Antibacterial Activity of Secondary Metabolites from *Bacillus* spp.isolated from Basrah ,Iraq

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KEYWORDS

Bacillus spp., secondary metabolites, antibacterial activity, MIC, VITEK

ABSTRACT

Twenty Bacillus spp. isolates were identified from sediment and water samples from different locations in Basrah Governorate, Iraq. The bacterial isolates were cultivated, identified by morphological, biochemical and by using VITEK BCL card. The antibacterial effects of Bacillus extracts against target bacteria included: Methicillin Resistant Staphylococcus aureus (MRSA), Kocuria kristinae, Pseudomonas aeruginosa, and Escherichia coli were examined by agar diffusion test as well as by measuring the Minimum Inhibitory Concentration (MIC) in microtiter dilution assay. The produced extracts showed a variable activity against target bacteria with a MICs ranged between (1.6-6.24) µg/ml against Pseudomonas aeruginosa, (1.6-3.12) µg/ml against Escherichia coli, (0.4-1.6) µg/ml against MRSA, and (0.2-0.8) µg/ml against Kocuria kristinae respectively. Our findings highlighted the importance of sediments and water bacterial isolates for production of compounds with interesting bioactivities that may contribute to drug research field.

الفعالية التضادية للايوض الثانوية لبكتيريا *Bacillus* spp. المعزولة من البصرة

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الكلمات الدالة

بكتيريا Bacillus spp، نواتج الأيض الثانوية،االتضادي الحيوي، التركيز المثبط الادنى, جهاز الفايتك

المُستلخص

تـم فـي هذه الدراسـة الحصول على عشـرون عزلة من بكتيريـا .Bacillus spp وقـد تم عزلها من عينات ترسـبات ومياه من مناطـق مختلفة في محافظة البصـرة في العراق . زرعت العـزلات البكتيرية وشـخصت بالاختبارات الكيميائية الحيوية وكذلك باستخدام نظام التشـخيص VITEK BCL cards . كما تم اسـتخلاص نواتج الاسـتقلاب أو الأيوض الثانوية لبكتيريا Bacillus spp. و درس تأثيرها التضادي تجـاه اربعة انـواع بكتيريـة هـي: Methicillin Resistant Staphylococcus aureus (MRSA), Kocuria kristinae, Pseudomonas aeruginosa, Escherichia coli باسـتخدام طريقتي الانتشار عبـر الآغـار وتحديد التركيـز المثبط الادنـى MIC وقد تراوحت قيـم MIC للبكتيريا المسـتهدفة بين: (1.6-6.24) مايكروغرام/مل لبكتيريـا Pseudomonas aeruginosa و (1.6-3.12) مايكروغرام / مل لبكتيريا (Escherichia coli 0.4-1.6) مايكروغـرام/ مل لبكتيريا MRSA و (0.2-0.8) مايكروغرام / مـل لبكتيريا Kocuria kristinae على التوالي . تسـلط هذه النتائج الضوء على اهمية بكتيريا الترسـبات والمياه في انتاج مركبات ذات فعالية بايولوجية تسـتحق الدراسـة والاهتمام والتي من الممكن ان تسـاهم في حقول ومجـالات كثيرة ومنها بحـوث الادوية.

Introduction

Bacillus spp. are Gram-positive bacteria found diversely in nature. In unsuitable conditions such as high temperature, radiation and harsh chemical reagents, they can form endospores for survival. Besides spore forming, Bacillus spp. are also able to produce secondary metabolite products, which are small molecules that are not directly involved in metabolism and growth of the organism, but they represent an additional function to compete against other organisms. (Boottanun et al., 2017). Bacillus isolates produce structurally diverse classes of secondary metabolites, such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, and isocoumarins .These structurally versatile compounds exhibit a wide range of biological activities, such as antimicrobial, anticancer, antialgal, antiperonosporomycetal (Mondol et al., 2013) Bacillus strains are known to produce a wide variety of biocontrol metabolites, including the ribosomally synthesized antimicrobial peptides (bacteriocins), as well as non-ribosomally synthesized peptides (NRPs) and polyketides (PKs) (Frinking and Marahiel,2004).

