Effect of Mersacidin produced from *Bacillus* sp.-AE isolated from soils in Northern Iraq

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Abstract

From (55) soils samples were collected, including soils from different regions of Nineveh and Duhok governorates, of which (29) isolates were isolated, with a rate of (52.7%) belonging to the genus *Bacillus*. Eleven (11) bacteriocin-producing isolates were selected, which were diagnosed as *Bacillus clausii*-AE, *Bacillus subtilis*-AE, *Bacillus cereus*-AE, *Bacillus thuringiensis* -AE, *Bacillus* sp. -AE depending 16S rRNA gene sequences. The *Bacillus* species clustered in 5 clusters: A: include 5 strains *B. subtilis*-AE at 100%, B: include 2 strains *B.cereus*-AE at 99.8%, C: include 2 strains *B. clausii*-AE at 99.6%, D: include 1 strains *B. thuringiensis*-AE at 99.2%, E. include 1 strain *Bacillus* sp.-AE at 94.4%. Inphylogenetic tree using Unweighted Pair Group Method with Arithmetic mean (UPGMA), using (Mega 7) program. The *Bacillus* sp.-AE has the highest effect on the bacterial type *Acinetobacter junii* -20AE, *Acinetobacter baumannii*- 19AE, *Acinetobacter baumannii* -22AE. The bacteriocin extracted from *Bacillus* sp.-AE was identified as a Mersacidin type Lantibiotics, which is a protein complex consisting of a peptide, the Rf value is 0.70 by TLC method, and the molecular weight is less than 5 kDa by SDS method. -PAGE GEL.

Keyword: Bacillus, Meroscidin, Acinetobacter, TLC, SDS.

Introduction

Members of *Bacillus* are Gram-positive bacteria, have endospore are widespread in many environments, Bacilli, motile, with peripheral flagella, aerobic, catalase -positive, the optimum temperature for its growth is 37˚C (Singh *et al*., 2020). It was first proposed to be classified by the scientist Fischer (1895) containing (94) species depending on the morphology, but the modern classification increased their number to(273)species (Amaresan *et al*.,2020). Members of the *Bacillus* family have received great interest in the medical and pharmaceutical industries due to their production of antimicrobial peptides and their use as therapeutic agents for several years as an alternative to the use of antibiotics due to the resistance of bacteria to many antibiotics (Lajis, 2020). Most of its species do not need complex media for growth (Cui *et al*., 2019). Bacterocins are protein or peptide antibiotics ,they produced by many Gram-positive and Gram-negative bacteria, including *Bacillus*, has a lethal effect or inhibits the growth of related species, also its have broad effect against different types of microorganisms, which increased the possibility of competition among strains producing it (Abdelli *et al*., 2019).The World Health Organization

(2017) published a list of multidrug-resistant pathogens worldwide that pose a threat to human health with an increase in the number of multidrug-resistant bacteria and also requires the development of new antimicrobials for these pathogens, ESKAPE, which includes *E.coli, Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus , Enterococcus faecium* And *Enterobacter* spp. (Motiwala *et al*.,2022). The current study aims to study phenotypic and molecular diagnosis of bacterial strains producing bacteriocin from the soil belonging to the genus *Bacillus*.

Materials and Methods

Isolation and diagnosis

Fifty-five(55) the soil samples were collected from different regions of Nineveh Governorate, including soils of (gardens, contaminated soil with oil, public places, contaminated soil with poultry waste), in addition to the governorate of Dohuk, which included soils for regions (Icaria, Zawita, Suara Tukah, Amniki, Ashawa). Serial dilution method on nutrient agar were used incubated under aerobic conditions (Jahnz *et al*., 1996; Al- Sammak, 2013), Study the growth of ranges of pH ranges (2,4,6,7,9) (Tille, 2017) and growth in different concentrations of salt 0.5, 1, 3, 5, 9 (MacFaddin, 2000) and different temperatures 30, 37, 45 °C (Lajis, 2020) the incubation period was 2,5,7 dayes (Tille, 2017). Five diagnosed species were obtained from the Department of Biology / University of Mosul, *Staphylococcus aureus, Acinetobacter baumannii, Acinetobacter junii, Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae* OP136161 and *Escherichia coli*, and two strains were obtained from include *Acinetobacter baumannii* ATCC19606 and *Salmonella typhii* ATCC6539.

Molecular Diagnostics

Bacterial DNA was extracted the genus *Bacillus* spp. using Presto ™ Mini g DNA Bacteria kit prepared from (Taiwan) Geneaid company with the addition of lysozyme to destroy the cell wall,In this study, a universal primer was used to determine the 16S r RNA gene, as shown in Table (1), which was prepared by Alpha DNA in a lyophilized form.

