to confirm this result The probiotic strain, isolated from soil bacteria, was able to produce the maximum amount of a bacteriocin-like compound at the onset of the stationary phase of the growth. The present results show that the purification bacteriocins extract from *P. putida* has more antibacterial activity than that obtained by Nugrahani et al. (2016) who found that the purification bacteriocins extract from *Lactobacillus casei strain* against *Staphylococcus Spp.* which reached between (6-11) mm in dimeter. While Gillor *et al*. (2009) found an initial purified antibacterial from *Brevibacillus laterosporus* against *Staphylococcus aureus* and clinical isolates of methicillin-resistant from air samples that inhibited reached between (11– 14) mm. In addition, Kadhim (2014) found that two isolates of *P. fluoresces* produced crude bacteriocin affected on growth of Gram-positive bacteria with a wide range of inhibition zone reached in (10-13) mm. The reason for this decrease has not been determined; this could be due to the activity of extracellular endogenous proteins induced during this growth phase (Yamamoto et al., 2003). Venigalla et al., (2017) who found that *S. aureus* did not show inhibitory response for purified bacteriocin produced by *Lactobacillus plantarum* JX138220, whereas larger bacteriocin inhibition areas were observed against *P. aeruginosa* (NCIM 2036). Islam, R. et al. (2020) found that *Lactobacillus Spp.* isolates possesses a wide spectrum of LAB inhibitory activity against *Escherichia coli, Staphylococcus aureus, Bacillus*

cereus, Bacillus subtilis, Acetobacter, E. coli fecalis, Salmonella typhi with different inhibition zone.

Estimation of Protein Concentration

The concentration of protein was calculated from the standard curve shown in Figure (8) and recorded the absorption and concentration of purified protein as shown in Table (9), which clarifies the production and Characterization of *P. putida* produced bacteriocin.

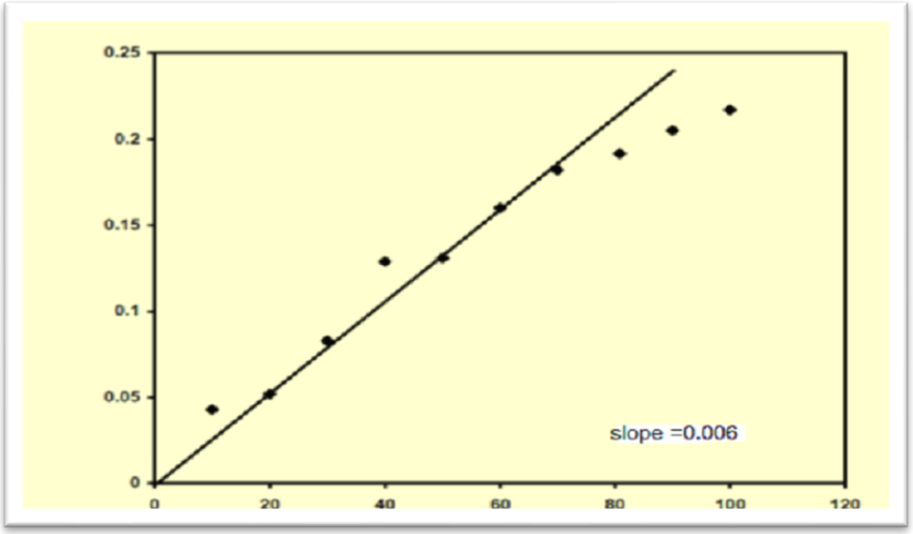

Figure 8. The stander curve of protein concentration.

Table 9. Production and Characterization of Bacteriocin Produced by *P. putida*.

Steps	Volume (ml)	Activity µ\ml	Protein concentration	Specific activity	Total activity	Fold of purification
Crude bacteriocin	100	0.2	0.182	1.09	20	1.0 ± 0.00 **b**
Ammonium sulfate	20	0.5	0.220	2.27	10	0.4 ± 0.03 **c**
Ion exchange	25	0.2	0.251	0.796	5	1.3 ± 0.03 **b**
Gel filtration	20	0.1	0.457	0.218	2	5.0 ± 0.17 **a**
P-value	---	---	---	---	---	0.0038 **

The different letters in last column that means having different significant.
**** (P≤0.01).**

