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College of Science for Women**



**Molecular and Antimicrobial Study of
Bacteriocin Extracted and Purified from
Staphylococcus haemolyticus Isolated
from Clinical Samples**

A Thesis

Submitted to the College of Science for Women / University
of Babylon in Partial Fulfillment of the Requirements for
the Degree of Master of Science Biology

By

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ وَلَوْلَا فَضْلُ اللَّهِ عَلَيْكَ وَرَحْمَتُهُ لَهَمَّتْ طَائِفَةٌ
مِّنْهُمْ أَنْ يُضِلُّوكَ وَمَا يُضِلُّونَ إِلَّا أَنْفُسَهُمْ ۗ وَمَا
يَضُرُّونَكَ مِنْ شَيْءٍ ۗ وَأَنْزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ
وَالحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُن تَعْلَمُ ۗ وَكَانَ فَضْلُ
اللَّهِ عَلَيْكَ عَظِيمًا ﴾

صَدَقَ اللَّهُ الْعَلِيَّ الْعَظِيمُ

سورة النساء: الآية (١١٣)

Dedication

We walk in The paths of life and remain who controls our minds in every path we take That Strong Woman With a beautiful Face and good deeds That Taught me Self –confidence and Spared no effort to always make me happy

My Loved Mother

To The One who always Encouraged me the Secret of my happiness and Success.....

My Dear Father

To Those Who always encouraged me and Rejoiced for my Success....

My Sisters and Brothers

To Those People who Challenge the Odds to achieve Their Goals Despite All Obstacles

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Summary

Summary

One of the most significant risks to global public health in the twenty-first century is antimicrobial resistance (AMR). An international danger to development and health is antimicrobial resistance (AMR). Therefore, it is necessary to focus to on the most creative antibacterial substances compared to conventional antibiotics that are active against multidrug-resistant pathogens. Thee aim of this study to extraction, purification and Characterization on Bacteriocin isolated from *Staphylococcus haemolyticus* which isolated from urine and seminal fluid.

In this study, a total of 150 (urine 75 and seminal 75) samples were collected from male patients attended to Al-Yarmouk Hospital and Teaching Baghdad Hospital of various age groups at the period between October to December 2022 in Baghdad. Out of all specimens, only 60/150 (40%) isolates were identified as *S.haemolyticus* by traditional methods,Cultural and morphological characteristic examination ,biochemical tests were conducted and confirmed the diagnosis by Vitek-2 system and molecular diagnosis by *Nuc* gene.

The results of antibiotic susceptibility by using Viteck-2 System , the results showed that *S.haemolyticus* had multi-resistance against antibiotics Benzylpenicillin, Oxacillin , Erythromycin , Fusidic acid , Tetracycline , Gentamicin, Clindamycin , Moxifloxacin , Trimethoprim/Sulfamethoxazole , Teicoplanin, Levofloxacin , Rifampicin , Vancomycin , Nitrofurantoin , at 60(100%),60 (100%),54 (90%),52 (86.6%),51 (85%),43 (71.6%),43 (71.6%),35 (58.3%),26 (43.3%),17 (28.3%), 16 (26.6%),16 (26.6%),12 (20%)and respectively, while the highest sensitive to Linezolid, Tigecycline was 60 (100%) and 58 (96.6) sensitive for Nitrofurantoin .

Summary

The prevalence of biofilm producer bacterial isolates was 57 / 60 (95%) that 9 / 57 (15.7%) produced strong biofilm, 30 / 57 (52.6 %) produced moderate biofilm, 18 / 57(31.5 %) isolates produced weak biofilm and 3 / 60(5%) non producer biofilm.

The study revealed that 34 / 60 (59.6%) isolates from Seminal and 23 / 60 (40.3%) isolates from urine samples harbored gene encoding intracellular adhesion *icaADB* genes.

All isolates of *S.haemolyticus* exhibited the lysis of human red blood cells by Beta-hemolysin on blood agar, Also by PCR amplification of the gene encoding hemolysin of *S.haemolyticus* with specific primers it could be observed that *hla_haem* genes were present in all isolates.