The potential of Bacillus species to synthesize a wide varieties of secondary metabolites with antimicrobial activity has been widely used in medicine and pharmaceutical industry. Using of Bacillus species as a biocontrol agent for human, animals and plant diseases control is one of their abilities . Bacillus species are known for the synthesis of secondary metabolites with remarkable diversity both in structure and function. For instance cerecin 7, Tochicin, Thuricin 7, thuricin 439 and entomocidus 9 and few may be ribosomal in origin including sublancin, subtilosin A, subtilin, and TasA are highly active against variety of microorganisms(Sansinenea and Ortiz, 2011). Recently, Bacillus spp. can be considered an inexpensive source for the commercial production of antibiotics. (Amin et al., 2015). In recent years, many investigations have utilized the antimicrobial properties of Bacillus strains (Hsieh et al.,2008 ; Al-Ajlani and Hasanin, 2010 ; Sawale et al. , 2014 and Al- Saraireh et al.,2015) .This study was conducted to identify Bacillus species isolated from different regions of Basrah Governorate, and to test their antibacterial properties.

Materials and Methods

Samples Collection and Preparation Collection and preparation of samples:

Fifty water and sediments samples; 25 each were collected inbetween January and April, 2016 from 7 locations in Basrah Governorate (Table 1). approximately, 20 g per sample, samples were incubated in waterbath at 80◦C for 30 min to kill the non- spore forming bacteria (PHE, 2015). Ten-fold serial dilution were prepared by adding 1g sediment or 1ml water to 10 or 9 ml distilled water respectively, and shaked vigoursly for 1 min, and then 1 ml was added to 9 ml of distilled water for 1 x 10-1 dilution. The process was repeated up to the dilution 10-4 and 10 -6 for water and sediment samples, repectively. Two water dilutions (10-3 and 10 -4) and three sediment dilutions (10-1, 10-3 and 10-6) were filtered through 0.45 µm Millipore filter. The filter papers were blotted on on Lauria – Bertani (LB) agar plates and incubated for 18 h at 35 ° C. the grown colonies were purified by streaking on LB plates and incubation at the privous conditions. Microscopic samples were taken from the suspected colonies for Gram and morphological tests. Subsequently, biochemical testes and VITEK BCL card system were applied on Gram-positive, rod shape and spore forming bacteria for identification.

The presumptive bacilli colonies which isolated from sediments and water were kept by both streaking on LB slants then keeping in the fridge at 40C for several months, and by lyophilization then keep in at -20 0C.

Location	No. of samples		Number of sampes per site
	water	sediments	
fish feeding tanks in marine science center	2	2	4
Khor Al-Zubair harbor	5	5	10
Um Qasr harbor	10	10	20
Abu Floos harbor	2	2	4
Abu Al-Khaseeb	4	4	8
Al-Faw harbor	1	1	2
Asseaba	1	1	2
Total	25	25	50

Identification

For identification both biochemical tests mentioned in Logan and De Vos (2015) as well as BCL card system were used, the biochemical tests were: starch analysis, Vogus Proskauer, cell diameter ≥ 1µm, swellen cell(containing spore), citrate and 6.5% NaCl growth.

BCL card system was used as the following: Cultures were grown on LB agar and incubated aerobically for 18 h at 35 °C for, then tested with BCL card in ASsadr General hospital in Amara-Meesan Governorate . A VITEK2 instrument was used for initial testing ,bacterial suspensions were prepared in 2-5 of sterile saline and adjusted to a McFarland standard of 1·8–2·2 using the VITEK2 DensiChek (bioMérieux). BCL cards were filled automatically in the VITEK vacuum chamber, sealed, incubated at 35·5°C and read automatically for 14 h and 15 min intervals. Data were analyzed automatically using the VITEK2 database version 1.02, and following the release of a later version (3.01), original data were compared with it (Halket et al., 2010).

Secondary metabolites(SM) production

The Bacillus isolates were grown for 48h in LB broth which-contained (per liter) 10 g tryptone, 5 g yeast extract, and 1 g sodium chloride . The pH was adjusted to 7 with 0.01 M HCl and 0.01 M NaOH. The temperature was maintained at 30◦C and the agigation was maintained at shaking 120 rpm for 48 h. The culture broth was centrifuged at 3500 rpm for 15 min ,and the supernatant was filter-sterilized by passing through 0.22 µm millipore filter paper. Thus, the cell free supernatant (CFS) was assessed for the antimicrobial activity against Pseudomonas aeruginosa, Methicillin – Resistant Staphylococcus aureus (MRSA) , Kocuria kristinae and Escherichia coli by using the agar well- diffusion method (Dusane et al.,2011). In this method, LB agar plates were flooded with 0.1ml of target bacterial broth by L-shape spreader then incubated at 35◦C for 15 min. then by using cork porer the plates were pored and the pores were filled with 50 µl of CSF. the plates were incubated at 35◦C for 18 h. the diameters (mm) of inhibition zones were determined by ruler and recorded.

