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Inhibitory Potential of Bacteriocins from *Corynebacterium*, *Staphylococcus*, and *Bacillus* sp. Against Multiple Resistant Bacterial Reference: A Comparative Study with Antibiotics

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Abstract

Bacteriocins are antimicrobial peptides produced by bacteria that can function as an alternative to conventional antibiotics. The main objective of this work is to utilize bacterial metabolic products as an alternative to antibiotics. This study assessed the inhibitory capacity of bacteriocins obtained from Bacillus sp., *Corynebacterium* sp., and *Staphylococcus* sp. against various antibiotic-resistant bacterial infections. Bacteriocins were isolated and refined from *Corynebacterium* sp., *Staphylococcus* sp., and *Bacillus* sp. They were later employed as antibiotic compounds against pathogenic bacterial strains which are including *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 4352, *Escherichia coli* ATCC 25922, *Bacillus cerus* ATCC 11778, *Pseudomonas aeroginosa* ATCC 27853, *Micrococcus luteus* ATCC 9341, and *Staphylococcus aureus* ATCC 65389, that were isolated from clinical specimens and identified as pathogenic species and antibiotics resistant. Additionally, a total of five antibiotics were utilized as controls against the tested isolates. These antibiotics include Ampicillin (10 µg), Spectinomycin (25 µg), Streptomycin (10 µg), Meropenem (10 µg), and Penicillin (10 µg). The broth dilution method was employed in a 96-well microtiter plate for bacteriocins, while the Kirby-Bauer disc diffusion method was used for antibiotics. The results indicated that the bacteriocins synthesized by all three species possess antibacterial activity exclusively against *Escherichia coli* ATCC 25922 and *Micrococcus luteus* ATCC 9341, suggesting their potential as alternatives to conventional antibiotics. Nevertheless, their influence on other microorganisms that tested was not observed. The antibacterial efficacy of bacteriocins can be affected by several factors, including the concentration of NaCl, temperature, bacterial enzymes, and pH level. The study revealed that the most favorable circumstances for the antibacterial efficacy of bacteriocins were a pH of 6, a temperature of 35°C, and a NaCl concentration ranging from 1.5M to 2.5M.

Key words: Antimicrobial agent, Antibiotic resistance, Bacteriocins, Corynebacterium sp., Staphylococcus sp., and Bacillus sp.

I. INTRODUCTION

A wide range of microorganisms have been extensively utilized in various industries such as food, paper, baking, textile, cleaning, pharmaceutical products, feed, and dairy industries. These microorganisms have the capacity to produce a wide range of valuable metabolic compounds (Ventosa *et al*., 1998; Rohban *et al*., 2009). Examples of these items include vitamin B12, citric acid, glutamic acid, aspartic acid, amino acids (such as lysine and phenylalanine), sake, rennin, wine, beer, and distilled spirits. All of these substances are derived from microorganisms (Jay, 1986; Madigan *et al*., 2012). Furthermore, it is crucial to note that select microbes, particularly those that generate halocin or bacteriocin, synthesize significant metabolic compounds. These chemicals have the ability to inhibit the proliferation of other microorganisms in the same ecological settings, so playing a vital role in promoting survival and maintaining the balance of nature within a certain habitat (Riley and Wertz, 2002). Halocins

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are antimicrobial peptides produced by ribosomes and derived from halophilic microorganisms, also known as bacteriocins. These peptides are produced via the metabolic processes of several bacterial species, such as lactic acid bacteria including *Pediococcus, Streptococcus, Lactobacillus, Leuconostoc, Lactobacillus,* and *Enterococcus*. Bacteriocins possess the ability to effectively inhibit the growth of other bacteria in the surrounding milieu, rendering them valuable as defensive agents. Lactic acid bacteria (LAB) having the capacity to create many chemical compounds that can be exploited as preservatives. The compounds under discussion include reutin, acids, antifungal peptides, diacetyl, hydrogen peroxide (H2O2), and bacteriocins (Heredia-Castro, *et al*., 2015). Bacteriocins have demonstrated effectiveness against both Gram-negative and Gram-positive bacteria, including *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Helicobacter pylori, Clostridium,* and *Listeria monocytogenus*. Bacteriocins exhibit exceptional resilience in various unfavorable environments, such as changes in salinity, pH, and temperature. Furthermore, their flawless safety record makes them very suitable for use in food preservation (Heredia-Castro, *et al*., 2015). The pharmaceutical industry has utilized a diverse array of bacterial chemicals for their antiviral, antibacterial, antioxidant, vaccine, anti-adhesive, and gene therapy properties (Vasavada, et al., 2006). It is noteworthy that a significant proportion of these compounds are derived from microbes that exhibit remarkable resilience in difficult circumstances. Consequently, these microorganisms have garnered considerable interest in recent times.(Williams, 2009).