Bacteriocin production can be detected using the agar well diffusion (AWD) method and filter paper disc (FPD) method. The result showed The FPD method revealed that about 95% of the *S.haemolyticus* were bacteriocin producers .while only 5% activity of the *S.haemolyticus* for producing bacteriocin it was observed by AWD.

The best conditions for the synthesis of bacteriocin were found in brain heart infusion broth (BHIB), which also included 1% glucose and 1% yeast extract was added to broth with a pH of 7 at a temperature of 37°C for 72 hours. the result also demonstrated the growth process' ability to produce bacteriocin was significantly impacted by the addition of nitrogen sources.

The effect result of incubation temperature showed that maximum production was attained between 30 to 37°C with inhibition zones measuring 32 mm to 39 mm for each strain of examined bacteria.

Summary

Effect of incubation periods displayed the greatest inhibition zones were 26 mm and 39 mm in seminal isolates after 48 and 72 hours, respectively, and the highest production was then shown with an inhibition zone of 37 mm, 45 mm in urine isolates.

The pH values between 6 and 8 give the maximum production of bacteriocin and bacterial growth of *S. haemolyticus*.

The result of relation between bacteriocin production and size of bacteria inoculum with 1.5×10^8 CFU/ml where the greatest zones of inhibition reached 25 and 38mm.

Bacteriocin precipitation by Ammonium sulfate had the highest activity at 70% saturation. While it was less effective with a saturation rate of 40-60%.

The molecular weight of *S. haemolyticus* bacteriocin about (29 kDa) were determined by Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Also, the physical properties of bacteriocins were examined, and the findings demonstrated that they were stable over a wide pH (4–9) and temperature range (40, 60 and 80°C) As for the study of the effect of storage on the effectiveness of *S. haemolyticus* bacteriocin was remained active following freezing for three month of storage with the maximum inhibition of zone (30 mm) from seminal fluid(S) isolates and (25 mm) from urine (U) isolates at -20°C .

The cytotoxic effect of Pure of Bacteriocin was assessed *in vitro* using the MTT test, which was used to measure the bacteriocin's cytotoxic activity on cell lines, the result of IC₅₀ values of the PC3and HdFn cells were 61.82- 122.5(3.763-168.2) µg/ml.

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List of Abbreviation

Meaning	Abberviate
Antimicrobial activity	AMA
Antimicrobial resistance	AMR
Ammonium persulfate	APS
agar well diffusion	AWD
Brian heart infusion broth	BHIB
Bacteriocin like inhibitory substance	BLIS
Continuous ambulatory peritoneal dialysis	CAPD
cell-free supernatant	CFS
Coagulase- negative staphylococci	CONS
Coagulase- positive staphylococci	COPS
distilled water	D.W
Di ethylaminoethyl-cellulose	DEAE-cellulose
Dimethyl sulfoxide	DMSO
Deoxyribonucleic acid	DNA
Extracellular DNA	eDNA
Environmental DNA	eDNA
Ethylene diamine tetra acetic acid	EDTA
Enzyme-linked immunosorbent assay	ELISA
Extracellular polymeric substance	EPS
fragment crystalizable	FC
Food Drug Adm.	FDA
Fibronectin-binding protein	FnBP
filter paper disc method	FPD
Gram positive bacteria	G+ve
Gastro intestinal tract	GIT
Gut microbiota	GM
Gram negative bacteria	G-ve
Human Dermal Fibroblasts, neonatal	HdFn
Alpha-hemolysin	Hla
inhibitory concentration	IC
ion exchange chromatography	IEC
lactic acid bacteria	LAB
Late- onset sepsis	LOS
Minimum bactericidal concentration	MBC
Multi drug resistance	MDR

