EVALUTION OF ANTIMICROBIAL ACTIVITY OF PARTIAL PURIFIED BACTERIOCIN FROM LOCAL ISOLATE OF *BACILLUS LICHENIFORIMS* HJ2020 MT192715.1

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ABSTRACT

This study was aimed to produce bacteriocin from *Bacillus. licheniformis* isolated from local soil of corn and sunflower fields and using as antimicrobial agent . Fourteen of local isolates of *Bacillus* sp. were obtained and ability of these isolates for growth on Brain heart infusion agar (BHI) at 55^0C were tested. Isolate C4 was revealed high growth density in comparison with other isolates. Isolate C4 was identified as *Bacillus licheniformis* according to morphological, cultural and biochemical tests, Moreover genetic analysis for 16S rRNA gene and given accession number MT192715.1 in GenBank of NCBI . Production of bacteriocin from this isolate was carried out in Luria Broth (LB) and partially purified by precipitation with 30-70 % saturation of ammonium sulfate followed by concentrated using poly ethylene glycol (PEG).The antimicrobial activity of partially purified bacteriocin was assayed against many species of food spoilage microorganism. Results were revealed that anitimicrobial activity of bacteriocin were between (0 - 360) units / ml. Stability of antimicrobial activity of partially purified bacteriocin toward *Staphylococcus aureus* were tested after incubation at different values of pH, temperature and some of enzyme which included proteolytic enzymes, α-amylase and lipase .The results indicated that residual inhibition activity of bacteriocin were varied according to conditions of incubation and type of treatment .

Key words: 16S rRNA, GenBank MT192715.1, Sunflower soil, Corn soil.

تقييم الفعالية المضادة للميكروبات للبكتريوسين المنقى جزئيا من العزلة المحلية من *Bacillus licheniforims* HJ2020 MT192715.1

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المستخلص

هدفت الدراسة الحاليه الى انتاج البكتريوسين من بكتريا *Bacillus licheniformis* المعزولة من الترب المحلية لحقول نبات الذرة الصفراء وحقول زهرة الشمس واستخدامه كمضاد للميكروبات التي تسبب تلف الغذاء . تم الحصول على 14 عزلة محلية من البكتريا العصوية *Bacillus* sp. . تم اختبار قابلية هذه العزلات في النموعلى نقيع القلب والدماغ(BHI) الصلب بدرجة حرارة 55 م ^{0} . اظهرت العزلة C 4 كثافة نمو عالية في هذا الوسط مقارنة بالعزلات الاخرى . شخصت العزلة C4على انها *Bacillus licheniformis* بناءًعلى الفحوصات المظهرية والمزرعية والكيموحيوية فضلا عن التحليل الجيني لجين 16S rRNA واعطيت رمزالوصول MT192715.1 اعتمادا على بنك الجينات التابع للمركز العالمي للمعلومات الحياتية (NCBI). انتج البكتريوسين من العزلة المنتخبة وباستخدام وسط(Luria Broth (LB ، ونقي جزئيا بالترسيب باستخدام كبريتات الامونيوم بنسبة اشباع 30 –70 % وركزباستخدام البولي اثلين كلايكول (PEG).اختبرت الفعالية التثبيطة للبكتريوسين المنقى جزئيا ضد مجموعة من الاحياء المجهرية والمسببة لتلف الغذاء. اظهرت النتائج بان الفعالية التثبيطية للبكتريوسين تراوحت بين 0– 360 وحدة / مل.كما اختبرت ثبات الفعالية التثبيطية للبكتريوسين المنقى على بكتريا المكورات العنقودية الذهبية *Staphylococcus* aureus بعد حضنه بارقام هيدروجينية وحرارية مختلفة ومع عدد من الانزيمات شملت على البروتيزات والالفا اميليز واللايبيز،اوضحت النتائج ان الفعالية التثبيطية المتبقية للبكتريوسين المنتج من العزلة الحالية كانت متباينة حسب ظروف الحضن ونوع المعاملة المستخدمة.