Table (1) Primers of 16Sr RNA gene

primer	Sequence 5 - → 3 -	Gene length (bp)	Source
16SrRNA-F	AGAGTTTGATCCTGGCTCAG	1465	Cotârlet *et al.*, 2010
16SrRNA-R	ATGGACCAGGCCACGATTTT		

F: forward R: reverse

Table (2) PCR program of 16SrRNA gene

Sequencing	Stage	Number of courses	(C°) Temperature	Time
1	DNA Initial Denaturation	1	95	6 min
2	Denturation	35	95	45 sec
3	Annealing		58	1 min
4	Extension		72	1 min
5	Final extension	1	72	5 min

Determination the 16S rRNA gene of *Bacillus* spp. and its evolutionary relationship

Amplifications of PCR Products were sent to the MACROGENE laboratory in South Korea, and the sequences were compared with NCBI website using BLAST. The relationship between the diagnosed strains under study was determined by comparing sequences with Clustal W for the strains under study and Phylogenetic Tree Analysis. Using Mega 7 program and the Unweighted Pair- Group Average Method (Kaur and Kaur, 2015).

Production of bacteriocin

A Cross Streak Method was used for the primary investigation of bacteriocin production of *Bacillus* spp. against some pathogenic gram-positive and gram-negative bacteria (Al-Sammak, 2013; Walaolak, 2008), a young colony was taken, and cultured as a central line in the heart-brain infusion plate incubated at 37° C for (18-24) h. From the pathogenic bacteria *Acinetobacter* spp., *Staphylococcus aureus* (MRSA), *Escherichia coli* A4, *Klebsiella pneumoniae* OP136161, *Salmonella typhi* ATCC6539, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* ATCC19606, *Escherichia coli* 43, *Staphylococcus aureus* 13 cultured at an angle of 90°C to the central growth line of *Bacillus* spp., incubated at 37°C for (18-24) h, to determine the inhibitory effect against pathogenic species by measuring inhibition zone.

***Bacillus* sp.-AE**

The *Bacillus* sp. -AE gave the best production of bacteriocin incubated in 10 ml of the Synthetic Kl medium at 37° C for (18-24) h, the medium consisting of: Glucose (5), $CaCl_2$ (20), $(NH_4)_2SO_4$ (4), $K2HPO_4$ (2.6), $MgSO_4$ (4), NaCl (2), $FeSO_4.7H_2O$ (0.002), $ZnSO_4.7H_2O$ (0.002), $MnSO_4.H_2O$ (0.0015). Dissolved it in (1 liter) of distilled water, the pH was adjusted at (7.2), They were incubated in a shaking incubator at a rotational speed of (200 cycles/min) under aerobic conditions at a temperature of (37 °C) for a period of 24-48 h (Teng *et al*., 2012; Lajis, 2020).

Extraction of bacteriocin

Synthetic Kl medium was taken and was ran centrifuge at a speed of (10,000 cycles/min) for a period of 15 min in 4 ° C (Yousef, *et al*., 2011), the precipitate was discarded and the cell- free bacterial culture

filtrate was taken supernatant mix with the same amount of solvent (2 chloroform: 1 methanol) volume: volume (Guyonnet *et al*., 2000), a separating funnel was used to obtain the aqueous layer milky colour contain the bacteriocin, was filtered by mily pore filter (0.45 mm), placed in oven at a temperature 60°C for 24 h . (Shubhrasekhar *et al*., 2013; Umar *et al*., 2021b).

Thin Layer Chromatography (TLC)

Using silica sheets with dimensions (20 cm * 20 cm), the bacteriocin extract was mixed with the solvent (2 Chlorofoam: 1 methanol) volume/ volume. The separation solution consisting of (65 chloroform: 25 methanol: 4 distilled water), left at room temperature for an hour, then the separated material was determined (Lamilla *et al*., 2018; Umar *et al*., 2021a). Several reagents were used to diagnose the type of bacteriocin: Ninhydrin reagent used to detect free amino groups present in proteins. Phenol-Sulfuric acid solution used to detect glycolipid. Iodine granules used to detect lipid. (Bezza *et al*., 2015; Umar *et al*., 2021b). After sprayed with reagent left to dry and heated at (110 °C) for 5 min, the flow rate (RF) calculated according to:

Flow rate = distance of substance / distance of solvent

Molecular weight determination of bacteriocins by SDS-PAGE GEL technique

The approximate molecular weight of the bacteriocin was estimated in order to support the phenotypic and biochemical diagnosis of the extract produced from *Bacillus* sp.-AE Sodium polyacrylamide sulfate gel electrophoresis method .An SDS gel kit prepared by Bio-Rad Miniprotein Canada also used a Protein Molecular marker of 10-200 kDa to determine the molecular weight of protein after migration (Alomari and Gowers, 2017).