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Muller-Hinton agar	MHA
Muller-Hinton broth	MHB
Minimum inhibitory concentration	MIC
Methicillin-resistant <i>Staphylococcus aureus</i>	MRSA
3-(4,5-Dimethylthiazol-2-yl)-2,5-dimphenyl tetrazolium Bromide	MTT
molecular weight	Mw
Nutrient Agar	NA
Nutrient broth	NB
Neutrophil extracellular traps	NETs
purified bacteriocin	PB
Peripheral blood mononuclear cells	PBMCs
phosphate buffer saline	PBS
Human Prostate cancer cell line	PC3
Pore-forming cytotoxin	PFT
Polysaccharide intercellular adhesin	PIA
polymer of β -1,6-N-acetylglucosamine residues	PNAG
Phenol-soluble modulins	PSMs
Roswell Park Memorial Institute – 1640 Medium	RPMI1640
Sodium Dodecyl Sulfate	SDS
Severe Skin and soft tissue infections	SSTI
Tetramethylethylenediamine	TEMED
Urinary tract infections	UTI

Chapter One
Introduction

Introduction:

Staphylococcus haemolyticus, an emerging nosocomial pathogen, is considered as the second most common species of coagulase-negative staphylococci (CoNS) and is associated with device-related infections in immunocompromised individuals (Meerabai *et al.*, 2020).

There are several clinical manifestations recorded with *S. haemolyticus* infections such as bloodstream infections, ocular infections, epididymo-orchitis, chronic prostatitis, UTI, etc. Furthermore, it is an important pathogen associated with hospital-acquired infections. Other complications were also recorded especially in immunocompromised patients such as septicemia, peritonitis, and otitis. The genome of *S. haemolyticus* causing hospital infections is characterized by the abundance of insertion sequences, and resistance to several antibiotics. (Hala *et al.*, 2022).

Resistance to two or more classes of antimicrobial agents such as penicillins, tetracyclines, aminoglycosides, cephalosporins, quinolones, macrolides and glycopeptides among *Staphylococcus haemolyticus* of clinical importance have been frequently reported in different parts of the world. (Alahmadi *et al.*, 2021).

As CoNS possesses less virulence factors than *S. aureus*, particularly those that cause infection, they are thought to be less harmful. However, CoNS are considered as opportunistic bacteria that colonize healthy people, as well as being the cause of the most common hospital infections, with a growing effect on human health and life (Ahmad-Mansour *et al.*, 2021).

Hemolysins of staphylococci are categorized into four different types, including alpha (α), beta (β), gamma (γ), and delta (δ). Pathogenicity of this toxin is due to hemolytic, dermonecrotic, and neurotoxic effects. (Nasaj *et al.*, 2020).

Biofilm is one of the most widespread and most successful life forms on Earth (Phusa, 2019) In nature, microorganisms commonly exist in the shelter of highly hydrated biofilms which creates a conducive environment for cells to adhere together and onto all kinds of surfaces (Srivastava and Bhargava 2016; Abebe ,2020)

Antimicrobial resistance (AMR) is a relevant health problem worldwide that needs the development of new strategies. The human–animal environment interface is currently being considered under the One Health approach to better understand the ecology and dissemination of (AMR) and to control the silent pandemic, as is considered by some authors (Swift *et al.*, 2019; Penna *et al.*,2021).

Bacteriocins, are interesting alternative to the use of conventional antibiotics. These peptides might become important biological weapons, especially against emerging drug resistant bacteria, and they might be used in different areas, such as the food industry, medicine, veterinary, and agriculture (De Freire Bastos *et al.*, 2020).

Bacteriocins are ribosomally synthesized antimicrobial peptides that are lethal to producer cells. These peptides are generally active against species closely related to the producing microorganism. Bacteriocins produced by Gram-positive bacteria exhibit a wider spectrum of inhibitory activity, which may favor a broader industrial application.(Simons *etal.*, 2020).

Bacteriocins are synthesized to confer a selective advantage to the producer in terms of niche colonization ability since these molecules often display activity against closely related bacterial species (Heilbronner *et al.*, 2021).

Bacteriocins have been found as potentially safe biopreservatives. The selective potential of bacteriocin 10A as a candidate food biopreservative to control the growth of food pathogenic and spoiling bacteria. (Noktehsanj Avval, *et al.*,2023).

Bacteriocins are peptidic toxins produced by bacteria, offer promising potential as substitutes or conjugates to current therapeutic compounds. These toxic peptides exhibit significant potency against certain bacteria including multidrug-resistant (MDR) species while producer strains remain insusceptible to the bactericidal peptides (Belstrøm.,2020).