Extraction of crude metabolites

The extraction of crude metabolites was undertaken according to Amin et al.,(2015) as the following : the isolates were cultured in100 ml of LB broth and incubated at 30◦C for 48 h with shaking then the medium was mixed with ethyl acetate 1:1 (v:v) and then stirred using a magnetic stirrer for six hours. The upper organic layer was separated using a separating funnel and centrifuged at 5000 rpm for 10 min. The ethyl acetate layer was then removed and transferred into a clean flask. The extract was pooled and dried in a rotary evaporator (Heidolph, Germany) at 50 °C. The yield from the extract was dissolved in 3 ml of ethanol for antibacterial susceptibility testing.

Separation of the secondary metabolites constituents

1. Thin layer chromatography (TLC)
Method

TLC method was performed on pre-coated silica gel glass plates (silica gel 60 F254 Merck, 0.25 mm layer thickness) (Kaaria et al., 2012) by using (5:78:17) v:v of acetic acid: ethanol 99% :distilled water as an eluent. Developed spots were visualized with ninhydrin (0.1% spray reagent of 2,2-dihydroxyin-1,3- dandion) and heating at 120 °C for detection of amino acids, bromocresol green (0.1 g bromocresol green in 500 ml ethanol, 5 ml of 0.1 M NaOH) for detection of organic acids; and bromothymol blue (0.1% bromothymol blue in 10% aqueous ethanol) for detection of lipids and phospholipids (Al- Sarairah et al., 2015).

2. Determination of total protein content

This test was carried out by using the total proteins kit by Biuret method according to the manufacture's procedures Biolabo company- Italy. This test was undertaken in higer research lab/ College of Agriculture/ Basrah University. Isolates with high protein yield were chosen for further tests.

3. Gas chromatography and mass spectroscopy(GC –MS) characterization of S.M.

The ethanol extract of purified S.M. was undergone for the detection of active molecules by GC-MS according to Mohan et al.,(2016). It was analyzed using Agilent 78908/ 5977A GC-MS ,this test was undertaken in the chemical lab of South Oil Company – Nihran Omar.

Determination of Minimum inhibitory Concentration (MIC)

MIC of SM was determined by the broth microdilution assay according to (Dusane et al., 2013). The target bacteria were : Pseudomonas aeruginosa, Methicillin – Resistant Staphylococcus aureus(MRSA) ,Kocuria kristinae and Escherichia coli.

Results and Discussion

Among 50 sediments and water samples, only 20 bacterial isolates were Gram positive, aerobic spore forming bacilli, , these 20 isolates were identified by using VITEK BCL card, which identified 14 isolates as Bacillus subtilis, whereas the remaining 6 isolates were identified biochemically according to Logan and De Vos (2015) as Bacillus amyloliquifaciens , the results of biochemical tests were illustrated in Table (2). The reson behind the failure of BCL card in identification of these 6 isolates may be attributed to the thick slime layer excreated by these isolates.

Table (2) : Results of biochemical tests for the 6 remaining Bacillus isolates

Biochemical test	result
Starch hydrolysis	+
Vogus Proskauer	+
Cell diameter≥1µm	-
Cell containing spore	+
Citrate utilization	+
6.5 % NaCl growth	+

The identified isolates were designated according to species name as BA for Bacillus amyloliquifaciens or BS for Bacillus subtilis followed by numbers from1-20.

From the 20 Bacillus isolates , 17 were isolated from marine sites(water +sediments) , while only three isolates were isolated from fresh water sites, as we see from these results that higher number of Bacillus isolates were obtained from marine locations , this may be attributed to the fact that the marine environment is poor in nutrients and highly salted, so, not all microorganiss can bloom in such extreme niches except those which tolerate these sever conditions like Bacillus , these reslts were in agreement with Mondol et al. , (2013) who stated that among diverse microbial species, isolates of marine Bacillus belong to phylogenetically and phenogenetically heterogeneous groups of bacteria. They are ubiquitous in the marine environment and can tolerate adverse conditions such as high temperature, pressure, salinity, and pH. Generally, Bacillus strains need more nutrition and space to be the fastest growing bacteria for which they compete with other organisms. Due to the diluting effect of the ocean drives, marine organisms produce potent bioactive compounds to fight off their competitors or to escape from micropredation. Metabolically marine strains are different from their terrestrial counterparts, and thereby, they may produce unique bioactive compounds, which are not found in their terrestrial counterparts .