الكلمات المفتاحية : 16S rRNA ; GenBank MT192715.1 ; Sunflower soil , Corn soil

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INTRODUCTION

Bacillus licheniformis is a bacterium commonly found in the soil , It is a rod to elliptical or cylindrical shape with length of 1.5 - 3 µm and width of 0.6 - 0.8 µm . It is central spore forming , gram-positive , optimal growth temperature is around 50 to 55 ^{o}C , though it can survive at much higher temperatures . It can exist in a dormant spore form to resist harsh environments, or in a vegetative state when conditions are good . Colony on agar become opaque with dull to rough surface , irregular edges , creamy color and somewhat sticky. It can grow in aerobic and anaerobic conditions . It can produce acid and weakly positive gas from glucose , it can hydrolyze starch , reduce NO_3^- to NO_2^- and liquefication of nutrient gelatin (7 , 18) . *Bacillus licheniformis* has proved itself as a multipurpose organism and has gained popularity along with *B. subtilis* . It is most commonly found in soil (9). This isolate was used to produce a polypeptide antibiotic known as bacteriocin as well as production of commercially feasible quantities of different industrial enzymes (11 , 20) . *B. licheniformis* is capable of producing bacteriocins in aerobic conditions (2 , 16) , Moreover at anaerobic conditions against anaerobic microorganisms (24). Production of antimicrobial peptides is widespread among diverse bacteria (6). Most known bacteriocin producing *Bacillus* strains are either from soil or food . Such as bacteriocin produced by *Bacillus cereus* isolated from food (22) and those that produced by *Bacillus cereus* 8 A isolated from soil (5). Keeping the significance of multiple uses of *Bacillus licheniformis* in view, present study was designed to isolate strain of *Bacillus* from Iraqi soil for the production of bacteriocin , then evaluate and characterize the produced bacteriocin as antimicrobial agent .

MATERIALS AND METHODS

Media and chemical reagents

All medium such as nutrient agar, nutrient broths, Brain Heart Infusion (BHI) agar, BHI broth, Lurain broth (LB), De Man, Rogosa and Sharpe agar (MRS) agar, Potato dextrose agar and MacConkey agar were purchased from HiMedia Biosciences company (india). other chemicals used throughout study were commercial products of highest purity grade and purchased from Sigma Chemicals Co. Millipore membranefilters 0.45µm were purchased from Sigma-Aldrich company. Solutions were prepared in de-ionized pure water from Al-Joud company (Iraq) .

Microorganisms

Some of microorganism tested against bacteriocin activity were provided from Ibn – Albetar and Ibnsina center / Industrial research and development Authority / Ministry of Industry and Minerals / Iraq and others from Food science Dept./ College of Agr. Engineering .sciences. / University of Baghdad. These microorganism were included *E.coli*, *Staphylococcus*, *Salmonella*, *Bacillus*, *Candida*, *Lactobacillus* and *Bifidobacterium.*

Isolation of *Bacillus* strains

Ten samples from different places of soil were collected from Iraqi corn and sunflower field belong to horticulture and field crops departments / College of agricultural engineering sciences / Baghdad university . In order to isolate the spore-forming bacteria, 10 gm of each sample of soil were suspended in 90 ml of sterilized distilled water and heating at 80 ^{0}C to kill non-spore-forming bacteria . Serial dilutions were done , then 0.1 ml of each diluents were transfered to petridish with duplicates , then BHI agar were add. After solidification, plates were incubated inverse direction under aerobic condition at 55 ^{0}C for 48 hour. Mesophiles and obligated anaerobic spore-formers bacteria will not grow at this conditions (7, 18). Single isolates which grown were purified by sub - culturing on BHI agar. Pick up these isolates which have similar characteristics of *Bacillus* such as irregular edges, creamy color and somewhat sticky.