The current study aimed to Producing, purification and characterization of bacteriocins using *Staphylococcus haemolyticus* isolated from clinical sample. Also used as an alternative to antibiotics in the treatment of antibiotic-resistant bacteria.

1. Isolating the *Staphylococcus haemolyticus* from clinical samples by using different culture media (selective and differential) and Identification of *S. haemolyticus* using biochemical test and Vitek 2 analyser.
2. Extraction genomic DNA from Bacteria by using special extraction DNA kit, and Molecular Identification of bacteria Isolated strains by using *Nuc* gene.
3. Phenotypic and Genotypic detection of hemolysin and Biofilm formation by *Staphylococcus haemolyticus*
4. Screening of isolates that have the ability to produce bacteriocins. and Choosing the most productive isolate of bacteriocins and studying the optimum conditions for production from pH, temperature, sugars and others.
5. Purification of bacteriocins using ammonium sulfate and Purification of the selected bacteriocin using an ion exchanger.

Chapter Two
Literature Review

2.1 *Staphylococcus SPP.*

Staphylococcus widely distributed in the environment and comprises important members of the major bacteria communities colonizing the skin and mucous membranes of humans and animals (Kranjec *et al.*, 2020). This genus comprises a high diversity of species, traditionally separated into two major groups based on their ability to clot the plasma. coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS). CoNS is the broadest group, with more than 80% of species (Becker *et al.*, 2015). Although staphylococci often maintain a commensal relationship with their hosts, staphylococcal infections could develop in specific cases (De Freire Bastos *et al.*, 2020). Some staphylococcal species are more related to human and animal infectious diseases, being *Staphylococcus aureus* one of the most important Individual bacterial strains living in highly competitive and polymicrobial environments have developed multiple types of interaction and self-defense mechanisms. The survival success can include different capabilities such as a higher specificity to a limited number of host attachment sites, a better ability to take up and metabolize micronutrients, or the production of antibacterial substances that inhibit competitors (Janek *et al.*, 2016).

Species are among the most frequently encountered bacteria in hospital settings and have been incriminated for many infections in humans. The CoPS include *Staphylococcus aureus* as the major pathogen. There is now increasing evidence that some of the CoNS species (*Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *Staphylococcus warneri*, *Staphylococcus capitis*) could also be pathogenic and cause nosocomial infections (Oladipo *et al.*, 2019).

2.2 *Staphylococcus haemolyticus*:

Staphylococcus haemolyticus is characterized by it is gram positive, non-motile, non spore forming facultative anaerobe and can grow on a wide range of carbohydrates such as glucose, maltose and sucrose, This bacteria are negative for coagulation enzyme tests, DNase, Ornithine decarboxylase, Phosphatase, Ureas (Paul *et al.*, 2009).

Staphylococcus haemolyticus is one of the CoNS that inhabit the skin as a commensal. Studies of the skin metagenome have propped the long-known assertion that *Staphylococcus* species prefer areas of higher humidity such as the plantar foot region, axillae, and umbilicus (Asante *et al.*, 2020). CoNS particularly *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, are considered important nosocomial agents of medical device-associated infections (Vuong and Otto, 2002).

The remarkable *S. haemolyticus* ability of to acquire antibiotic resistance, especially to oxacillin, limits the available therapeutic options for catheter-related infections caused by *S. haemolyticus* methicillin-resistant isolates and may predispose to sepsis and increase patient's morbidity and mortality (Eltwisy *et al.*, 2020)

Across the world, approximately 2.4 million children lose their lives in the first month of birth each year and India contributes majorly to this. India has a neonate mortality rate of 21.7% (Brzychczy-Wloch *et al.*, 2013). *S. haemolyticus* is a part of skin microflora and one of the main species of CoNS (Saida *et al.*, 2009).

This species accounts for 10–20% of clinical CoNS infections and is the second-highest species of CoNS in frequency and importance among isolates from clinical infections (Szczuka *et al.*, 2015). There are several clinical infections recorded with *S. haemolyticus* including bacteremia, meningitis, eye infections, skin infections, peritonitis, urinary tract infections, and male genital dysfunction (Do carmo ferreria *et al.*, 2011).