The capacity of microorganisms to produce biosurfactants is essential for their survival and reproduction in challenging environments typified by elevated salinity, temperature, pressure, and limited macronutrient supply. The wide array of settings has necessitated the selection and modification of bacteria in response to fluctuating situations. (Valentine, 2007). Bacteriocin is a peptide generated by particular bacteria, such as lactic acid bacteria. Because of its antagonistic capabilities against other bacterial species, it is frequently used as a method of preserving food products. Eleven strains were found and categorized into distinct genera, each assigned a unique extension number. These categories include *Halobacillus sp. HQ426913, Halobacillus sp. HQ834847, Halobacillus sp. HQ834853, Halobacillus sp. HQ704870, Virgibacillus sp. HQ834848, Salinicoccus sp. HQ704871, Staphylococcus sp. HQ426917, Nesterenkonia sp. HQ704868, Salinicoccus sp. HQ704873, Pontibacillus sp. HQ834851, and Virgibacillus sp. HQ426915* the samples were acquired from the coastal area of Karnataka, India. The microbial strains were classified by SY, J., et al., (2013) as valuable producers of hydrolytic enzymes, such as protease, lipase, amylase, β-galactosidases, and inulinases, which have important commercial uses. (SY, *et al*., 2013). The objective of this study was to examine the inhibitory capacity of bacteriocins derived from *Corynebacterium aurimucosum*, *Staphylococcus epidermidis,* and *Bacillus haynesii* against multiple resistant bacterial pathogens. The broth dilution method is utilized on a 96-well microtiter plate to assess the inhibitory capacity of bacteriocins against reference isolates. The findings clarify that the bacteriocins derived from the three aforementioned species exclusively impact *Escherichia coli* ATCC 25922 and *Micrococcus luteus* ATCC 9341. Nevertheless, there is no impact on other bacterial isolates used as references.

II. MATERIALS AND METHODS

2.1. Preparation bacteriocins-producing bacterial species and pathogenic-bacterial species:

The three isolates employed in this investigation, namely *Corynebacterium aurimucosum*, *Staphylococcus epidermidis*, and *Bacillus haynesii*, were obtained from the leather industry as a part of our prior study (Abdulhusein, 2023). All disease-causing species were acquired from the Microbiological laboratory at the College of Veterinary Medicine, University of Al-Shatrah. Additional investigations were conducted to refine the bacterial species and biochemical tests were also performed. The pathogenic strains and bacteria that produce bacteriocin were cultivated in Nutrient Broth Medium and kept in an incubator at a temperature of 37°C for duration of 24 hours.

2.2. Experimental Procedures

2.2.1. The Activation process of bacterial isolates:

University of Thi-Qar Journal of agricultural research

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Our isolates were grown on enrichment medium (blood agar, chocolate agar, and nutritional agar) to activate them. Bacterial suspensions were prepared for each isolate, and 10 µL of each suspension was applied and dispersed on the enrichment media. All petri plates were then incubated at 37oC for 48 hours.

2.2.2. Isolation and purification of isolates

Distinct mediums were employed to purify each isolate, followed by additional biochemical assays. The turbidity of all bacterial suspensions was standardized to a concentration of 108 CFU/g by using sterile physiological saline. (Bilgehan, 2004).

2.2.3. Production of Bacteriocin

The bacteriocins have been synthesized from *Corynebacterium aurimucosum*, *Staphylococcus epidermidis*, and *Bacillus haynesii* using the specified methodology by Rajesh *et al* (2012), with specific alterations. The three stated isolates were initially cultivated in a nutrient broth medium and subsequently incubated at a temperature of 37oC overnight to generate a crude bacteriocin. The incubated solution was then centrifuged at a force of 8000 times the acceleration due to gravity for a duration of 15 minutes at a temperature of 4°C. The purpose of this procedure was to eradicate all bacterial cells and acquire the supernatant devoid of cells. To mitigate the suppressive effect of acids on bacterial growth, the pH of the environment was adjusted to 7.0 by introducing 1N NaOH. Afterwards, the liquid part was acquired and underwent filtration using a 0.22 µm Millipore membrane filter. (Rajesh *et al*., 2012).