Table (3) showed the results of antibacterial activity for Bacillus isolates by agar diffusion method, from these results one can observe that the antibacterial activitey of Bacillus isolates toward gram positive bacteria were higher than gram negative bacteria, these results may attributed to the presence of lipopolysaccharide layer in the cell wall of gram negative bacteria which acts as abarrier that prevent the antimicrobial agents from effect on these group of bacteria, this is in agreement with Delcoue,(2009) who reported that the core region of LPS plays a major role in providing a barrier to hydrophobic antibiotics and other compounds, and the strains which express full length LPS have an intrinsic resistance to these compounds.

Table(3) Results of antibacterial activity for Bacillus isolates by agar diffusion method

Isolate No.	Diameter of inhibition zone (mm)			
	K. kristinae	MRSA	E. coli	P. aeruginosa
BA1	12	-	10	8
2BA	10	9	7	-
3BS	12	-	-	-
4BS	9	7	7	-
5BS	11	9	7	-
6BS	16	14	12	10
7BS	10	8	8	-
8BS	15	14	12	12
9BS	13	11	9	9
10BS	13	13	11	10
11BA	11	10	9	9
12BS	11	11	10	8
13BA	15	14	-	11
14BS	14	12	-	-
15BS	10	9	8	8
16BA	15	13	11	-
17BA	12	10	8	8
18BS	13	11	11	9
19BS	13	10	9	8
20BS	14	13	10	8

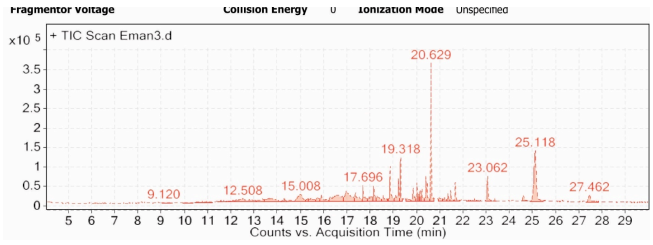
Table(4) Results of antibacterial activity for Bacillus isolates Secondary metabolites against target bacteria

Isolate No.	Diameter of inhibition zone (mm)			
	P. aeruginosa	E. coli	MRSA	K. kristinae
BA1	10	12	16	16
BA2	9	10	12	12
BS3	11	12	12	14
BS4	9	9	11	12
BS5	7	9	10	11
BS6	12	14	14	15
BS7	7	9	9	11
BS8	15	15	17	20
BS9	10	10	12	12
BS10	12	14	12	16
BA11	11	12	14	14
BS12	10	10	13	15
BA13	13	14	16	17
BS14	12	16	16	18
BS15	10	14	13	14
BA16	13	10	12	12
BA17	12	12	14	16
BS18	10	13	15	15
BS19	10	12	14	13
BS20	11	10	12	14

As shown in Table(4), the antibacterial effect of secondary metabolites of Bacillus spp. in gram positive target bacteria was higher than it in gram negative bacteria, the most effected gram positive target bacteria was K. kristinae followed by MRSA, wheras gram negative bacteria was less effected by SM , P. aeruginosa was the less affected bacteria followed by E. coli. These results were in agreement with (Poole, 2005) who indicated that the reason behind resistance of P. aeruginos to the antimicrobial agents may be attributed to both impermeable outer membrane and the presence of proteinaceeous channels in cytoplasmic membrane which exclude the antimicrobial agents out of cell to protect the cell from the effect of these agents.

TLC results showed that Bacillus isolates S.Ms contain amino acids , the GC-MS analysis had confirmed this chemical nature as shown in Table(5) which showed the presence of the

Amino acids leucine and the derivative norvaline in addition to esters and hydrocarbons, figure (1) showed the GC- MS analysis of secondary metabolites from Bacillus spp.