2.3 Ecology of *S. haemolyticus*

Staphylococcus haemolyticus is one of the coagulase-negative staphylococci (CoNS) that inhabit the skin as a commensal. It is increasingly implicated in opportunistic infections in immunocompromised patients, particularly in hospitalized patients and those with medical implants worldwide (Eltwisy *et al.*, 2020). The most significant species of CoNS which frequently cause infections in humans are *S. epidermidis* (blood infections, catheter related infections, surgical wounds, osteomyelitis etc.), *S. haemolyticus* (urinary tract infections, endocarditis, wounds, septicemia etc.), and *S. saprophyticus* (septicemia and urinary tract infections) (Al-hilu and Al-shujairi, 2020). Coagulase-negative *S. haemolyticus* is an important pathogen in healthcare-associated and medical device-related infections, and after *S. epidermidis*, is one of the causes of CoNS infection, Despite the increased prevalence of this pathogen in the nosocomial environment, little is known about its pathogenicity and related virulence factors (Araujo-Alves *et al.*, 2022). Furthermore, *S. haemolyticus* strains were isolated from dogs and dogs' owners suggesting a possibility of zoonotic transmission (Ruzauskas *et al.*, 2014).

2.4 Pathogenesis of *S. haemolyticus*

Staphylococcus haemolyticus produces several toxins and invasive enzymes that help in bacterial pathogenesis by changing the host immune responses and inducing damage in the host cells (Eltwisty *et al.*, 2020). In the absence of appropriate diagnosis and management of infections caused by *S. haemolyticus*, resistant strains of this pathogen can spread to other hospital settings, and probably to the community (Hosseinkhani *et al.*, 2016). Hemolysin protein is cytolytic toxin produced by

Staphylococcus haemolyticus that effects the human erythrocytes and has nonspecific membrane toxicity to other mammalian cells. (Al-Always and Al-Khafaji, 2016). *S. haemolyticus* has a great ability to survive in the hospital environment, especially on medical devices, becomes a Crucial factor in nosocomial infections caused by multiresistant staphylococci.

Also, CoNS has been reported as pathogenic causes of infections in human and veterinary medicine, especially immunocompromised patients, critically ill, long-term hospitalized and in those harboring invasive medical devices such as catheters (Eltwisy *et al*, 2020). Clinicians and microbiologists are frequently confronted with determining whether retrieved CoNS are contaminants that intrude during sampling or sample processing, are regular skin or mucous membrane commensals, or are clinically relevant, because many CoNS species form part of the skin and mucous membrane microbiota, the line between innocuousness and pathogenicity may be indistinct and comes down to virulence strategies employed by the various species as well as host defense mechanisms. Although, today, the CoNS are recognized as causative agents of clinically significant infections (Pereira-Ribeiro *et al.*, 2022). show in figure (2-1). Apart from phylogenetic findings and classifications, a simplified but more useful and well-accepted scheme, mainly based on clinical and diagnostic aspects, is still used in human medicine: staphylococci are divided into CoPS, almost exclusively represented by *S. aureus*, and CoNS. (Becker *et al.*,2014)

