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Detection, purification and characterization of a bacteriocin produced by *Bacillus subtilis* NK16 exhibits a significant antimicrobial activity against clinical *Staphylococcus* spp.

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Abstract:

Bacteriocin is an important antimicrobial peptide that can be used in industrial and medical fields due to its characteristics of antibacterial, food preservation and anticancer activities. Fifty isolates of *Bacillus* sp were collected from different soil samples which were already recognized via morphological and biochemical identification process. The isolates were screened for bacteriocin production effective against *Staphylococcus* spp in order to select the highest producing isolate. The isolate NK16 showed the maximum bacteriocin production (80 AU/ml) which was further characterized as *Bacillus subtilis* NK 16 through using API identification system (API 20E and API 50CHB). Then, next step was to detect the optimal conditions for maximum bacteriocin production which were found to be brain-heart infusion broth as the best production medium with pH 6, 30^{o}Cand 2% inoculum size. Bacteriocin was partially purified by precipitation with ammonium sulphate and then separation with sephadex G-150 gel filtration. The specific activity of the resulted partial purified bacteriocin was increased to 853.33 AU/mg with 38 fold purification and 24% yield. The study of bacteriocin characterization revealed that the activity of bacteriocin was stable after 10 min at 20, 30, 40^{o}C whereas 50% of the bacteriocin activity was lost after exposure to 50^{o}C and decreased to approximately 20 AU/ml at 60,70 and 80 C^{o}. In addition, bacteriocin activity showed stability at pH 6 and 7 for 30 min while it was decreased by approximately 50% at pH 5 and 8, and completely inhibited at pH 4 and 9. On the other hand, the investigation of mode of action showed that bacteriocin has a bactericidal activity. Antimicrobial activity tests of the partial purified bacteriocin displayed a significant activity against most clinical *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates, whereas it was less effective against *Staphylococcus saprophyticus* isolates.

Key words: *Bacillus subtilis*, bacteriocin, purification, characterization, antimicrobial activity and *Staphylococcus* spp

Introduction:

From the mid-1940s until relatively recent years, we have been fortunate to be living through what has been termed “the Golden Age of

Antibiotics". During this period, all of the major infections caused by bacteria and fungi have been defeated by the use of antibiotics. This period has also seen dramatic improvements in surgical procedures which have revolutionized medicine and saved countless lives [1].Unfortunately this golden age is now at the end due to the development of antibiotic resistance amongst pathogens. Such resistance was noticed immediately after the introduction of penicillin and has grown worse over the last half of this century. The development of antibiotic resistance is particularly problematic in the case of MRSA (Methicillin Resistant *S. aureus*), which is endemic in most hospitals worldwide [2].

Since *S. aureus* was discovered in the 1880s, it has been considered as a potential pathogenic Gram-positive bacterium, causing serious infections, such as postoperative wound and minor skin infections. Nowadays, it is regarded as the second most common etiologic agent in blood stream and lower respiratory tract infections. Penicillin-resistant *S. aureus* started to appear in hospitals only two years after the introduction of penicillin in the medical use. Within a few years later, the appearance of penicillin-resistant strains of *S. aureus* in the general community was reported [3and4].In Iraq, a considerable amount of literature has been published on penicillin-resistant strains of *S. aureus,* In this context, Kareem [5] reported that out of 74 isolates were collected from Medical City in Baghdad, 61 isolates were MRSA. In addition, Al-Dahbi [6] showed that 106 isolates of *S. aureus* were collected from 250 nasal swabs of health care workers and patients from Al- Kadhamia Teaching Hospital and Al- Numan Hospital in Baghdad that were found completely resistant to Penicillin G (100%) and highly resistant to Cefoxitin (alternative to Methicillin) (94.3%).However, the reality is that antibiotic resistance will continue to remain a major problem in future medicine. The obvious way for this problem is to develop new antibiotics which pathogens have not previously distinguished and therefore have not developed resistance [7]. Bacteriocin could be the prospective antibiotics for *S. aureus* after the significant activity displayed by this compound against this pathogen [7]. Bacteriocins or antimicrobial peptides represent one of the best-studied microbial defense systems that serve as a good example for explaining evolution and ecological behavior of microorganisms. Furthermore, potential bacteriocins can serve as a natural alternative product to therapeutic antibiotics for treating bacterial infections as well as natural antimicrobials for food preservation [8]. Bacteriocins properties make them typical alternatives to antibiotics and they can inhibit the growth of bacteria of the same species (narrow spectrum) or other genera (broad spectrum) and their range of activity often depends on the mechanisms of action of each bacteriocin [9, 10]. They are usually heat-stable, small peptides made of short chains of about 20-60 amino acid residues; however, longer chains can also be found [10]. Most bacteriocins are products of Gram-positive bacteria, as reported in BACTIBASE dataset. Some bacteriocins from Gram-negative bacteria have been described and are even fewer from Archaea domain [11].

In this work, we have screened a number of *Bacillus* isolates collected from soil for the production of bacteriocin against clinical isolates of *Staphylococcus spp*. The production, purification, properties and evaluation of *in vitro* antimicrobial activity of the bacteriocin produced were reported.

Materials and Methods:

Microorganisms

Aerobic, spore forming *Bacillus* spp. were selectively isolated from different soil sources collected from Baghdad University on nutrient agar plates based on the method previously described by Nicholson and Setlow [12]. Colonies were then subjected to the identification process based on Bergey's classification of determinative bacteriology that involved morphological characteristics and regular biochemical test in order to characterize the genus [13]. After screening experiments, the higher bacteriocin producer isolate was further characterized by API system (API 50CHB, API 20E) for the identification of Gram-positive and endospore-forming bacteria according to online API database (bio Mérieux, Inc).