2.3. Study the antimicrobial activity of bacteriocins against pathogenic reference isolates

The antibacterial activity of bacteriocin was assessed against reference isolates. Each bacterial concentration was separately adjusted to 10^8 CFU/mL using a physiological solution. 100 mL portions of each modified bacterial suspension were deposited onto petri dishes containing Muller Hinton Agar. On these inoculated petri plates, 3 L aliquots of each bacteriocin were dispensed. The widths of the inhibition zones (in mm) caused by the bacteriocin compound against each test isolate were measured after 24 hours of incubation at 37°C. (Price and Shand, 2000). The experimental results were used to identify isolates that produce bacteriocin, isolates that are susceptible to bacteriocin, and isolates that are resistant to bacteriocin. Subsequently, certain haloversatile strains were cultivated and produced bacteriocins, which were then evaluated against pathogenic strains.

2.4. Minimum inhibitory concentrations of bacteriocins against pathogenic isolates

The minimum inhibitory concentration (MIC) of bacteriocins produced by the previously stated isolates against pathogenic isolates was evaluated using the broth dilution method on a 96-well microtiter plate. Serial dilutions of each bacteriocin were prepared in a 96-well micro dilution plate using Nutrient broth. The dilutions were done in a two-fold manner, with the concentrations being 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, and 1/1024. Subsequently, a suspension of the pathogenic bacteria test isolate was created by utilizing sterile Nutrient broth Medium and adjusting it to a uniform turbidity of 10^8 CFU/mL. A 100 µL portion of a pathogenic test isolate bacterial culture was applied to each well. Subsequently, 100 µL of each bacteriocin produced was introduced into wells spanning from A1 to H1. Subsequently, a volume of 100 µL was extracted from each well and sequentially transferred to the subsequent well till reaching well A10. Subsequently, a volume of 100 µL was administered. The volume per well is 200 µL. Following a 24-hour incubation period at a temperature of 37°C, a volume of 30 µL of a resazurin solution with a concentration of 0.015% will be introduced into every well. Afterwards, the 96-well plates will be incubated for 4 hours at a temperature of 25°C. Subsequently, the wells will be examined for the presence of visible growth, indicated by (turbidity and a pink color). (Gutiérrez-Arnillas *et al*., 2016). The Minimum Inhibitory Concentrations (MICs) of bacteriocins will be determined by identifying the lowest concentration that inhibits the growth of the test isolates, as indicated by the absence of blue color in the wells. The experiments will be conducted three times. The Minimum Inhibitory Concentration (MIC) value was determined as the lowest concentration at which bacterial growth was not observed. (EUCAST, 2000; Hammer et al 1999).

2.5. Study the activity of bacteriocin under different conditions

The bacteriocins isolated from all three tested isolates were evaluated for their effectiveness against bacterial references. The evaluation involved incubating the bacteriocins at various temperatures

University of Thi-Qar Journal of agricultural research

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(ranging from 5 to 60 ℃), treating them with different concentrations of sodium chloride (ranging from 1 to 4M), and subjecting them to protease and lipase enzymes. (Abdulhusein *et al*., 2023).

III. RESULTS AND DISCUSSION

Bacteriocins produced by *Corynebacterium aurimucosum*, *Staphylococcus epidermidis*, and *Bacillus haynesii* have been put to evaluation against seven pathogenic reference species. Results of this investigation revealed that all tested bacteria exhibited resistance to the bacteriocins, with the exception of *Escherichia coli* ATCC 25922 and *Micrococcus luteus* ATCC 9341. These two bacterial strains were found to be susceptible to all three types of bacteriocins, as indicated in Tables 1, 2, and 3.

Table (1) The Antibacterial activity of metabolic bacterial compounds produced from *Corynebacterium aurimucosum* against pathogenic isolates

No	Isolates	The concentrations of Bacteriocin											
		1	2	3	4	5	6	7	8	9	10	P.C*	N.C**
1	*Enterococcus faecalis* ATCC 29212	+	+	+	+	+	+	+	+	+	+	+	-
2	*Klebsiella pneumoniae* ATCC 4352	+	+	+	+	+	+	+	+	+	+	+	-
3	*Escherichia coli* ATCC 25922	-	-	-	-	-	-	-	-	-	+	+	-
4	*Bacillus cerus* ATCC 11778	+	+	+	+	+	+	+	+	+	+	+	-
5	*Pseudomonas aeroginosa* ATCC 27853	+	+	+	+	+	+	+	+	+	+	+	-
6	*Micrococcus luteus* ATCC 9341	-	-	-	-	-	-	-	-	-	+	+	-
7	*Staphylococcus aureus* ATCC 65389	+	+	+	+	+	+	+	+	+	+	+	-
	(P.C*)= Positive control (only bacteria+ media without bacteriocin), (N.C**) = Negative control (only medium without bacteria and bacteriocin), (+***)= Bacteria is a live and non affects by bacteriocin, (-****)= All bacteria in the well are dead.												