Table(5) analysis of secondary metabolites from Bacillus spp. isolates by GC- MS

Compound Label	RT	Name	Formula	MFG Formula	DB Formula	Hits (DB)
Cpd 4 :2-Amino-2-methyl-1,3-propanediol	13.644	2-Amino-2-methyl-1,3-propanediol	C4H11NO2	C4H11NO2	C4H11NO2	6
Cpd 6: Phenol, 4)-2-aminoethyl)-	15.015	Phenol, 4-)2-aminoethyl)-	C8H11NO	C8H11NO	C8H11NO	10
Cpd 7 :1,3,5-Trioxane	15.471	1,3,5-Trioxane	C3H6O3	C3H6O3	C3H6O3	2
Cpd 8 :2H-Thiopyran, 5,6-dihydro-	15.71	2H-Thiopyran, 5,6-dihydro-	C5H8S	C5H8S	C5H8S	5
Cpd 9: Diethylene glycol, O,O-di(pivaloyl)-	15.902	Diethylene glycol, O,O-di(pivaloyl)-	C14H26O5	C14H26O5	C14H26O5	1
Cpd 10: Pyrrole, 2-methyl-5-phenyl-	16.276	Pyrrole, 2-methyl-5-phenyl-	C11H11N	C11H11N	C11H11N	10
Cpd 11: Phenethylamine, N-benzyl-p-chloro-	16.586	Phenethylamine, N-benzyl-p-chloro-	C15H16ClN	C15H16ClN	C15H16ClN	10
Cpd 12 :1-(1'-pyrrolidinyl)-2-butanone	16.983	1-)1'-pyrrolidinyl)-2-butanone	C8H15NO	C8H15NO	C8H15NO	10
Cpd 13 :2-Cyclohexen-1-one, 3,5-dimethyl-	17.375	2-Cyclohexen-1-one, 3,5-dimethyl-	C8H12O	C8H12O	C8H12O	3
Cpd 14 :3-Ethoxy-4-methoxyphenol	17.699	3-Ethoxy-4-methoxyphenol	C9H12O3	C9H12O3	C9H12O3	10
Cpd 16 :1H-Pyrazolo[3,4-d]pyrimidin-4-amine	18.041	1H-Pyrazolo[3,4-d]pyrimidin-4-amine	C5H5N5	C5H5N5	C5H5N5	10
Cpd 17 :2-Cyclopenten-1-one, 2-hydroxy-3,4-dimethyl-	18.162	2-Cyclopenten-1-one, 2-hydroxy-3,4-dimethyl-	C7H10O2	C7H10O2	C7H10O2	10
Cpd 18: Indolizine, 5-methyl-	18.328	Indolizine, 5-methyl-	C9H9N	C9H9N	C9H9N	10
Cpd 19: Methyl-6-deoxy-6-fluoro-2,3,4-tri-O-methyl.beta.d-galactopyranoside	18.569	Methyl-6-deoxy-6-fluoro-2,3,4-tri-O-methyl.beta.d-galactopyranoside	C10H19FO5	C10H19FO5	C10H19FO5	2
Cpd 20: l-Norvaline, n-propargyloxycarbonyl-, nonyl ester	18.863	l-Norvaline, n-propargyloxycarbonyl-, nonyl ester	C18H31NO4	C18H31NO4	C18H31NO4	10
Cpd 21 :1-Propanone, 1)-5-methyl-2-thienyl)-	19.114	1-Propanone, 1)-5-methyl-2-thienyl)-	C8H10OS	C8H10OS	C8H10OS	1
Cpd 22: Pentadecanoic acid, ethyl ester	19.228	Pentadecanoic acid, ethyl ester	C17H34O2	C17H34O2	C17H34O2	3
Cpd 23: Ethyl 13-methyl-tetradecanoate	19.314	Ethyl 13-methyl-tetradecanoate	C17H34O2	C17H34O2	C17H34O2	10
Cpd 24: l-Leucine, N-cyclopropylcarbonyl-, butyl ester	19.855	l-Leucine, N-cyclopropylcarbonyl-, butyl ester	C14H25NO3	C14H25NO3	C14H25NO3	10
Cpd 25: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3)-2-methylpropyl)-	20.021	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3)-2-methylpropyl)-	C11H18N2O2	C11H18N2O2	C11H18N2O2	10
Cpd 27 :5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1,'2-'d]pyrazine	20.165	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1,'2-'d]pyrazine	C14H22N2O2	C14H22N2O2	C14H22N2O2	2
Cpd 28: Heptanoic acid, 2-ethyl-	20.234	Heptanoic acid, 2-ethyl-	C9H18O2	C9H18O2	C9H18O2	10
Cpd 29: Ethyl 9-hexadecenoate	20.417	Ethyl 9-hexadecenoate	C18H34O2	C18H34O2	C18H34O2	10
Cpd 30 :4-Fluorobenzylamine, N,N-diheptyl-	20.465	4-Fluorobenzylamine, N,N-diheptyl-	C21H36FN	C21H36FN	C21H36FN	4
Cpd 31: Hexadecanoic acid, ethyl ester	20.628	Hexadecanoic acid, ethyl ester	C18H36O2	C18H36O2	C18H36O2	10
Cpd 34 :9-Octadecen-1-ol, (E)-	21.679	9-Octadecen-1-ol, (E)-	C18H36O	C18H36O	C18H36O	10
Cpd 35 :9-Octadecenoic acid, ethyl ester	23.066	9-Octadecenoic acid, ethyl ester	C20H38O2	C20H38O2	C20H38O2	10
Cpd 37: Phthalic acid, di(6-methylhept-2-yl) ester	25.111	Phthalic acid, di(6-methylhept-2-yl) ester	C24H38O4	C24H38O4	C24H38O4	10
Cpd 38] :1,2,4]Triazolo[1,5-a]pyrimidin-7-ol, 5-methyl-	25.128	]1,2,4]Triazolo[1,5-a]pyrimidin-7-ol, 5-methyl-	C6H6N4O	C6H6N4O	C6H6N4O	5
Cpd 39: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	27.46	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C14H16N2O2	C14H16N2O2	C14H16N2O2	2