The selected bacterial isolates of *Bacillus* sp were stored at -20^{o}C in Brain Heart Infusion (BHI) broth, containing 20% (v/v) glycerol.

S. aureus isolate which was used as an indicator strain in this study was provided by the Department of Biotechnology/ College of Science at University of Baghdad.

Screening of *Bacillus* isolates for bacteriocin production

Bacillus isolates were screened for the production of bacteriocin against *S. aureus* in liquid culture in order to select the higher bacteriocin producing isolate as follows: Erlenmeyer flasks (100 ml) each contained 50 ml of sterile BHI broth medium were inoculated with1 ml (2% inocula) inoculum of each *Bacillus* isolate contained approximately 1×10^8 cells/ml. Then flasks were incubated in an orbital shaker at 30°C and 150 rpm for 48 hrs. After the incubation, samples were taken for the analyses of bacteriocin [14].

Preparation of bacterial inoculums

Inoculums of *B. subtilis* NK16 was prepared as follows: a few loopfuls growth from an overnight culture on nutrient agar was inoculated into a 100 ml Erlenmeyer flask containing 20 ml of BHI broth. This culture was incubated for 24 hrs in an incubator at 37°C. After the incubation, a haemocytomtere was used to adjust the number of cells to be approximately 1×10^8 cells/ml for *B. subtilis* NK16.

Cultivation methods and media

BHI broth was used for the cultivation of *B. subtilis* NK16 inoculated at a level of 2% (v/v) and then incubated in an orbital shaker at 30°C and 150 rpm for 48hrs. After the incubation, samples were taken for the analyses of bacteriocin. For more reliability, each run was conducted either in triplicate or duplicate and the results were represented as the arithmetic average [15].

Optimization of culture conditions

Several optimization experiments were performed in order to determine the medium and culture conditions that support the maximal production of bacteriocin. The experiments involved testing different media (nutrient broth [16], BHI [16], MLB broth [14], LB broth [17], MTY broth [18], R2 broth [19], Buffered Peptone water broth [19], trypticase Soy Broth [20], MRS broth [16] and Muller-Hinton broth [16]), inoculum size, temperature, pH and incubation period.

Analytical methods

Determination of growth

The growth of *B. subtilis* NK16 isolate was measured as the dry weight of cell material. A known volume of the culture was filtered with vacuum through pre-dried and pre-weighed filter paper (0.2µm, Whatman). The filter paper was thereafter placed in an oven at 60°C for 24 hrs and then weighted. The difference in weights represented the mass of cells in the samples of the culture. This method was used with pure culture of *B. subtilis* NK16 while in the elicited cultures this method was not followed because of the difficulty of

separating two microorganisms which are growing in the same fermentative liquid.

Determination of bacteriocin activity

Bacteriocin activity was determined using the critical dilution assay which is similar to the minimum inhibitory concentration technique (MIC) for antibiotic assessment. This method involved preparation of a twofold dilution series of the *B. subtilis* NK16 culture to be tested and then bacteriocin activity was determined against *S. aureus* in each dilution using agar well diffusion assay.200 µl of an overnight growth culture of *S. aureus* containing approximately 1×10^7 cells /ml was mixed with 25 mL of a sterile Muller Hinton agar kept at 45-50°C in a water bath. The mixtures were kept at the same temperature until poured into sterile plastic Petri dishes and allowed to solidify. Circular wells of 5mm in diameter were cut using a sterile cork borer and then low melting temperature Muller Hinton agar was used to seal the bottom of the wells. 100 µl aliquots of the filtered CFS were dispensed in the wells and then plates were incubated for 24 hrs at 37°C. Following the incubation, the growth inhibition zone around each well was examined. The highest dilution generating an inhibition zone indicated the strength of bacteriocin activity. Thus, the bacteriocin activity is a proportional to the reciprocal of the highest dilution factor producing a detectable inhibition zone (DF) [21]. The bacteriocin activity which is known as arbitrary unites (AU) was calculated using the following equation:

$$\mathrm{AU/ml} = \frac{1}{DF}\frac{1000}{volums\ spotted\ in\ \mu l}$$

Protein Estimation

The protein concentration in the samples was estimated by the method of Bradford [22].

Purification of bacteriocin

Precipitation with ammonium sulphate

Bacteriocin was precipitated by ammonium sulphate at different saturation levels (40, 50, 60, 70, 80) %. Ammonium sulphate was added to CSF slowly with gentle stirring at 4°C. The precipitate was separated by centrifugation for 30 min at 10000 rpm. Then the precipitates were redissolved in an appropriate volume of phosphate buffer (0.1M, pH 7.2). Next, the dissolved precipitates were dialyzed separately, in phosphate buffer using dialysis membrane tubes (1 kDa MW cutoff). Then, the dialysis bags contained precipitates were placed in 0.5 liter of phosphate buffer overnight at 4°C. The buffer was replaced four times. The antibacterial activity of the dialyzed protein was determined by agar well diffusion assay using *S. aureus* as an indicator strain [23].

Sephadex gel filteration

The resulting bacteriocin was loaded on a column (3 × 20 cm) of sephadex G-150 gel filtration. Elution of proteins was performed with phosphate buffer (0.1 M, pH 7.2). The flow rate was adjusted to 36 ml/hour and fractions of 3 ml were collected. Seventy six fractions were collected and the absorption of these fractions was measured at 280 nm. The fractions were tested for antibacterial activity against *S. aureus* as an indicator strain by well agar diffusion assay. Fractions showed antimicrobial activity were mixed together in one tube and protein concentration with Bradford method and bacteriocin activity were determined [14].