Enterococcus faecalis ATCC 29212, *Klebsiella pneumoniae* ATCC 4352, *Bacillus cerus* ATCC 11778, *Pseudomonas aeroginosa* ATCC 27853, *Pseudomonas aeroginosa* ATCC 27853, *Staphylococcus aureus* ATCC 65389, *Escherichia coli* ATCC 25922, and *Micrococcus luteus* ATCC 9341 have been detected as antibiotic resistance (Caglayan 2018b). The results found that all above mentioned species are resistance to all concentrations of bacteriocin extracted from *Corynebacterium aurimucosum* expects *Escherichia coli* ATCC 25922, and *Micrococcus luteus* ATCC 9341 showed resistance to the tenth concentration of bacteriocin, whereas it was susceptible to the first nine concentrations of bacteriocin. Other our previous study explained that the bacteriocin from *Corynebacterium aurimucosum* affected *Corynebacterium aurimucosum* (PSS2b), *Kocuria polaris* (PSS8a), *Bacillus haynesii* (PSS11b) *Bacillus piscis* (PSS17a), *Pseudomonas iranica* (PSS21a), and *Paenibacillus illinoisensis* (PSS7a) by using agar diffusion method. However, there is no effect on other tested halophilic isolates *Staphylococcus warneri* (PSS1a), *Staphylococcus epidermidis* (PSS3a), *Pseudomonas oryzihabitus* (PSS14a), *Bacillus subtilis* (PSS16a), *Micrococcus luteus* (PSS21b), *Bacillus paralicheiniformis* (PSS23b), *Peribacillus frigoritolerans* (PSS4a) (Abdulhusein, 2023). Our findings indicate that not all isolates have the ability to make bacteriocin, and even when bacteriocin is produced; it may not have an effect on all types of bacteria. Furthermore, other factors can influence bacteriocin formation, including temperature, pH, and NaCl concentration.

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Table (2) The Antibacterial activity of metabolic bacterial substances produced from *Staphylococcus epidermidis* against pathogenic isolates

No	Isolates	The concentrations of Bacteriocin											
		1	2	3	4	5	6	7	8	9	10	P.C*	N.C**
1	*Enterococcus faecalis* ATCC 29212	+	+	+	+	+	+	+	+	+	+	+	-
2	*Klebsiella pneumoniae* ATCC 4352	+	+	+	+	+	+	+	+	+	+	+	-
3	*Escherichia coli* ATCC 25922	-	-	-	-	-	-	-	-	+	+	+	-
4	*Bacillus cerus* ATCC 11778	+	+	+	+	+	+	+	+	+	+	+	-
5	*Pseudomonas aeroginosa* ATCC 27853	+	+	+	+	+	+	+	+	+	+	+	-
6	*Micrococcus luteus* ATCC 9341	-	-	-	-	-	-	-	-	-	+	+	-
7	*Staphylococcus aureus* ATCC 65389	+	+	+	+	+	+	+	+	+	+	+	-
	(P.C*)= Positive control (only bacteria+ media without bacteriocin), (N.C**) = Negative control (only medium without bacteria and bacteriocin), (+***)= Bacteria is a live and non affects by bacteriocin, (-****)= All bacteria in the well are dead.												

The bacteriocin produced by Staphylococcus epidermidis only had an impact on Micrococcus luteus ATCC 9341 and Escherichia coli ATCC 25922, with no observed effects on other examined bacterial strains. The initial inquiries revealed the presence of two separate categorizations of bacteriocins: lantibiotics and non-lanthionine low molecular weight bacteriocin-like substances. These bacteriocins have a positive charge and are highly effective in acidic circumstances, especially when the pH is below 5. Additionally, it was shown that the bacteriocins exhibited a higher attraction towards bacterial cells at pH 6 compared to pH 2 (Jack *et al*., 1995). Other previous work by Azemin in 2015 isolated bacteriocins from some species which are including *Halomonas sp.*, *Vibrio sp.*, *Staphylococcus sp.*, *Micrococcus sp.*, *Streptococcus sp.*, *Bacillus cereus*, *Escherichia coli*, *Serratia*, *Proteus*, *Aeromonas*, *Enterobacter*, and *Plesiomonas*, have ability to inhibit the growth of other tested microorganisms within a pH range of 7-8.5, a temperature range starting from room temperature up to 55°C, and a NaCl concentration of 2.9 M. A previous study has shown that bacteriocins maintain stability at a temperature of 100 degrees Celsius for a length of 10 minutes. As a result, they are utilized in methods for preserving food (Moradi *et al*., 2021). Furthermore, a previous study demonstrated that bacteriocins exhibit antibacterial effects within a temperature range of 10°C-60°C, a pH range of 6.0-8.0, and salt concentrations ranging from 3% to 20%, effectively targeting all the isolates that were studied (Caglayan and Birbir, 2018).