The pooled bacteriocin purification fractions obtained from ion-exchange chromatography using DEAE-cellulose were then subjected to gel filtration chromatography in the following phase. The specimen was introduced into the Sephadex G-75 column. A plot was generated to illustrate the relationship between the absorbance values and the corresponding fractions, resulting in the identification of a single protein peak. Each fraction was measured to comprise 5 mL at a wavelength of 280 nm. A graph was constructed to illustrate the relationship between the absorbance and the fraction number, which revealed the peak corresponding to the pure bacteriocin 1 protein (Figures 9 A and B). The bacteriocin peaks exhibited optimal efficacy against both Gram-negative and Gram-positive pathogenic bacteria, with putadicin demonstrating the highest level of activity. The initial protein concentration of the crude bacteriocin extract was around 500 µg/ml. After purification of the bacteriocin-like molecule, the total protein concentration increased to approximately 4669 µg/ml, with a corresponding absorbance value of 0.43, indicating excellent purification.

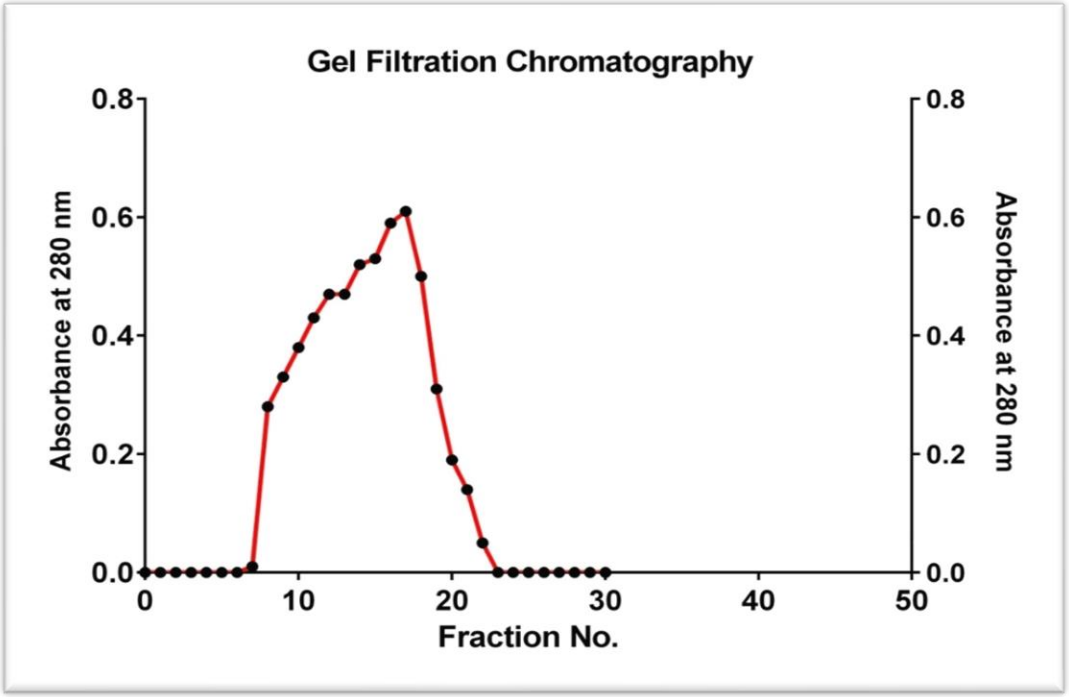

Figure 9. Purification steps chromatography

A- Ion exchange chromatography

B- B- Gel filtration chromatography

The result showed that there is significant (P≤0.01) difference between the different steps of purification that appeared in table (9). The purification was done by using Sephadex G-75 scale exclusion chromatography. Enhanced partial purified bacteriocin activity produced by Lactobacillus viridescence which exhibited total activity 6×107AU/mL, while the total activity 3×107AU/mL. shown after completing the gel chromatography purification. (Sure et al., 2016).

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic analysis showed that the apparent molecular masses of the bacteriocin purification steps were P≤ 11 kDa. Bacteriocin and native polyacrylamide gel electrophoresis showed one thick large band in each area, which agree with many studies (Saeidi N et al., 2011; Ghrairi T. *et al*., 2014) as shown in Figure (10).