Characterization of bacteriocin

Susceptibility to proteases and thermal, pH stability

The sensitivity of the bacteriocin produced from *B. subtilis* NK16 to proteolytic enzyme trypsin was tested. Trypsin was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) in

a test tube contained the bacteriocin solution with an activity of 80 AU/ml for a final concentration of 1 mg trypsin/ ml. The control contained the bacteriocin solution with an activity of 80 AU/ml without trypsin. Tubes were incubated at 37 °C and bacteriocin activity was measured at zero time and after 30 and 90 mins using the well diffusion assay method [24, 25]. Furthermore, in order to test the thermo stability of bacteriocin, samples were exposed to different temperatures (30, 40, 50, 60, 70, 80, 90)^{o}C for 10 mins followed by cooling on an ice-bath**.** The residual activity was then determined by agar-well diffusion technique against indicator strain. In addition, bacteriocin preparation was treated with either 0.1N HCl or 0.1 N NaOH to achieve the desired pH values between 4 and 9. The pH adjusted crude extracts were incubated for 30 mins. After incubation, aliquots were neutralized and activity was measured by agar-well diffusion technique against indicator strain.

Mode of bacteriocin action

The mode of action of the bacteriocin against *S. aureus* was investigated. 0.5 ml of the partial purified bacteriocin with total activity of 640 AU/ml was added to10 ml of an overnight culture of *S. aureus* grown in nutrient broth at 37 °C (OD 600 nm =0.6 of *S. aureus*). Control culture was prepared without adding bacteriocin. Changes in the turbidity at 600 nm and viable cells count (cfu/ml) were measured at zero time and after 10, 30, 60, and 120 min of incubation. Viable cells count was determined on nutrient agar plates [26].

Assessment of in vitro antibacterial activity of bacteriocin

The activity of bacteriocin at a concentration of 300, 400, 500, 640 AU/ml was tested against 29 clinical isolates of *Staphylococcus spp* including 21isolates of *S. aureus*, 4 isolates of *S. epidermidis* and 4 isolates of *S. saprophyticus*. These isolates were collected from different hospitals in three cities (Baghdad, Al Kut and Baqubah) in Iraq. The inhibitory spectrum was determined using the agar well diffusion assay.

Statistical analysis

The results of antimicrobial activity of bacteriocin produced by Bacillus sp NK 16 against the clinical gram positive and negative isolates were statistically studied by using two-way variance analysis (ANOVA) for the separation of mean differences. A probability level of P value ≤ 0.01 was used in testing the statistical significance of the experimental data [27].

Result and Discussion:

Bacillus is an interesting genus to investigate the antimicrobial activity, since it produces a large number of peptide-antibiotics representing several basic chemical structures [28]. Although many species of *Bacillus* can synthesize antimicrobial peptides, the data on bacteriocins is limited to only few species of *Bacillus* [29]. In this study, 50 *Bacillus* sp. isolates were isolated from different soil samples collected from different parts in Baghdad. An identification process was followed to select the *Bacillus* sp. isolates based on morphological examination and biochemical characterizations according to Bergey's manual of systematic bacteriology which confirm that all isolates belong to the genus of *Bacillus* [30].

The 50 *Bacillus* isolates were subjected to screening process using *S. aureus* as an indicator strain in order to select the higher bacteriocin producing isolate that can be used for further experiments in this study. The screening was achieved in BHI broth and for more reliability all isolates were cultivated under the same conditions in terms of inoculum size, cells number, pH, incubation period and shaking speed. Based on the results, culture supernatant of *B. subtilis* NK16

isolate showed the maximum activity against *S. aureus*. Inhibition zone formed in well diffusion assay by bacteriocin produced by this isolate was 18mm against *S. aureus.* Thereafter, *B. subtilis* NK16 isolate was chosen, and therefore was subjected to Analytical Profile Index (API 20E and API 50CH) Strep System. The results of API 20E and API 50CHB were compared and identified online using the identification software via online API database (bioMérieux, Inc) which revealed that *B. subtilis* NK16 isolate was *Bacillus subtilis.*

The next step was to investigate the optimized cultural parameters that are required for elevating bacteriocin production such as media, temperature, pH and inoculum size. In this study, different media were used in order to select the one that can support the maximum production of bacteriocin. It has been hypothesized that biosynthesis of peptides requires a rich medium [31], hence the effect of media composition on bacteriocin from *B. subtilis* NK16 production was tested. Based on the results presented in Figure (1A), the maximum production of bacteriocin was found in BHI with an activity of approximately 80 AU/ml. Whereas, in the other media, production of bacteriocin was either low or completely suppressed.

In addition, bacteriocin production was determined after growing *B. subtilis* NK16 in BHI broth prepared with different initial pH. As can be seen in Figure (1B), production of bacteriocin was obtained at pH ranging from 5 to 8. Maximum bacteriocin production of 80 AU/ml was observed in cultures with an initial pH of 6 and 7. In addition, no production was found in culture with an initial pH of 4 and 9.

In general, the cultivation temperature plays a crucial role in the microbial growth and synthesis of secondary metabolites. Therefore, it was necessary to investigate the optimum temperature that maximizes the production of bacteriocin. Figure (1C) shows that the maximum production of bacteriocin (80 AU/ml) was obtained at 30°C. However, when the temperature of the incubation was less or more than 30°C, bacteriocin production was consequently decreased.