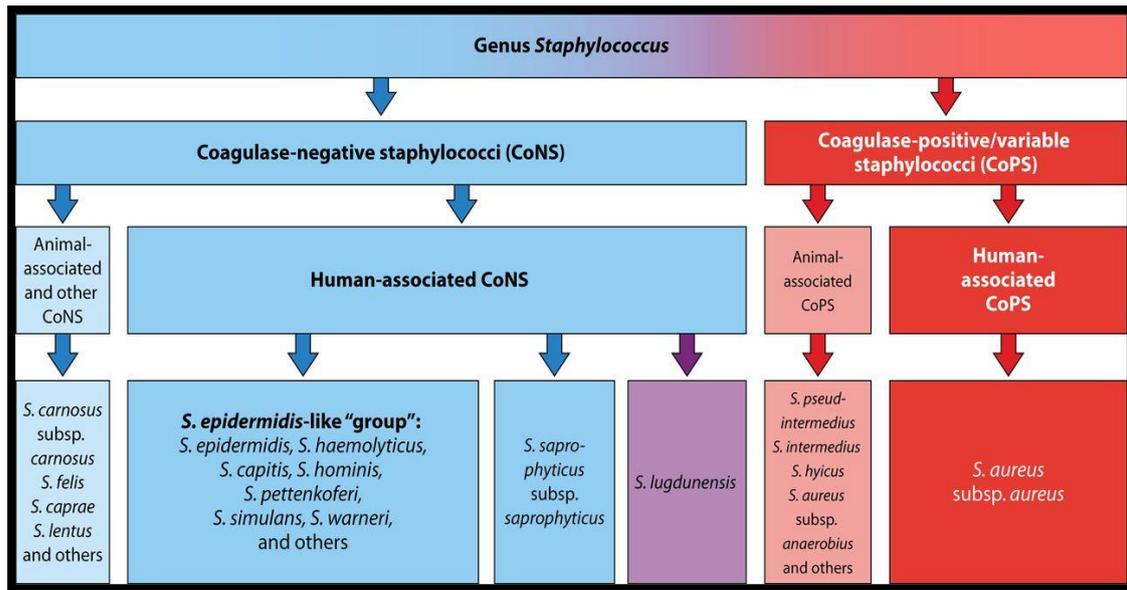


Figure (2-1): Clinical and epidemiological schema of Staphylococcal species, and the categorization of coagulase as a major virulence and its resulting impact on human health (Becker *et al.*, 2014).

2.5 Virulence Factors of *S. haemolyticus*

Genome sequencing of common *S. haemolyticus* strain C10A has illustrated the brief detection of multiple antibiotic resistance and virulence genes (Chan *et al.*, 2015). However, many of these virulence factors remained virtually unexplored (Da *et al.*, 2017). Surface substances and cytolytins have crucial effects on the virulence of *S. haemolyticus* (Czekaj *et al.*, 2015) Bacterial adherence and internalization are mediated by biofilm and fibronectin-binding proteins (FnBP). Following the entry to the host cell, toxins and enzymes are released by *S. haemolyticus* which mediate tissue damage, activation of proinflammatory cytokines, and apoptosis of host cell following:

2.5.1. *S. haemolyticus* Surface Proteins required for bacteria adherence besides the biofilm formation, *S. haemolyticus* secretes fibronectin-binding proteins (FnBP) that play an important role in bacterial adherence

to the extracellular matrix, bacterial internalization into the host cell, and invasion (Eltwisty *et al.*,2020). The adherence property of clinical *S. haemolyticus* is different from commensal *S. haemolyticus*. Commensal *S. haemolyticus* have high adherence to fibronectin and collagen, while clinical *S. haemolyticus* have low adherence to fibronectin and collagen (Wolden *et al.*,2020).

2.5.2. Toxins and Enzymes Some *S. haemolyticus* isolates secrete enterotoxins and/or hemolysins (Pinheiro *et al.*,2015). Staphylococci enterotoxins act as superantigens that activate the immune cells to produce their cytokines resulting in food poisoning and other diseases such as sepsis and multiorgan dysfunction (Taylor and Llewelyn, 2010). Several enterotoxin genes were recorded in *S. haemolyticus* such as *sea*, *seb*, *sec*, *seg*, and *sei* and one or more genes could be recorded in the isolates found from blood cultures (Pinheiro *et al.*,2015) and peritonitis in continuous ambulatory peritoneal dialysis (CAPD) patients (Barretti *et al.*,2009). In addition, enterotoxin-producing *S. haemolyticus* was isolated from the clinical samples of newborns (Vasconcelos *et al.*,2011).

Furthermore, *S. haemolyticus* PSMs (phenol-Soluble modulins) induce neutrophil chemotaxis resulting in a pronounced pro-inflammatory effect (Da *et al.*,2017). The previous findings highlight the importance of toxins in the invasion of *S. haemolyticus* and the development of bacteremia and sepsis.