Results and discussion

From fifty-five(55)the soil samples collected from different regions of Nineveh and Dohuk governorates, (29) isolates (7.52 %) belonging to the genus *Bacillus* and the highest isolation of of Ashawa area in Dohuk with a percentage of 83.3%, followed by soils from general areas in Nineveh 66.6 %, contaminated soil with oil 60%, contaminated soil with poultry waste 50%, gardens 44.4%, and soil for Zawita area 40%, while the lowest percentage of bacteria isolates was from the soils of the Suara Tukah region with a percentage of 28.5%, and it did not show any isolation percentage from the soils of the icaria ,Amniki in Dohuk As shown in Table (3) the heat treatment of isolation sources caused the elimination of vegetative cells and the preservation only spores (Barrow and Feltham, 2003; Thapa *et al*., 2021). Biochemical tests were used to diagnose members of the genus *Bacillus* under study and the phenotypic characteristics of the colonies, as well as the microscopic characteristics of the cells and their interaction with Gram stain (Riedel *et al*., 2019).

Table 3: Percentages of *Bacillus* spp. isolates under study, depending on the source of the isolate

Sample type	Isolation site		Number of samples	Number of isolates	Isolates	Ratio
soil samples	Gardens	Soils of Nineveh Governorate	9	4	*Bacillus* spp.	%44.4
	Contaminated soil with oil		5	3	*Bacillus* spp.	%60
	Public places		15	10	*Bacillus* spp.	%66.6
	Contaminated soil with poultry waste		6	3	*Bacillus* spp.	%50
	Icaria	Soils of Duhok Governorate	1	0	-	%0
	Zawita		5	2	*Bacillus* spp.	%40
	Suara Tukah		7	2	*Bacillus* spp.	%28.5
	Amniki		1	0	-	%0
	Ashawa		6	5	*Bacillus* spp.	%83.3
total			55	29		%52.7

Molecular Diagnosis for *Bacillus* species

Universal primer 16S rRNA was used for the molecular diagnosis and bacterial identification of (11) isolates belonging to the genus *Bacillus* using RCR technique, which appeared at 1465 base pairs (Celandroni *et al*., 2019). Results were compared within the National Center for Life Technology Information (NCBI) using (BLAST), it was found that the strains showed a percentage of similarity with the reference strains registered to varying degrees for *Bacillus* strains, which ranged between (84-95.5)% depending on strain. The16 S rRNA gene was used to diagnose a wide range of bacterial species or genera, due to the fact that this gene is present in all bacterial species (Alajlani, 2022).

Phylogenetic Relationship between the strains under study using 16 S rRNA gene

Using the Clustal W program and the UPGMA method within the Mega7 program (Tamura and Nie ,1993), as shown in Figure (1) The *Bacillus* species clustered in 5 clusters:

A: include 5 strains *B. subtilis*-AE at 100%, B: include 2 strains *B.cereus*-AE at 99.8%, C: include 2 strains *B. clausii*-AE at 99.6%, D: include 1 strains *B. thuringiensis*-AE at 99.2%, E. include 1 strain *Bacillus* sp.-AE at 94.4%. were determined in the tree diagram. The 16 S rRNA gene proved highly efficient in diagnosis and classification, which indicates its high genetic affinity, stability of the 16SrRNA gene, and its important role in determining the identity of bacteria.