Inoculum size also represents an essential factor that usually affects the microbial growth rate and consequently the secondary metabolites production. Therefore, this factor was investigated using different inoculum sizes ranging from 1 to 5% (v/v). As shown in Figure (1D), the best inoculum size for the maximum bacteriocin production was 2% yielding an activity of approximately 80 AU/ml under the experimental conditions used in this work.

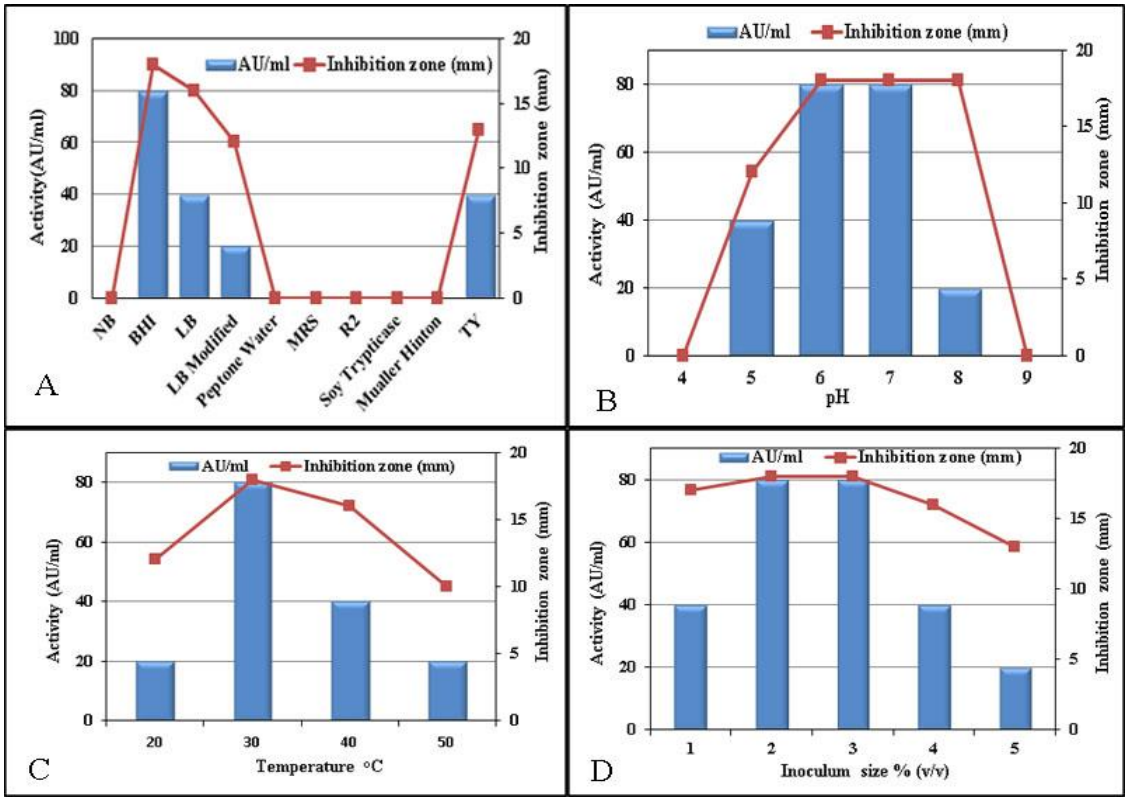

Fig. (1): Optimized cultural parameters for elevating bacteriocin production which were different media (A), different pH (B), different temperature (C) and different inoculum size (D)

The growth of *B. subtilis* NK16 isolate in BHI broth was investigated. The typical time course of growth (dry weight) and bacteriocin production by *B. subtilis* NK16 is presented in Figure (2).The exponential phase started where biomass concentration noticeably increased from 0.16 to 2.96 mg/ml after 31hrs of incubation which marks the start of the stationary phase. Then, *B. subtilis* NK16 population was increased slightly to 3.3 mg/ml after 41hrs of cultivation. Thereafter, population growth was ceased, and the growth curve becomes horizontal. The production of bacteriocin was started during the exponential phase after 9 hrs of incubation. At the end of the exponential phase (at 24 hrs of incubation), the activity of bacteriocin was approximately 80 AU/ml which is maximum production obtained under the conditions used in this experiment. No further increasing was observed in the production of bacteriocin during the stationary phase suggesting that bacteriocin produced by *B. subtilis* NK16 is a primary metabolite and associated with growth. In this context, several studies have mentioned that numerous bacteriocins are produced during the active growth phase [32].

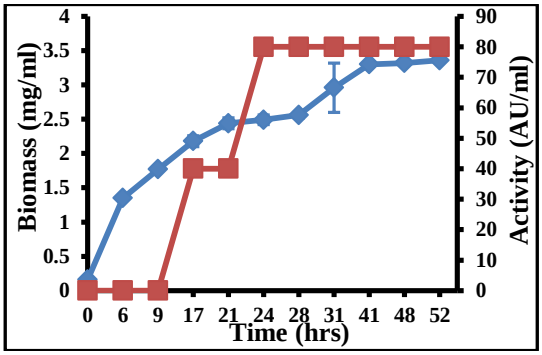

Fig. (2): Time course of cell growth (■) and bacteriocin production (♦) by *B. subtilis* NK16 in a shaker incubator at 30 C° and 150 rpm.