2.5.3. Cytotoxicity and Apoptosis of the Host Cells *In vitro* studies showed that *S. haemolyticus* infection could alter the host immune response through its effect on the host cells (Eltwisty *et al.*,2020). Krzyminska showed that *S. haemolyticus* causes injury and loss of mitochondrial membrane potential in macrophages (Krzyminska *et*

al.,2012) The previous findings show one strategy for *S. haemolyticus* persistence and dissemination in the host through inhibition and host macrophages. Similarly, the study by Eltwisty showed that *S. haemolyticus* infection causes damage and apoptosis of primary human skin fibroblast cells, and it induces the release of proinflammatory cytokines from the PBMCs cocultured with the skin fibroblast (Eltwisty *et al.*,2020). Collectively, the previous reports show that *S. haemolyticus* infection causes damage to the host cells through apoptosis. In addition, *S. haemolyticus* infection is cytotoxic to the macrophages through induction of caspase dependent apoptosis (Krzyminska *et al.*,2012).

2.5.4. Biofilm Formation

The biofilm permits the adherence and persistence of bacteria in foreign materials. Furthermore, bacteria organized in biofilms are protected from antimicrobials and from the host immune system (Mack *et al.*, 2007), are currently one of the major causes of device-related infections by forming biofilms, particularly in immunocompromised patients (Heilmann *et al.*, 2019). Among the CoNS species, *S. haemolyticus* is the leading cause of late-onset sepsis (LOS) in neonates and also plays a very important role in hospital-acquired infection worldwide (Zaha *et al.*,2020). and many isolates have the ability to form biofilms (Pereira *et al.*,2014; Pereira-Ribeiro *et al.*,2019). This pathogen can exchange resistance genes and enhance the biofilm forming ability for spreading of infections. (Pereira-Ribeiro *et al.*, 2022) .

A part of the early biofilm maturation process, the bacteria also start to produce extracellular polymeric substance (EPS), which eventually forms the biofilm matrix. EPS mainly consists of polysaccharides, proteins, teichoic acids, and eDNA. In polysaccharide-dependent biofilms, the

main EPS component is polysaccharide intercellular adhesin (PIA). (Silva-Santana *et al*, 2021).

A biofilm can be produced by a single species or by multiple species, including fungi, bacteria and virus, embedded in a secreted extracellular matrix. This complex biofilm matrix acts as a protective layer allowing bacteria to be more resistant to antibiotics and disinfectants (Yong *et al*, 2019). The steps of biofilm formation start with the attachment of bacteria to the biotic or abiotic surface, following to the proliferation and expression of intercellular adhesion traits leading to the formation of multilayered cell clusters; maturation of the biofilm and dissolution and dissemination with the blood stream leading to infection in other regions on the body, where after initial attachment of *Staphylococci*, they multiply and grow into multilayered cell clusters requiring intercellular adhesion. Intercellular adhesion is mediated by biopolymers, such as polysaccharides and proteins (Heilmann *et al*, 2019; Pereira-Ribeiro *et al*, 2022). show in figure (2-2).