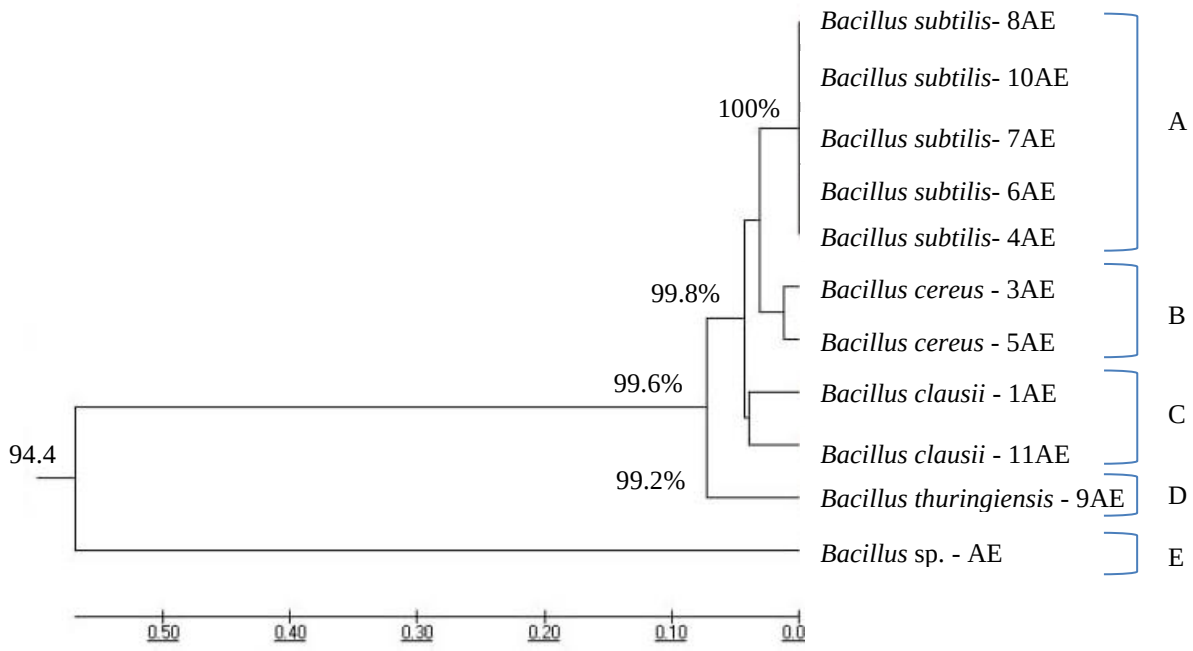

Figure (1) The relationship between *Bacillus* species depending on the 16S rRNA gene using Clustal W and UPGMA method within the Mega7 program

Production of bacteriocins from *Bacillus* sp.-AE

Members of the genus *Bacillus* are among the most important species that have the ability to produce bacteriocins (Lee *et al*., 2011) the research was still on going to investigate new bacteriocin- producing isolates, as a result of the increasing resistance to antibiotics by pathogenic bacterial species. The cross streaking method was used to investigate the ability of *Bacillus* species to produce bacteriocins using brain- heart agar media. (Al- Sammak, 2013, Ogunbanwo *et al*., 2003).

Table (4) shows the inhibitory effectiveness of species belonging to the genus *Bacillus* spp. on both Gram-positive and Gram-negative bacteria under study, the researchers (Thapa *et al*., 2021). The indicated that most bacilli have a clear and distinct effect against strains of pathogenic bacteria, due to its production of bacteriocin, by observing the lack of growth of pathogenic bacteria along the perpendicular line of the producing bacteria, the strain *Bacillus* sp. -AE has the highest effect on the growth of *A. junii*-20 AE, *A. baumannii* -19AE, *A. baumannii* -22AE and a moderate effect on the rest of the strains of the species *Acinetobacter* spp. and *Staph. aureus* (MRSA) and *E. coli* A4and *Sal. typhii* ATCC6539 and *Pseudo. aeruginosa*47 (Xie *et al*., 2009; Simons *et al*., 2020) ,the strains belonging to *K. pneumoniae* OP136161 , *A. baumannii* ATCC19606 , *Staph. aureus* 13 , *E. coli* 43 did not show any effect by *Bacillus* sp.-AE (Gillor *et al*., 2005).

Table (4) Bacteriocin activity produced by *Bacillus* spp. against some gram-positive and gram-negative bacteria using cross streaking method.

N	Test bacteria	Antibacterial activity of *Bacillus* species										
		***B. clausii* -1AE**	***B. cereus* - 3AE**	***B. subtilis* - 4AE**	***B. cereus* - 5AE**	***B. subtilis* - 6AE**	***B. subtilis* - 7AE**	***B. subtilis* - 8AE**	***B. thuringiensis* - 9AE**	***B. subtilis* - 10AE**	***B. clausii* -11AE**	***Bacillus* sp. AE**
1	*Acinetobacter juniii* -20AE	-	-	+	-	+	-	-	+	-	-	+++
2	*Acinetobacter baumannii* -22AE	+	+	-	+	-	-	+	+	+	+	+++
3	*Acinetobacter baumannii* -19AE	+	+	+	+	+	+	-	-	-	+	+++
4	*Acinetobacter baumannii* ATCC19606	-	-	-	-	-	-	-	-	-	-	-
5	*Acinetobacter baumannii* -26AE	-	-	-	-	+	+	+	-	-	-	++
6	*Acinetobacter baumannii* -25AE	+	-	-	-	-	+	+	-	-	+	++
7	*Acinetobacter baumannii* -24AE	-	-	-	-	-	+	+	+	+	+	+
8	*Acinetobacter baumannii* -23AE	+	-	-	-	-	-	-	-	-	+	++
9	*Acinetobacter baumannii* -21AE	-	+	+	+	+	-	-	-	+	-	+
10	*Acinetobacter junii* -18AE	+	+	+	+	-	-	-	-	-	+	++
11	*Acinetobacter junii* -13AE	-	++	-	++	-	-	-	++	-	-	++
12	*Acinetobacter junii* -17AE	++	++	-	-	-	+	+	-	-	-	++
13	*Acinetobacter junii* -16AE	++	-	-	+	-	+	+	-	-	+	++
14	*Acinetobacter junii* -15AE	++	+	+	-	++	-	-	++	-	-	++
15	*Staphylococcus aureus* (MRSA) 14	-	-	-	-	+	-	-	-	+	+	++
16	*Escherichia coli A4*	-	-	+	+	+	-	-	+	-	-	++
17	*Klebsiella pneumoniae* OP136161	-	-	-	-	-	-	-	-	-	-	-
18	*Salmonella typhii* ATCC6539	+	+	-	+	-	-	-	++	-	+	++
19	*Staphylococcus aureus* 13	-	-	-	-	-	-	-	-	-	-	-
20	*Escherichia coli* 43	-	-	-	-	-	-	-	-	-	-	-
21	*Pseudomonas aeruginosa47*	+	-	-	+	-	+	+	+	-	-	++