Purification of Bacteriocin

Based on the results presented in Figures (3) and (4), maximum bacteriocin precipitation was obtained at 80% saturation level. The bacteriocin activity was 320 AU/ml with specific activity of 237.04 AU/mg. The precipitated bacteriocin was then loaded in sephadex G-150 column. As can be seen in Figure (5), three separated peaks were obtained in the separation profile.

The bacteriocin activity was detected in ten fractions corresponding to the second and third peak in fractions No. 56 to 65. The active fractions were collected and the specific activity of the partial purified bacteriocin was increased to 853.33 AU/mg resulting in 38 fold purification with 24% yield (Table 1).

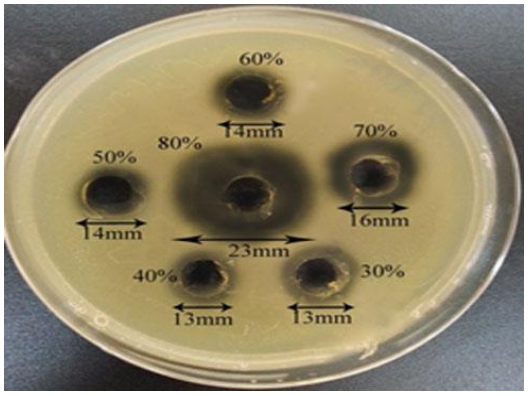

Fig.(3): Inhibition zone of partial purified bacteriocin of *B. subtilis* NK16 produced in a shaker incubator at 30 C° and 150 rpm precipitated at different concentration of ammonium sulphate.

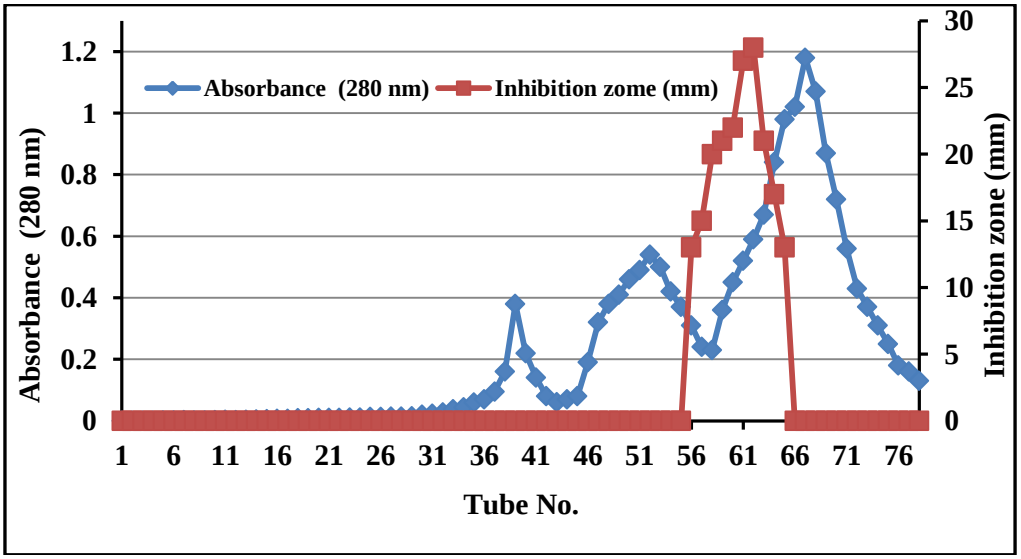

Fig. (4): Purification of bacteriocin by Sephadex G-150 column. Column was equilibrated and eluted with sodium phosphate buffer, pH 7 at a flow rate of 0.6 ml/min.

Table (1): Summary of purification of bacteriocin from crude culture filtrate of *B. subtilis* NK16

Step	Volume (ml)	Total activity (AU/ml)	Protein conec. mg/ml	Total activity (Au)	specific activity (AU/mg)	Yield %	Fold purification
Crude	100	80	3.6	8000	22.2	100	1
Precipitation with Ammonium sulfate 80%	10	320	1.35	3200	237.04	40	11
Gel filtration Sephadex G-150	3	640	0.75	1920	853.33	24	38

Characterization of bacteriocin

As can be seen in Figure (5), bacteriocin activity was reduced when treated with trypsin. The results showed that bacteriocin activity was decreased to 50% (40 AU/ml) after 30 min of incubation and it was completely lost after 90 mins confirming the protein status of bacteriocin produced by *B. subtilis* NK 16. Several studies demonstrated the sensitivity of bacteriocins to proteolytic enzymes such as Trypsin. In this context, Cherif [33, 34] reported the loss of bacteriocin activity produced by *Bacillus thuringensis* after treatment with proteolytic enzymes.

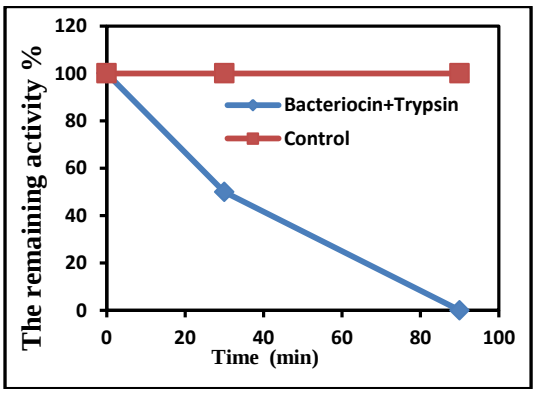

Fig.(5): enzyme sensitivity of bacteriocin from *B. subtilis* NK 16. Bacteriocin of *B. subtilis* NK 16 incubated with 1 mg/ml of trypsin for 30 and 90 min. followed by bioassay for bacteriocin activity by agar-well diffusion assay.