Identification of *Bacillus* isolate

The purified isolate that gives high growth on BHI agar at 55 ^{o}C were identified by morphological, biochemical tests and genetic analysis of *16S rRNA* gene sequence. The morphological tests were included a microscopic and culturing characteristics, gram stain, shap, aggregation, and spore position, while cultural characteristics of colonies were included appearance, color, edge and surface height when growth on BHI agar. Biochemical tests were included growth

in anaerobic condition, growth at 55 ^{o}C and 15 ^{o}C, catalase reaction, gelatin liquefication, starch hydrolysis, nitrate reduction, citrate utilization, Voges-proskauer reaction, acid production from glucose, xylose and l-arabinose (7, 18). Genetic analysis of the selected isolate was also carried out by amplification of 16S rRNA gene using PCR technique (27). Sequence of nucleotide were also analyzed in Korean Microgen Company and compared with those Genbank database in NCBI . Genetic analysis include extraction DNA of selected isolate using promega protocol and kit provided from this company. Purity of DNA extract (1 µl) was determined by Nanodrop spectrophotometer and absorbance at 260 nm and 280 nm were measured using following equation (Purity of DNA extract = Absorbance at 260nm / Absorbance at 280 nm) . Amplification of 16S rRNA was done using forward primer (F27) 5'AGAGTTTGATCCTGGCTCAG'3 and Reverse primer (R1492) 5'TACGGTTACCTTGTTAC GACT T'3 according to (27) . Adding 12.5 µl of Master mix , 1µl of these primers and 8.5 µl of nuclease free and 2ng/ µl of DNA extract . Eppendruff tube was transfer to thermocycler and following program were used : First denaturation of DNA target was carried out at 95°C for 5 minutes followed by second denaturation at 95^{0}C for 30 sec. , then primer annealing at 60 ^{0}C for 45 sec . Extension at 72 ^{o}C for 1min . Reaction was repeated for 30 cycles . Final extension were done at 72 ^{0}C for 7 min. Finally reaction mixture was held at cooling temperature 4 ^{0}C for 10 minutes . PCR amplification was detected by agarose gel electrophoresis . 1 % of 100 ml agrose was prepared by dissolve 1 gm of agrose in 100 ml of 1 X TBE buffer . Mixture was heated in microwave oven for 2min and 1 µl of ethidium bromide(10mg /ml) was added . Gel was poured at 50-60 ^{0}C in electrophoresis mold . Putting comb in gel to make samples slot and leave gel to solidification . Remove comb and add 1 X TBE buffer to cover gel surface.Transfer 5µl of PCR product and ladder (100 - 1500 bp) provided from promega company in gel wells , then run of electrophoresis at 60 mA and 90 volt . Coming down of dye were noticed . Finally visualized the isolated bands under UV light .The output of amplified gene were sent to Korean Macrogen company to determine sequencing of nitrogen base and BLAST program was used (4) to compare with GenBank database of NCBI.

Bacteriocin production

production of Bacteriocin from *Bacillus lichenformis* HJ2020 MT192715.1 was carried out in Lurain broth (LB) medium (Tryptone 10.0 , Yeast extract 5.0 ; NaCl 5.0 g/L) , pH of medium was neutralized to 7.0 and sterilized in autoclave at 121 ^{o}C for 15 minutes at 15 pound / inch^{2} .Two percent of Inoculum contain 1x10^{6} CFU ml^{-1} of *Bacillus licheniformis* HJ2020 MT192715.1 were transfer to 250 ml of conical flask contain 100 ml of sterilized LB medium and placed in shaking incubator at 37^{o} C with the agitation of 150 rpm for 48 hr. After that the cells were harvested by centrifugation at 8000 Xg for 15 minutes at 4 ^{o}C and cell free supernatant(CFS) was considered as crud bacterocin. (19)

Partially Purification of bacteriocin

Bacteriocin as Cell Free Supernatant (CFS) was concentrated by precipitation using 30 -70% saturation of ammonium sulphate with continuous stirring for overnight at 4°C . Precipitate was harvested by centrifugation at 6000 xg for 15 min at 4^{o}C and re-suspended in 25 ml of 5 mM phosphate buffer pH 7 , then dialyzed with phosphate buffer 0.1M pH 7 for overnight with replacing buffer for three times using dialysis tube with 1 KDa molecular weight cut off (MWCO). Suspension was concentrated by dialyzing in poly ethylene glycol (from Fluka company) . Finally concentrated bacterocin was sterilized by subjected to Millipore membrane filter (0.45) µm and considered as partially purified bacteriocin, (19).