Table (3) The Antibacterial activity of metabolic bacterial compounds produced from *Bacillus haynesii* against pathogenic isolates

No	Isolates	The concentrations of Bacteriocin											
		1	2	3	4	5	6	7	8	9	10	P.C*	N.C**
1	*Enterococcus faecalis* ATCC 29212	+	+	+	+	+	+	+	+	+	+	+	-
2	*Klebsiella pneumoniae* ATCC 4352	+	+	+	+	+	+	+	+	+	+	+	-
3	*Escherichia coli* ATCC 25922	-	-	-	-	-	-	-	-	+	+	+	-
4	*Bacillus cerus* ATCC 11778	+	+	+	+	+	+	+	+	+	+	+	-
5	*Pseudomonas aeroginosa* ATCC 27853	+	+	+	+	+	+	+	+	+	+	+	-
6	*Micrococcus luteus* ATCC 9341	-	-	-	-	-	-	-	-	+	+	+	-

University of Thi-Qar Journal of agricultural research

Thi-Qar Journal of agricultural research

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7	*Staphylococcus aureus* ATCC 65389	+	+	+	+	+	+	+	+	+	+	+	-
	(P.C*)= Positive control (only bacteria+ media without bacteriocin), (N.C**) = Negative control (only medium without bacteria and bacteriocin), (+***)= Bacteria is a live and non affects by bacteriocin, (-****)= All bacteria in the well are dead.												

Similarly, the bacteriocin from *Bacillus haynesii* affects *Micrococcus luteus* ATCC 9341 and *Escherichia coli* ATCC 25922; whereas there is no any effect on other tested pathogenic bacterial reference. The action mechanism of the majority of bacteriocins is inadequately understood (Hacker *et al*., 2015).

The bacterial isolates utilized in this investigation were shown to be resistant to all antibiotics tested, including ampicillin (10 µg), spectinomycin (25 µg), streptomycin (10 µg), meropenem (10 µg), and penicillin (10 µg). Table (4).

Table (4) The reference isolates, antibiotics, and Inhibition Zones (mm).

Isolates		Amoxicillin 10µg	Penicillin 10 µg	Streptomycin 10 µg	Ampicillin 10 µg	Meropenem 10 µg	Spectinomycin 25 µg
		Inhibition Zones (mm)					
1	*Enterococcus faecalis* ATCC 29212	-	-	-	-	-	-
2	*Klebsiella pneumoniae* ATCC 4352	-	-	-	-	-	-
3	*Escherichia coli* ATCC 25922	-	-	-	-	-	-
4	*Bacillus cerus* ATCC 11778	-	-	-	-	-	-
5	*Pseudomonas aeroginosa* ATCC 27853	-	-	-	-	-	-
6	*Micrococcus luteus* ATCC 9341	-	-	-	-	-	-
7	*Staphylococcus aureus* ATCC 65389	-	-	-	-	-	-

The results found that the activity of bacteriocin had lost after treated with protease enzyme. However, there is no effect on the bacteriocin by lipase enzyme. Also the antibacterial activity of bacteriocins was high against both of *Escherichia coli* ATCC 25922 and *Micrococcus luteus* ATCC 9341 at low and high temperature (4, 10, 20℃- 45, 55, 60℃). 35℃ was the optimum temperature for bacteriocin activity. Moreover, the results found the activity of bacteriocin was high at pH 6 and NaCl 1.5 to 2.5 M. Antibiotic resistance has been known as a ubiquitous global issue that has shown a notable increasing in recent years, significantly impacting the healthcare sector. The misuse of antibiotics is an important factor contributing to the development of antibiotic resistance. Additionally, bacterial activities such as transformation, transduction, and bacteriophage activity (conjunction) play a role in facilitating horizontal and vertical gene transfer among bacterial species, adding to this issue. All tested isolates were detected as antibiotics resistance species and only two isolates were sensitive to bacteriocins while the others show resistance to bacteriocins even at high concentrations. This result appeared that the bacteriocins can be used as alternative drug in the food production sector in order to minimize the number of bacteria.

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