Thermal stability of bacteriocin is an important criterion that can help to determine whether the bacteriocin belongs to the class of heat-labile or heat –stable protein [35]. As can be seen in Figure (6), the activity of bacteriocin remained stable after 10 min at 20, 30, 40^{o}C as no effect was observed on its activity. However, 50% of the bacteriocin activity was lost after exposure to 50^{o}C for 10 min. In addition, the activity of bacteriocin was obviously decreased to approximately 75% (20 AU/ml) upon exposure to 60, 70 and 80^{o}C for 10 min. Bacteriocin activity disappeared at 90C^{o} for 10 mins. From the results, it can be concluded that the bacteriocin is heat –labile. In this context, Lee [36] reported that partially-purified bacteriocin from *Bacillus polyfermenticus* was inactivated by heating treatment at 70°C for 30 min. Furthermore, Bizani and Brandelli [37] mentioned that the activity of bacteriocin produced from *Bacillus cereus* 8A was lost when heated at 87^{o}C. Activity of the bacteriocin from *B. subtilis* NK16 was also tested for pH stability. As can be noticed in Figure (7), bacteriocin activity showed stability at pH 6 and 7 in which the bacteriocin kept its stability of 80 AU/ml for 30 min. However, bacteriocin activity was decreased to approximately 50% (40 AU/ml) at pH 5 and 8, whereas, no activity was observed at pH 4 and 9. The range of pH stability for bacteriocin activity differs from one to another. Some bacteriocins have a wide range activity of pH while others are active at a narrow range. In this context, purified bacteriocin from *B. amyloliquifaciens* showed an activity at pH ranging from 4 to 8,thuricin S produced by the *B. thuringiensis* was found stable at a variety of pH levels ranging from 3 to 10 and bacteriocin from *Aeromonas hydrophila* found stable at pH ranging from 3 to 8 [15, 38, 39].

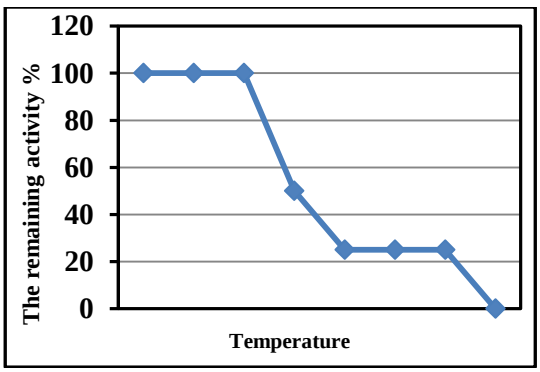

Fig.(6):The residual activity of bacteriocin produced by *B. subtilis* NK16 after exposed to different temperature for 10 min.

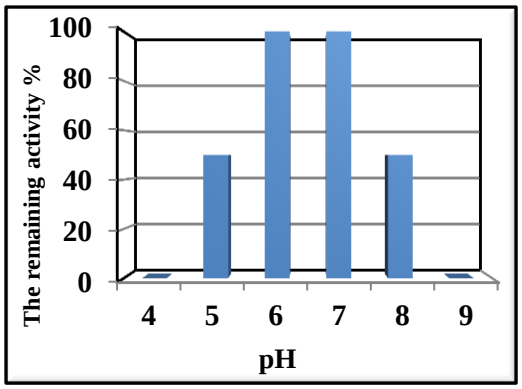

Fig.(7): pH stability of bacteriocin from *B. subtilis* NK16 after exposed to different pH for 30 min.

Mode of action of bacteriocin

Most of bacteriocins produced from *Bacillus* species show a bactericidal effect with or without cell-lysis [40]. In the present study, the mode of action of the partially purified bacteriocin produced by *B. subtilis* NK16 was studied using *S. aureus* as an indicator strain. Figure (8) shows a rapid decline in the number of viable cells and optical density at 600 nm in the tube which contained *S. aureus* culture with bacteriocin. The number of cells in this tube was decreased to approximately zero within 2 hrs. Whereas, no effect was observed on the growth of *S. aureus* in the control tube. These results suggest that the bacteriocin has a bactericidal effect rather than bacteriostatic. Bactericidal mode of action leads to the death of a pathogen; therefore, it is able to eradicate the main population of undesirable microorganisms [41].