Antimicrobial activity of bacteriocin

Antimicrobial activity of partially purified bacteriocin was tested against several Gram-positive bacteria, Gram-negative bacteria and yeast included *E.coli*, *Staphylococcus*, *Salmonella*, *Bacillus*, *Lactobacillus*, *Bifidobacterium* and *Candida*. These microorganisms were cultured in appropriate media and bacterocin antimicrobial activity was determined using agar well diffusion method as described by (25 , 32). Suspension

of these indicator isolates were adjusted to 0.5 MacFarland standard and inoculated in appropriate media. Allowed to dry and a sterile cork borer was used to made well of 6mm diameter on the agar plates. Wells were filled with 50 µl of partially purified bacterocin of *B. lichenformis* HJ2020 MT192715.1 . Plates were incubated for overnight at optimal temperature of test organism. After incubation, diameter of zone inhibition was measured.The activity of the bacteriocin preparation expressed in units per milliliter (U/ml) and calculated by following formula:

Antimicrobial activity of bacteriocin (U/ml) = (1,000/50) x inhibition zone diameter of highest dilution (mm) x 1 / D

where 50 is the volume (µl) of utilized sample , 1000 to convert of utilized sample volume from(µl) to (ml) and D is the highest dilution that inhibited the growth of indicator strain at 24 h of incubation (10). All the experiments were performed in duplicate and results were mean of the observations.

Effects of pH, temperature and enzymes on stability of bacterocin from *B.lichenformis* HJ2020 MT192715.1 against *Staphylocoous aureus*

pH of partially purified bacterocin was adjusted to (3, 5, 8, 10, and 12) , and incubated at 37 ^{o}C for 2 hr , then readjusted to 7 with 1N of HCL or IN of NaOH . This step was done to determine the residual antimicrobial activity of bacteriocin using treatment of pH 7 as control (11, 17, 19). Also the partially purified bacteriocin solution was incubated at 5, 35, 50, 80, 100 °C for 30 min. and at 121 ^{o}C for 15 min. then cooled to room temperature and residual antimicrobial activity were determined. Partially purified bacteriocin with out heat treatment used as control at pH 7. To analyze sensitivity of bacterocin toward various enzymes , partially purified of bacteriocin solution was treated with 1mg ml^{-1} final concentration of the following enzymes at 37 C for 2 h : α-amylase . lipase , α-chymotrypsin proteinase-K , pepsin and trypsin (All enzymes used were from Sigma –Aldrich company) . Samples were then boiled at 100 ^{o}C for 2min to denature enzymes , then cooled to room temperature and residual antimicrobial activity were determined using untreated partially purified bacteriocin as control . All treated samples were tested for residual activity against *Staphylococcus aureus 1x10^{6}* CFU ml^{-1} (17 , 32) .

RESULTS AND DISCUSSION

Identification of isolated strain

Fourteen isolates of *Bacillus* sp. from Sunflower and Corn soil samples were obtained when incubation on BHI agar at 55 ^{o}C. The isolate C4 was revealed a highest growth density on BHI agar in comparison with other isolates Table(2).

Table 1. Corn and sunflower soil isolates growth on BHI agar at 55 ^{0}C / 48 hr

Strain from corn soil	Growth density	Strain from sunflower soil	Growth density
C1	+	S1	++
C2	+	S2	+
C3	++	S3	+
C4	+++	S4	++
C5	+	S5	+
C6	++	S6	+
C7	+	S7	++

Preliminary identification of isolate C4 was based on its morphological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology. Isolate C4 revealed white-creamy colony with irregular edge when growth on BHI agar at 55 ^{0}C. Arrangements of colonies were bilateral or separate groupings . Morphological test of C4 isolate revealed that this isolate was gram positive and motile , it seem to be elliptical – cylinderical shape and endospore forming was central position .These results were agree with (23).Biochemical test of isolate C4 revealed that this isolate can able to hydrolyze starch, catalase positive , gelatin liquefaction was positive and can grow at 15 and 55^{O}C (Table 3).