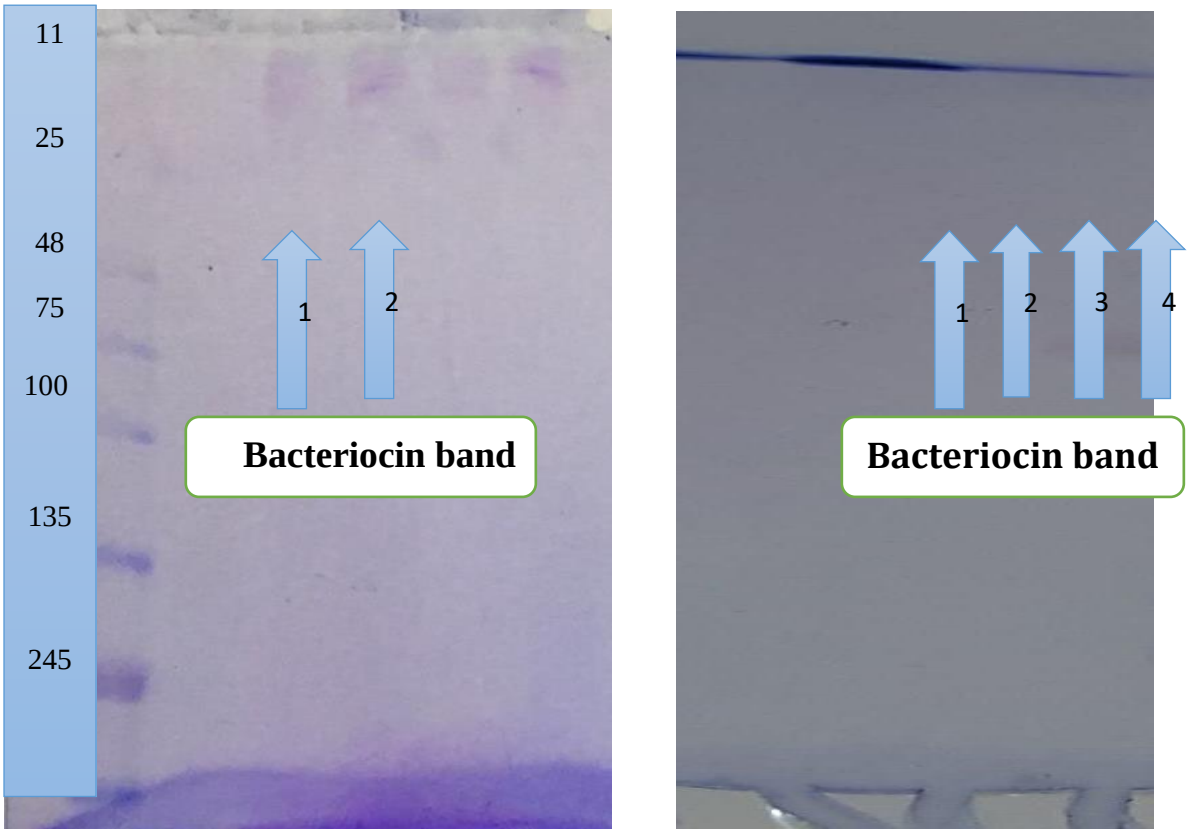

Figure 10. A. Show electrophoresis for bacteriocin with SDS; B. Show bacteriocin SDS-electrophoresis (L: ladder, 1: crude, 2: ammonium sulphate, 3: Daylaza, 4: pure bacteriocin)

High Performance Liquid Chromatography (HPLC)

As the external standard, the active fraction of purified bacteriocin was applied to the HPLC column gel filtration along with nisin. Reinjection of this predominant peak gave in the previous

gel chromatogram only one peak at the same peaks observed. Based on the standard elution profile, peaks of both methods yielding comparable results may have a mass P ≤11 kDa. This peptide was finally purified on a reversed-phase semi-preparative-HPLC. The purified peptide exhibited a single peak at 220 nm of UV absorption and was positive for antimicrobial activity. This peptide and reverse-phase high-performance liquid chromatography is analyzed by SDS-PAGE. Few studies have reported the use of hydrophobic resin interactions to extract selective peptides such as (Sebei et al., 2007; Appleyard AN et al., 2009 and Singh P.K. et al., 2012; Katharopoulos. et al., 2016)

Conclusion

In conclusion, our research demonstrates that the gene 16S rRNA serves as a powerful phylogenetic tool, exhibiting exceptional discrimination capabilities in species differentiation. Furthermore, conducting thorough comparisons of evolutionary distances via the use of conserved genes may contribute to a more precise delineation of the genetic foundation for classifying into distinct genes and families. The proposed approach has significant potential for the reassessment of bacterial taxonomy.

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