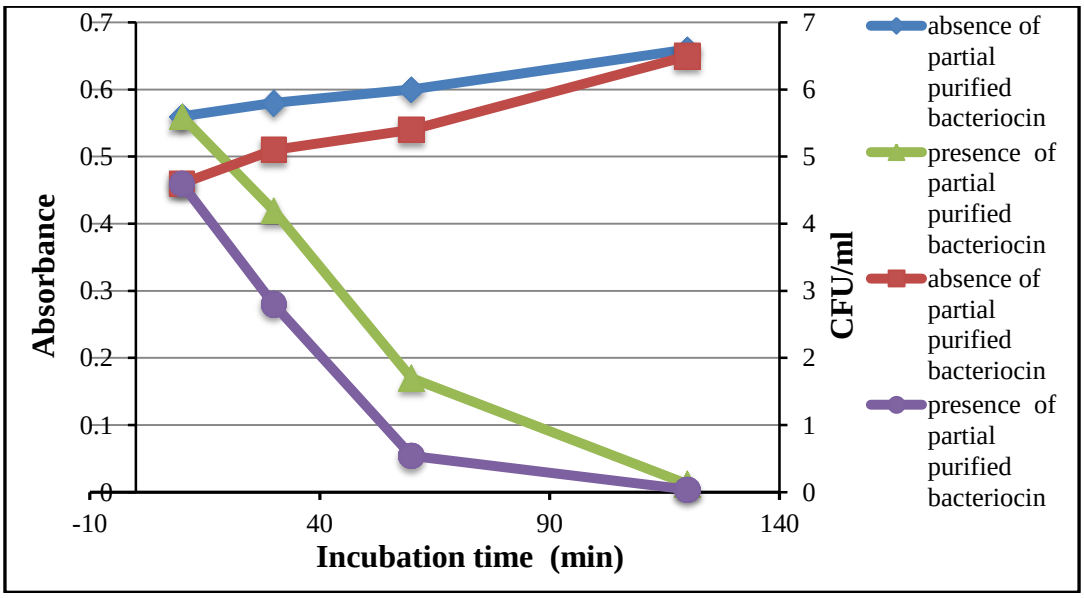

Fig.(8): Mode of action of partially purified bacteriocin produced by *B. subtilis* NK16 against *S. aureus.* Viable cell counts (CFU/ml) in the absence (■) or presence (●) of partial purified bacteriocin. Optical density at 600 nm in the absence (♦) or presence (▲) of partial purified bacteriocin.

Assessment of bacteriocin activity against *Staphylococcus spp*

The partial purified bacteriocin produced by *B. subtilis* NK 16 was tested against several clinical isolates of *Staphylococcus* spp collected from different hospitals in Baghdad, Kut and Baqubah cities in Iraq. As can be noticed in Table (2), bacteriocin displays a significant activity against most *S. aureus* and *S. epidermidis* isolates, whereas it was less effective against *S. saprophyticus* isolates. Based on the results, bacteriocin was effective against 63% of clinical *Staphylococcus* spp isolates used in this study.

A statistical analysis was performed using Analysis of variance (ANOVA)

models in order to measure the statistically significant differences of the effect of three different concentrations (640, 500 and 400 AU/ml) of bacteriocin on clinical *Staphylococcus* spp isolates tested in this study. As shown in Table (3), the statistical analysis results of the sensitivity of different Gram positive bacterial isolates to three different bacteriocin concentrations (640, 500, 400 AU/ml) revealed no significant differences observed as the P values were 0.145, 0.077 and 0.43 at bacteriocin concentrations 640, 500 and 400 AU/ml respectively when Probability at $P \leq 0.01$.

Finally, certainly, these results have significance important as it contributes to the growing body of literature on finding an effective antibiotic against this microorganism. As mentioned earlier, *S. aureus* is considered as an important pathogenic bacterium which can cause several infections such as postoperative wound, minor skin infections and lower respiratory tract infections. However, there is a large volume of published studies describing the resistance of this bacterium to penicillin and its derivatives as well as many known antibiotics that are usually used to treat common *S. aureus* infections which is certainly considered as a serious problem in combating this pathogen [42, 43, 44].

Table (2): Activity of partial purified bacteriocin against several clinical isolates of *Staphylococcus* spp collected from different hospitals in Iraq.

No	Isolates	Clinical isolates source	Inhibition zone (mm)			
			300 AU/ml	400 AU/ml	500 AU/ml	640 AU/ml
1	*S. aureus*A1	Al-Imam Ali hospital (Baghdad)	-	13	26	30
2	*S. aureus*A2	Al-Imam Ali hospital (Baghdad)	-	-	-	-
3	*S. aureus*A3	Al-Imam Ali hospital (Baghdad)	-	-	-	15
4	*S.aureus*A6	Al-Imam Ali hospital (Baghdad)	-	13	18	26
5	*S. aureus*A15	Al-Imam Ali hospital (Baghdad)	-	-	-	-
6	*S. aureus*A24	Al-Imam Ali hospital (Baghdad)	-	-	-	-
7	*S. aureus*A26	Al-Imam Ali hospital (Baghdad)	-	-	-	17
8	*S. aureus*B7	Al ShaheedAlsader hospital (Baghdad)	-	16	24	32
9	*S. aureus*B10	Al ShaheedAlsader hospital (Baghdad)	-	13	20	33
10	*S. aureus*B7	Al ShaheedAlsader hospital (Baghdad)	-	16	24	32
11	*S. aureus*B11	Al ShaheedAlsader hospital (Baghdad)	-	-	-	-
12	*S. aureus*B13	Al ShaheedAlsader hospital (Baghdad)	-	-	-	-
13	*S. aureus*B14	Al ShaheedAlsader hospital (Baghdad)	-	-	16	21
14	*S. aureus*B10	Al ShaheedAlsader hospital (Baghdad)	-	13	20	33
15	*S. aureus*C1	Baqubah hospital (Baqubah city)	-	-	-	13
16	*S. aureus*C2	Baqubah hospital (Baqubah city)	-	-	-	-
17	*S. aureusC3*	Baqubah hospital (Baqubah city)	-	-	-	-
18	*S. aureus*C8	Baqubah hospital (Baqubah city)	-	-	-	19
19	*S. aureus*C14	Baqubah hospital (Baqubah city)	-	-	-	-
20	*S. aureus*D6	Al-kut hospital (Al kut city)	-	-	14	21
21	*S. aureus*D10	Al-kut hospital (Al kut city)	-	15	22	34
22	*S. epidermidis B1*	Al ShaheedAlsader hospital (Baghdad)	-	11	19	28
23	*S. epidermidis B9*	Al ShaheedAlsader hospital (Baghdad)	-	-	13	19
24	*S. epidermidis B12*	Al ShaheedAlsader hospital (Baghdad)	-	13	26	35
25	*S. epidermidis B15*	Al ShaheedAlsader hospital (Baghdad)	-	-	13	19
26	*S.saprophyticus C5*	Baqubah hospital (Baqubah city)	-	-	-	-
27	*S. saprophyticus C10*	Baqubah hospital (Baqubah city)	-	-	-	-
28	*S. saprophyticus C11*	Baqubah hospital (Baqubah city)	-	-	-	12
29	*S. saprophyticus C12*	Baqubah hospital (Baqubah city)	-	-	-	-

Table (3): Statistical analysis for bacteriocin effect produced by *B. subtilis* NK 16 in three concentrations against several clinical isolates of *Staphylococcus* spp collected from different hospitals in Iraq.