- No effect, +++ High effect, ++ Medium effect, + Low effect

Optimal conditions for production of Bacteriocin from *Bacillus* sp.-AE

The strain *Bacillus* sp.-AE was chosen to determine the optimal conditions for the production of bacteriocins, which it gave the highest productivity in the Synthetic Kl medium during an incubation period of 48 hours (Al-Abbasi, 2018). The highest productivity for the Bacteriocin at pH 6,7,9

as shown in figure 2, and at NaCl salt concentrations of 0.5%, 1%, and 3% as shown in figure 3, and a temperature of 37°C as shown in figure 4, and an incubation time of 2,5,7 days as shown in figure 5, against three strains of AE *Acinetobacter junii*-20AE, *Acinetobacter baumannii*-19AE, *Acinetobacter baumannii*-22AE by using well diffusion method.

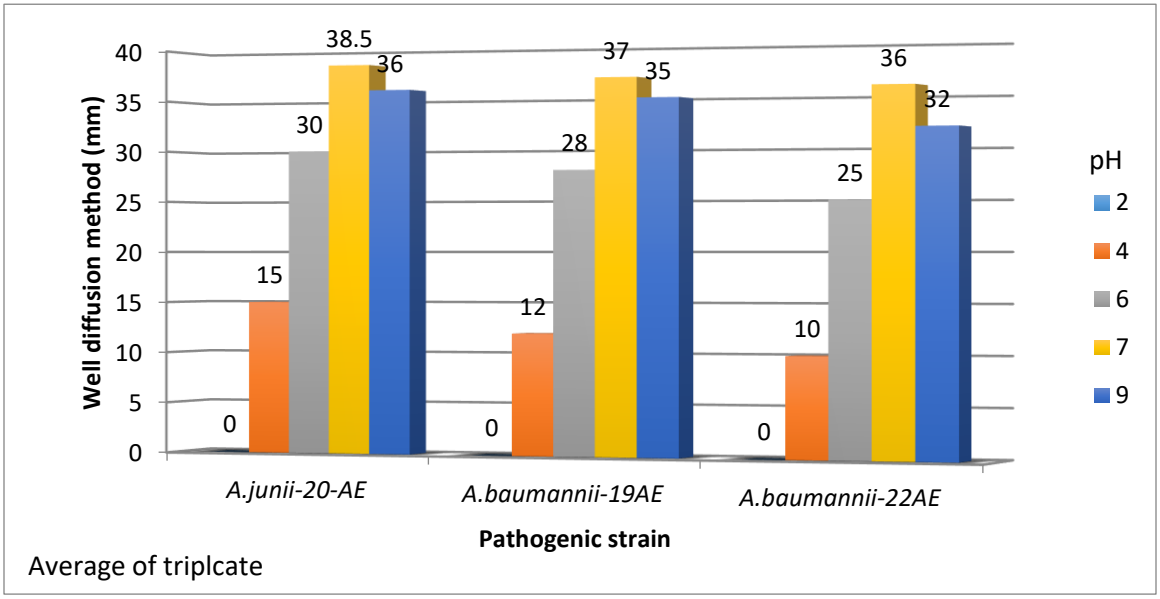

Figure (2) Effect of pH on the production of Bacteriocin from *Bacillus* sp.-AE against some pathogenic strains