Table 2 . Morphological and Biochemical test of isolate C 4

Morphological	
Test	**Result**
Shape	**Elliptical -cylinderical**
Arrangements	**Bilateral or separate groupings**
Gram reaction	**+**
Endospore formation	**Central**
Colony color	**White – creamy**
Motility	**+**
Biochemical	
Test	**Result**
Catalase	**+**
Gelatin liquefaction	**+**
Starch hydrolysis	**+**
Nitrate reduction	**+**
Citrate utilization	**+**
Voges-proskauer	**+**
Acid production from	
D-glucose	**+**
D-xylose	**+**
L-arabinose	**+**
Growth in anaerobic agar	**+**
Growth at 55 ^{0}C	**+**
Growth at 15 ^{0} C	**+**

These results were agree with (7, 18), where they pointed out the general characterizations of *Bacillus* genes and revealed that this isolate belong to *B. lichenformis* according to (12). Moreover genetic analysis was achieved depending upon identification of 16S rRNA gene, Firstly the extraction of DNA for selected isolate C4 has been done and the purity was determined by measure the ratio of absorbance at 260nm to absorbance at 280nm. Therefore the purity of DNA extract of isolate C4 attained to 1.9 . This result conforms with many studies which pointed out that the purity of DNA extraction for prokaryote was considered highly purified if the ratio of absorbance at 260 nm to absorbance at 280 nm was equal ≥ 1.8 (31). Then amplification of 16S rRNA gene by PCR technique was achieved . Results of electrophoresis were revealed that there is a single band of amplified gene(Fig1)**. 1500bp**

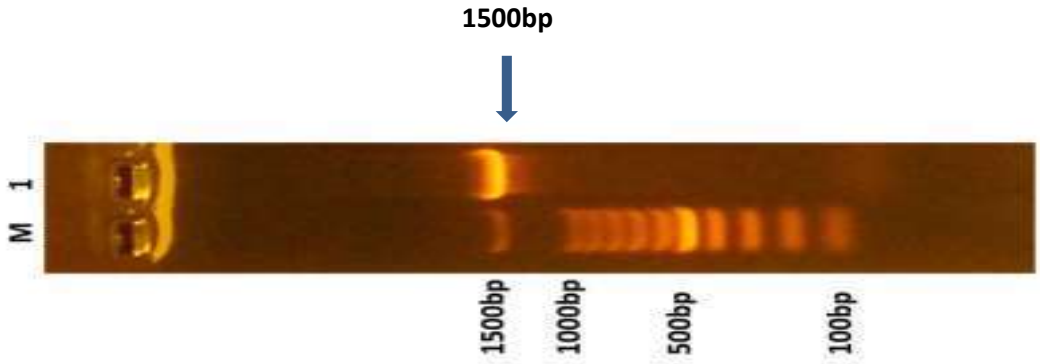

Fig 1. PCR amplification product of 16S rRNA gene on 1 % agrose for isolate C4 , Lane M: ladder (100bp); lane 1 sample

This result indicate a successful engagement of primers with gene target 16S rRNA with out other parts of DNA . Molecular weight of amplicon was determined and attained to about 1500 bp (Fig 1) .Many studies point out using gene 16S rRNA with successfully for identification of many species of bacteria and it gives a decisive results (1, 26) . Another study (3) indicate length of amplified target (16S rRNA) of *Bacillus licheniformis* attained to 1.4 Kbp , While (28) found molecular weight of amplified gene of 16S rRNA for *Bacillus* sp. were 1082 bp and (13) pointed that molecular weight of 16S rRNA of *Bacillus subtilis* ZHR(GenBank no. MG735442.1) were 1150 bp after trimmed some of low quality sequences especially irregular peaks . Also (14) indicated that molecular weight of 16S rRNA of *Bacillus subtilis* MH049477.1 was 1265 bp. Differences in molecular weight of amplified gene for 16S rRNA may be due to differences between sources of isolates, Moreover differences between DNA extraction protocol or program conditions which used in PCR technique (26). Products of PCR amplification and primers were sent to Macrogen company to study sequence of nucleotides for 16S rRNA gene . Results revealed that sequence of 16S rRNA gene contain 1415 nucleotide after trimmed some of sequences which had low quality especially irregular peaks (Table 3). Formation of these parts may be due to primers fusion or it represent short results of amplification which disappeared during electrophoresis on agarose gel . Alignment of