These findings were in agreement with Sawale et al., (2014) who extracted a polypeptide compound from halophilic Bacillus spp. this extract showed antimicrobial activity against both bacteria and fungi. We chose the more five protein producing isolates to carry out the MIC determination test, these isolates were designated BS8, BS14, BS6, BS3, and BA1 respectively. The results of MIC inhibition were shown in table (6)

Table (6) MIC of secondary metabolites from five Bacillus isolates. against target bacteria (µg/ml)

Bacillus isolate	P.aeruginosa	E.coli	MRSA	K.kristinae
BS8	1.6	1.6	0.4	0.2
BS14,	3.12	1.6	0.8	0.8
BS6	3.12	3.12	0.4	0.4
BS3	6.24	3.12	0.8	0.4
BA1	6.24	3.12	1.6	0.8
Nalidixic acid	40	10	5	5
Tetracyclene	80	80	10	5

The results of MIC inhibition test showed that the S.M extracted from Bacillus spp. were more effective against Gram positive bacteria than Gram negative isolates. Moreover , the S.M were effective against bacteria more than the both traditional antibiotics (Nalidixic acid and Tetracyclene) which had used for comparison. Our findings are in agreement with Anju et al,. (2015) who reported that the growth inhibitory effect against a variety of microorganisms can be attributed to either the bacteria or its metabolites. Also, these findings were in agreement with Al-Sarairah et al., (2015) and Amin et al.,(2015) who indicated that members of Bacillus spp. may be considered a promising source for isolation secondary metabolites with potential application in pharmaceutical and agricultural industry

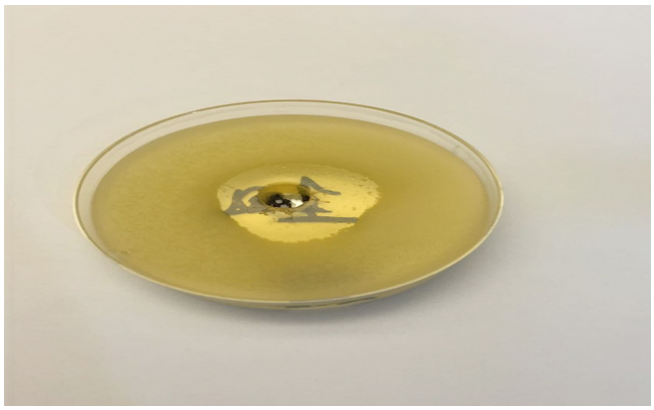


Figure (2) The antibacterial activity of Bacillus spp. secondary metabolites against target bacteria (Kocuria kristinae)

Conclusion

Throughout this study, 20 Bacillus spp. isolates exhibited a promising bioactivity against target bacteria . Our findings highlighted the importance of sediments and water bacterial isolates for production of compounds with interesting bioactivities that may contribute to drug research field.

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