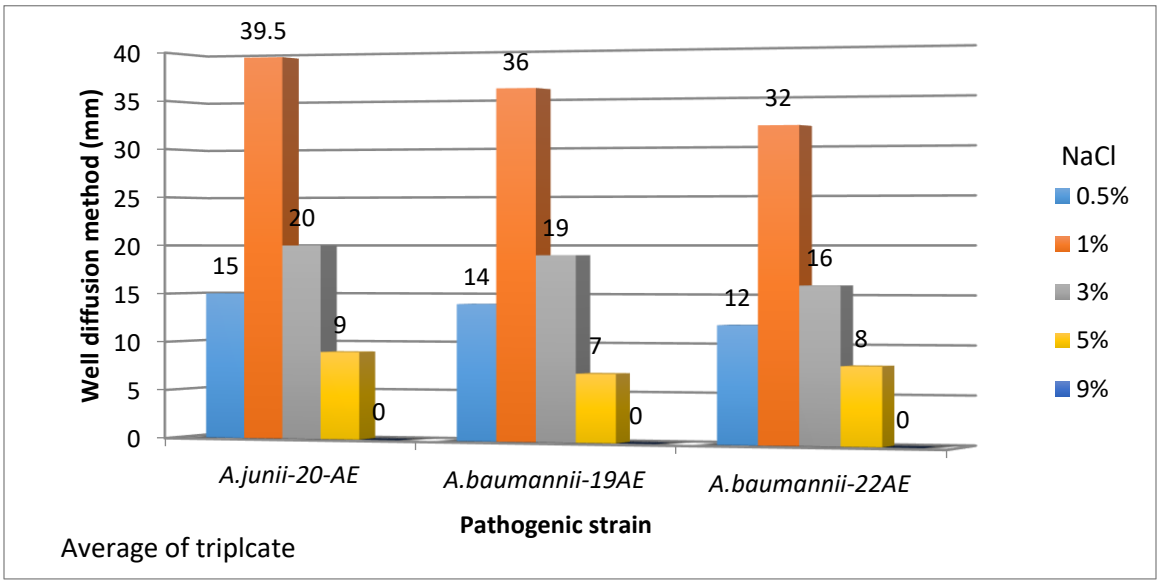

Figure (3) Effect of NaCl% on the production of Bacteriocin from *Bacillus* sp.-AE against some pathogenic strains

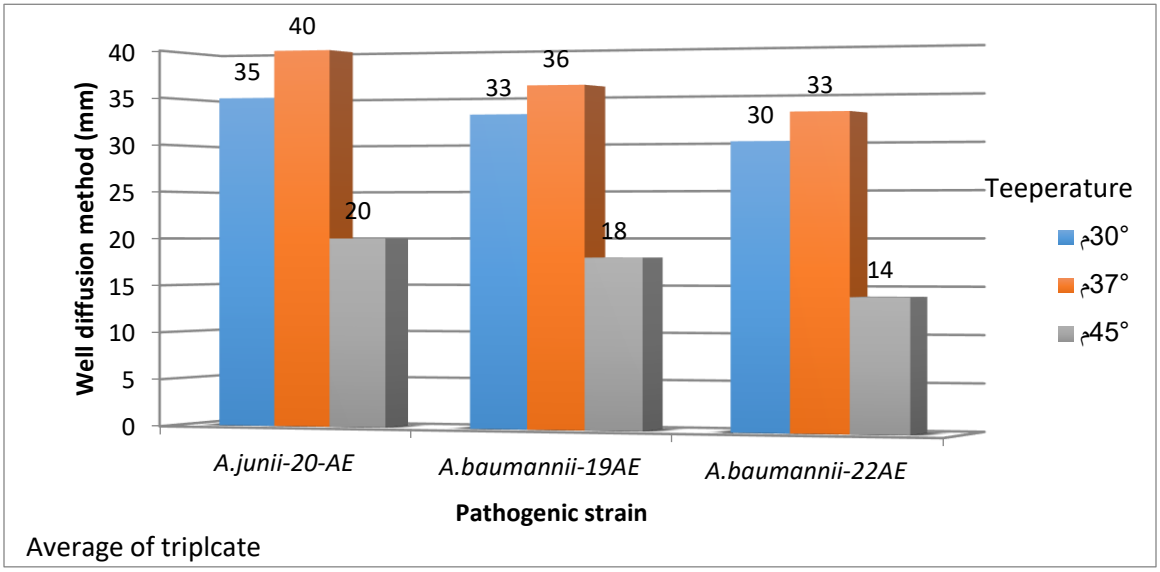

Figure (4) Effect of temperatures on the production of Bacteriocin of *Bacillus* sp.-AE against some pathogenic strains