this sequence through matching with reported 16S rRNA gene sequences in NCBI Genbank using BLAST program showed that isolate C4 have similar sequence with 100 % to those sequences of more than 20 strain of *Bacillus licheniformis* (Table 4). For this reason the local isolate C4 was considered belong to *Bacillus lichenformis* and given a strain name HJ2020 with accession number MT192715.1 according to Genbank data base of NCBI.

Table 3. The sequence of nucleotides (FASTA) of *16S rRNA* gene for isolate C4

Gene	Sequencing of *16S rRNA* (FASTA Sequencing)	Total nitrogen base
1_27F 16SrRNA	GTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAG TAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCT AATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGC TACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAA AACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACG GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTC TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGG GGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGAC GCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAG CAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAA GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA CGCGAAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCC CCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATT CAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCA GAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCA GTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCG CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC ACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAG CCGCCG	1415 bp

Table 4. Ratio of similarity of local isolate C4 with *Bacillus* strains in NCIB – blasted record

Item	Strain	Identi. %	Accession
1	*Bacillus licheniformis* strain HJ2020 16S ribosomal RNA gene,partial sequence	100	MT192715.1
2	*Bacillus licheniformis* strain ANA17 16S ribosomal RNA gene, partial sequence	100	MT122807.1
3	*Bacillus licheniformis* strain LXJ95 16S ribosomal RNA gene, partial sequence	100	MN746177.1
4	*Bacillus licheniformis* strain CGZ927 16S ribosomal RNA gene, partial sequence	100	MN900518.1
5	*Bacillus licheniformis* strain P8_B2 chromosome, complete genome	100	CP045814.1
6	*Bacillus licheniformis* strain MDSp5 16S ribosomal RNA gene, partial sequence	100	MN493794.1
7	*Bacillus licheniformis* strain BioE-BL11 16S ribosomal RNA gene, partial sequence	100	MN493718.1
8	*Bacillus licheniformis* strain strain KNU11 chromosome, complete genome	100	CP042252.1
9	*Bacillus licheniformis* strain HN-5 16S ribosomal RNA gene, partial sequence	100	MK648261.1
10	*Bacillus licheniformis* strain CLMTCHB29 16S ribosomal RNA gene, partial sequence	100	MH197076.2
11	*Bacillus licheniformis* strain PB3 chromosome, complete genome	100	CP025226.1
12	*Bacillus licheniformis* strain UN1 16S ribosomal RNA gene, partial sequence	100	MK088263.1
13	*Bacillus licheniformis* strain D4-10-2-2 16S ribosomal RNA gene, partial sequence	100	MK063871.1
14	*Bacillus licheniformis* strain D4-10-1-3 16S ribosomal RNA gene, partial sequence	100	MK063868.1
15	*Bacillus licheniformis* strain BRM043913 16S ribosomal RNA gene, partial sequence	100	MH305328.1
16	*Bacillus licheniformis* strain HTI601-1 16S ribosomal RNA gene, partial sequence	100	MG835984.1
17	*Bacillus licheniformis* strain NYL26 16S ribosomal RNA gene, partial sequence	100	MG833400.1
18	*Bacillus licheniformis* strain Sohag3 16S ribosomal RNA gene, partial sequence	100	MH605438.1
19	*Bacillus licheniformis* strain MGYG-HGUT-02357 genome assembly, chromosome	100	LR698983.1
20	*Bacillus licheniformis* strain T7 16S ribosomal RNA gene, partial sequence	100	MH000674.1