Isolate	No	mean	SD	F value	P value	comment
640 AU/ml						
S. aureus	21	15.52	13.82			
S. epidermidis	4	25.25	7.76			No significant
S. saprophyticus	4	3	6	1.86	0.145	
500 AU/ml						
S. aureus	21	8.76	10.66			
S. epidermidis	4	17.75	6.18			No significant
S. saprophyticus	4	0	0	2.36	0.077	
400 AU/ml						
S. aureus	21	4.71	6.87			
S. epidermidis	4	6	6.97			No significant
S. saprophyticus	4	0	0	0.97	0.43	

Probability at P≤ 0.01

Conclusion:

The current situation, where antibiotic resistance is becoming increasingly common, has created a therapeutic challenge to the medical community where the development of new approaches and alternative treatments for such infections is becoming increasingly essential. Bacteriocin could be the suitable alternative antimicrobial for several pathogens particularly against those which showed resistance to most usefulness antibiotics such as *Staphylococcus spp***.** In general, bacteriocins are a narrow spectrum antimicrobial agent. However, many researchers believe that the narrow spectrum antimicrobial activity of bacteriocins could be a positive point. One of the limitations of using broad spectrum antibiotics is that they kill almost any bacterial species which is specifically not resistant to the drug. Therefore, broad spectrum antibiotics may develop resistance in both pathogen and commensal bacteria. In fact, Bacteriocins provide an alternative solution with their relatively narrow spectrum of activity, they can be considered as a designed drug which targets the specific pathogen. Thus, in the future, bacteriocins can be used as an alternative and novel therapeutic agent against pathogens.

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كشف و تنقية وتوصيف البكتريوسن المنتج من عزلة *Bacillus subtilis* NK16 والذي اظهر فعالية ضد عزلات *Staphylococcus* spp المرضية

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الخلاصة:

يعد البكتريوسن مضاد بكتيري ببتيدي مهم يستخدم في العديد من المجالات الصناعية والطبية بسبب خواصة المضادة للبكتريا، فضلا عن فعاليته المضادة للسرطان. تم جمع خمسون عزله من بكتريا *Bacillus* sp من عينات مختلفة من التربة والتي تم التحقق منها من خلال تشخيص ودراسة بعض الصفات الظاهرية وألفحوصات الكيموحيوية. تم اختبار قدرةعزلات بكتريا *Bacillus* sp على انتاج البكتريوسين ومن ثم اختيار العزلة الاكثر انتاجا حيث اظهرت العزلة NK16 اعلى انتاج للبكتريوسين بمقدار 80 وحدة /مل. استخدمت تقنية API identification systemباستخدامAPI 20E و API 50CHBمن اجل تشخيص هذه العزله على مستوى النوع حيث تبين انها تعود لبكتريا *Bacillus subtilis*. الخطوة التالية كانت لتحديد الظروف المثلى لاعلى انتاج من البكتريوسين ووجد ان الوسط السائل نقيع القلب والدماغ كانافضل وسط للانتاج مع رقم هيدروجني (pH) ، درجة حراره 30 م وحجم اللقاح % 2. تمت نقية البكتريوسين جزئيا بعدة خطوات اشتملت الترسيب بكبريت اتالامونيوم بنسبة اشباع % 80 ومن ثم الفصل بالترشيح الهلامي بواسطة عمود Sephadex G-150. اظهرت النتائج ان فعالية البكتريوسن قد ارتفعت الى 853.33 وحده/ملغم مع عدد مرات تنقيه 38 مره وبحصيله مقدارها 24%. بينت نتائج التوصيف ان البكتريوسين احتفظ بفعاليته ولمدة 10 دقائق في درجة حراره 20 و 30 و 40 م بينما فقدت 50% من فعالية البكتريوسين نتيجة التعرض لدرجة حرارة 50 م وانخفضت الفعاليه الى 20 وحدة/مل عند درجة حرارة 60 و 70 و 80 م. بالاضافة لذلك، احتفظ البكتريوسين بكامل فعاليته عند الرقم الهيدروجيني 6 و 7 لمدة 30 دقيقة واختفت كليا عند الرقم الهيدروجيني 4 و 9. من جهة اخرى اظهرت النتائج ان البكتريوسين كان ذو تأثير قاتل للبكتريا. تم اختبار فعالية البكتريوسين المنقى جزئيا كمضاد بكتيري وقد اظهرت النتائج ان البكتريوسين كان اكثر فعالية ضد معظم بكتريا *S. aureus* و *S. epidermidis* المرضية بينما كان اقل تاثيرا ضد عزلات *S. saprophyticus*.

الكلمات المفتاحيه: باسيليس سيتلس : البكتريوسين: تنقيه: توصيف: الفعاليه الضد مايكروبيه: ستافلوكوكس .