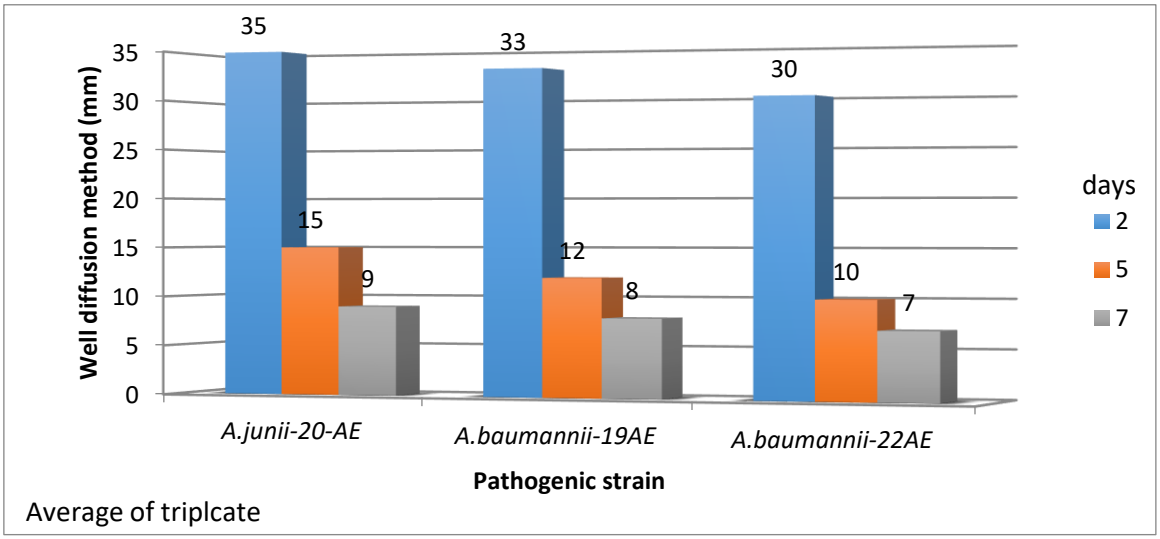

Figure (5) Effect of the incubation period (day) on the production of Bacteriocin from *Bacillus* sp.-AE against some pathogenic strains

Thin Layer Chromatography (TLC) technique for separation and identification of bacteriocin

One spot was obtained with a flow rate Rf = 0.70, when using a solvent system of 65 mL chloroform: 25 mL methanol: 4 mL distilled water, while the flow rate of the other bacterial isolates for the separated spots It ranged between 0.56 -0.84, as spots appeared when adding the reagents and after exposing the TLC plate to ultraviolet radiation, as shown in Table (5).

Table (5) The chemical composition of Bacteriocin extracted from strains of the genus *Bacillus* spp. using TLC

Bacteria	R.F	Sugar	Lipid	protein	Type of Bacteriocin
B. clausii -1AE	0.83, 0.56	-	+	+	Lipopeptide
B. cereus -3AE	0.66	-	-	+	peptide
B. subtilis -4AE	0.63,0.68	-	+	+	Lipopeptide
B. cereus -5AE	0.67	-	-	+	peptide
B. subtilis -6AE	0.57,0.84	-	+	+	Lipopeptide
B. subtilis -7AE	0.60,0.67	-	+	+	Lipopeptide
B. subtilis -8AE	0.63,0.84	-	+	+	Lipopeptide
B. thuringiensis -9AE	0.67	-	-	+	peptide
B. subtilis- 10AE	0.66	-	-	+	peptide
B. clausii -11AE	0.56,0.80	-	+	+	Lipopeptide
Bacillus sp.-AE	0.70	-	-	+	peptide

+ presence, - absent

The separated spots when using TLC to investigate the Bacteriocin produced from 11 diagnosed isolates belonging to the genus *Bacillus* were chemically sprayed with previously prepared reagents to detect sugar, lipid and protein, and their location was detected by exposing the silica plate to ultraviolet light when spraying with a phenol reagent. Phenol -Sulpheric acid Brown spots did not appear for all bacterial isolates on the TLC plate, and this indicates that the extract does not contain sugar groups in its composition, and the plate was exposed to iodine vapor Iodine showed spots in a brownish-yellow color to some of them, and this indicates the presence of lipids within their composition, as shown in figure (6) and in table (6), but when using the Ninhydrin reagent, a purple spot appeared for all bacterial strains, indicates the presence of protein or free amino acids within the composition of Bacteriocin according to the type of isolate, and this agreed with the researchers (Bezza *et al*., 2015).

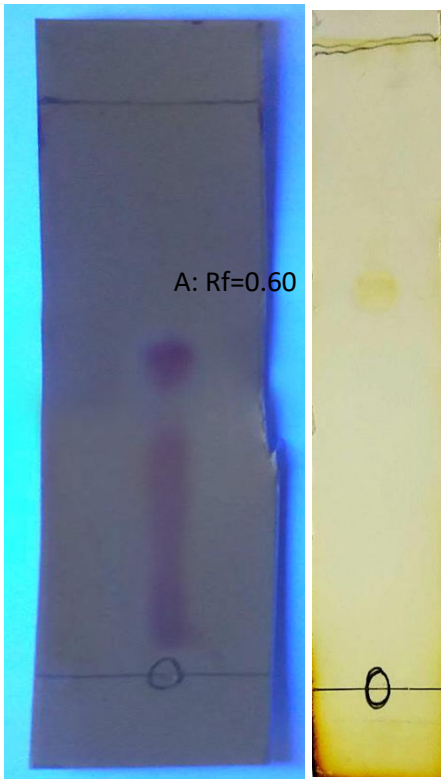

Figure (6) The location and colors of spots of Bacteriocin extracted from the strain