Antimicrobial activity of bacterocin

Inhibitory spectrum of bacteriocin was determined by agar well-diffusion according to method of (25) against different indicator strains (Table 5). Results were revealed that the Inhibitory activity was attained to 220

and 360 U ml^{-1} against to pathogenic strains, including clinical isolates of *Escherichia coli* and *Salmonella typhi* respectively, while it attained to 42, 60, and 80 U/ml against to *B.subtilis, B. cereus* and *Candida albican* respectively. The differences of mode of action of bacteriocin on microorganisms may be attributed to it's effect on cell membranes permeability or integrity which was disrupted and caused leakage of intracellular components including Na+K+-ATP, AKP, nucleic acids and proteins (33) or may be due bactericidal and cytoxicity effect of bacterocin which lead to kill the cell according to type of microorganism (30). Results also revealed that there is no activity was detected against *Lactobacillus and Bifidobacterium* which were generally recognized as beneficial bacteria or safe bacteria. These results were similar to those shown by *B. licheniformis* P40 (8).

Table 5. Inhibitory spectrum of bacteriocin from *Bacillus licheniformis* toward some of microorganism

Indicator strain	Activity (U ml^{-1})
***Escherichia coli* 0157:H7**	**220**
Staphylococcus aureus	**360**
Salmonella typhi	**200**
Pseudomonas aeroginosa	**233**
Bacillus cereus	**60**
Candida albicans	**80**
Lactobacillus plantarum	**0.0**
Bifidobacterium befidie	**0.0**
Bacillus subtilis	**42**

Effects of pH , temperature and enzymes on stability of bacterocin from *Bacillus lichenformis* HJ2020MT192715.1against*Staphylocoousaureus*

The stability of partial purified of bacteriocin produced from *Bacillus licheniformis* HJ2020 MT192715.1 was tested with different values of pH , temperature and sensitivity toward several proteolytic enzymes, Moreover α-amylase and lipase against *Staphylococcus aureus* with density of 1 X 10^{6} CFU ml^{-1} . Results were summarized in Table (6). Bacteriocin was loss about 25-40% of it's activity when incubated in acidic pH between (3-5), while it lost about 80 % of it's activity at pH 10 and there is no activity at pH 12 . These results were agree with (21) which pointed that the activity of bacteriocins was completely disappeared at pH 12 and (16) was suggested that the bacteriocin activity affected by pH and disappeared at pH 9. Heat stability of bacterocin also was tested and the results show that it retained all activity when incubated at 5 - 35 ^{o}C for 30 min . while it loss about 25-50 % of it's activity after incubation at 50- 80 ^{o}C and loss all activity when incubated at 100^{o}C / 30 min or treated with autoclave at 121 ^{0}C for 15 min at 15 psi. Reduction of bacteriocin activity and lost all of it's activity at high temperature attributed to denaturation will occur for proteinaceous nature of bacterocin (6). Results also revealed that bacteriocin was stable when treated with α- amylase and lipase because that bacteriocine might not contain glycosidic or lipidic structure, So these enzymes (glycosidaeses and lipases) have no effect on proteinaceous nature of bacterocine (29). While it sensitive to proteolytic enzyme and lost about 10 -50 % of it's activity when treated with these enzymes Table (6). These results confirm once again that this bacteriocin from *Bacillus licheniforims* HJ2020 MT192715.1 is proteinaceous nature and this agree with (5, 11, 17).

Table 6. Effects of pH , Temperature and Enzymes on antimicrobial activity of bacteriocin from *Bacillus licheniformis* HJ2020 MT192715.1 against *Staphylococcus aureus*

Treatments	pH	Residual activity %	Temperature	Residual activity %	Enzyme	Residual activity %
	3	**60**				
	5	**75**	**5 0C/30 min.**	**100**	**Amylase**	**100**
	7	**100**	**35 0C/ 30 min**	**100**	**lipase**	**100**
	8	**90**	**50 0C/ 30 min**	**75**	**Chymotrypsin**	**90**
	10	**20**	**80 0C/ 30 min**	**50**	**Proteinase K**	**50**
	12	**0.0**	**100 0C/ 30min**	**0.0**	**Pepsin**	**80**
			121 0C / 15 min/ 15 psi	**0.0**	**Trypsin**	**75**

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