***B. subtilis* -6AE using TLC**

A: Ninhydrin reagent, B: Iodine

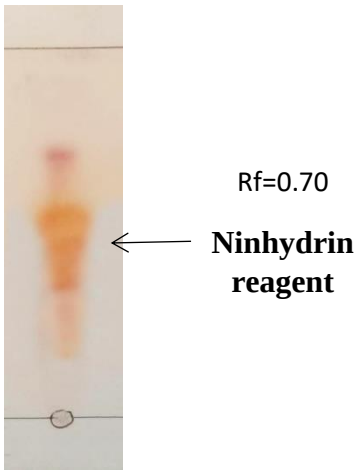

Figure (7) The location and colors of spots of mersacidin extracted from *Bacillus* sp. -AE using TLC

The relationship of incubation time with the amount of bacteriocin extracted from the strain *Bacillus* sp.-AE

The direct method was used to extract the bacteriocin from the strain *Bacillus* sp.-AE, which showed the highest productivity, and the highest weight obtained was 14.2 g/L using synthetic Kl medium, while the rest of the strains showed less productivity, as shown in Table (6)

Table (6) The weight of the bacteriocin produced from *Bacillus* using synthetic Kl medium (g/L) according to the incubation period per day

Incubation period /days	The weight of bacteriocin g/l	strains	T
2	**14.2**	***Bacillus* sp.-AE**	**1**
5	2.5	*B. clausii* - 1AE	2
7	1.8	*B. cereus* - 3AE	3
2	10.1	*B. subtilis* - 4AE	4
7	1.8	*B. cereus* - 5AE	5
2	9.0	*B. subtilis* - 6AE	6
2	6.0	*B. subtilis*-7AE	7
5	1.7	*B. subtilis*-8AE	8
7	2.0	*B.thuringiensis*-9 AE	9
7	1.1	*B. subtilis*-10AE	10
5	2.5	*B. clausii* -11AE	11

Molecular weight determination of bacteriocin by SDS-PAGE GEL

The bacteriocin was purified from silica plates by thin layer chromatography technique used for determination of MW molecular weight (Lamilla *et al*., 2018). Electrophoresis was performed using a polyacrylamide gel technique SDS, as it works on the dissociation of proteins due to the electrostatic repulsion between the SDS molecules associated with them, and that the number of its molecules is directly proportional to the molecular weight of the

protein, so it has an equal density of charge and therefore the separation depends on the molecular weight MW of the protein using SDS PAGE, proteins with less weight are faster flow from the largest by weight through the separation gel (Satoh *et al*., 2000; Alomari and Gowers, 2017) and compared the result depending on the distance traveled by the standard protein marker protein (10-200 kDa) as shown in the Figure (8) as it moves towards the positive electrode, the approximate molecular weight of the bacteriocin (less than 5 kDa), so its diagnosed with Class I of the Bacteriocin classification Depending on the molecular weight, small active peptides that contain modified non-essential amino acids such as lanthionine to confer structural stability to temperature, pH, and protein degradation. Therefore, they are called Lantibiotics close to type B bacteriocin was small spherical negatively charged or neutral peptides with a small molecular weight, such as the antibiotic Mersacidin, as indicated from (Riley and Chavan, 2007).

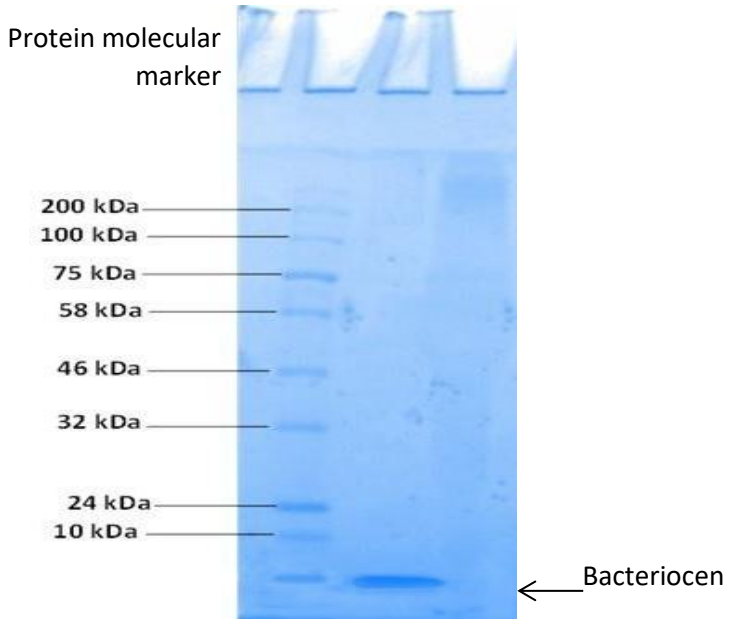

Figure (8): Molecular weight of mersacidin produced from strain *Bacillus* sp.-AE by SDS-PAGE GEL

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