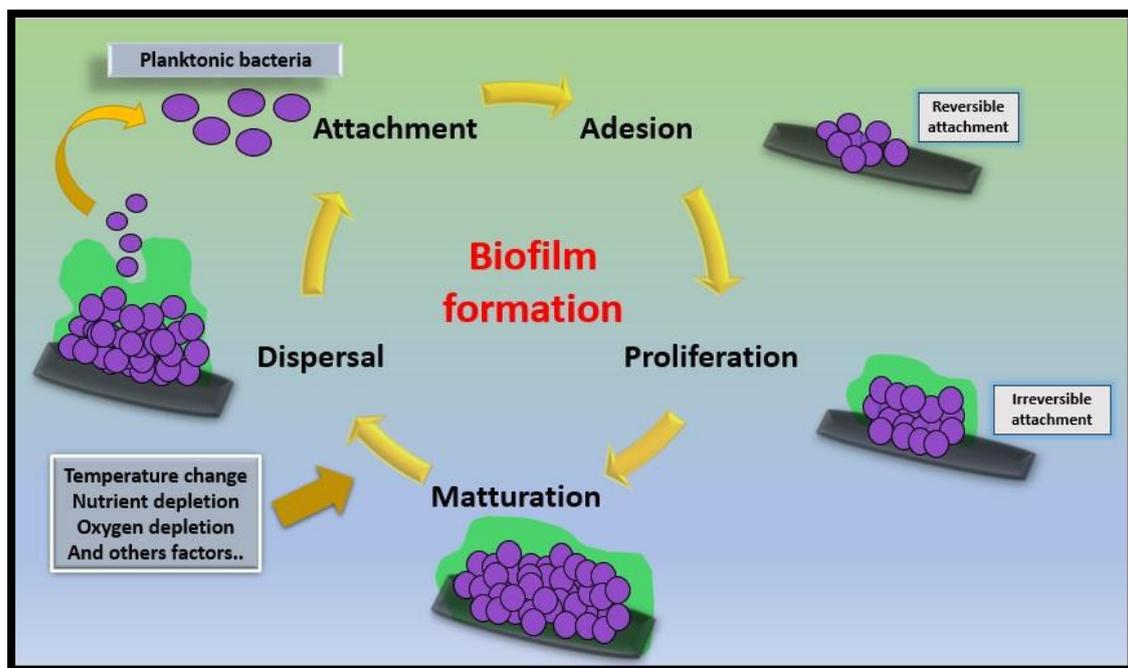


Figure (2-2): General steps of *Staphylococcus sp.* biofilm formation (Pereira-Ribeiro *et al.*, 2022).

Staphylococcus haemolyticus has a feature of ability to form biofilms, which play an essential role in the establishment of infections. The produced exopolysaccharides can inhibit the growth of other bacteria and decrease their ability to form biofilms. (Eltwisy *et al.*,2020) The ability to adhere and colonize implanted biomaterials in addition to biofilm formation is considered the main virulence factors of this specie. It is an increasing cause of nosocomial infections associated with indwelling medical devices, particularly affecting immunocompromised patients and premature babies (Eltwisy *et al*, 2020; Wolden *et al*, 2020).

The ability to colonize the surfaces of medical devices and biofilm formation is the critical *S. haemolyticus* pathogenicity factor (Sued *et al*, 2017; Argemi *et al*, 2019; Pereira-Ribeiro *et al*, 2019). A characteristic feature of *S. haemolyticus* is the formation of biofilms, which are crucial for the development of infections (Eltwisty *et al.*, 2020). Biofilm resistance to antimicrobials is multi factorial and there are several resistance mechanisms that act together to provide increased biofilm resistance. Biofilm growth of staphylococci also increased horizontal transfer of plasmid carried antibiotic resistance and heritable antibiotic resistance through spontaneous mutation (Yong *et al*,2019). *Staphylococcus haemolyticus* is commonly identified as one of the CoNS that takes virulence genes, such as, *icaADBC*, *aap*, *atl*, *fbp*, *bap*, *fnb*, *sesB* are involved in biofilm formation. Data showed a correlation with the presence of *icaA*, *aap*, *atl* and *fbp* genes colonizing abiotic surfaces that the process of biofilm formation is complex and may not be related to the *icaA* gene (Pereira-Ribeiro *et al.*, 2019; Yong *et al*, 2019).

2.6 Mechanism of Antibiotic Resistance

Over recent years the *S. haemolyticus* that has high resistance to antimicrobials, thus helping the clinician in a more effective treatment (Asante *et al.*, 2020). Different investigators have described an increasing frequency of multidrug-resistant strains of *S. haemolyticus* (Cavanagh *et al.*, 2014). *S. haemolyticus* is notably more resistant to antibiotics than any other CoNS, and the widest spectrum of resistance was observed among strains isolated from the hospital environment (Czekaj *et al.*, 2015). The presence of resistance genes in *S. haemolyticus* and its spread in the hospital environment constitutes a potential risk since this bacterium can store the resistance genes and transmit them to other species (Hosseinkhani *et al.*, 2017). Furthermore, they could transfer the drug resistance to other staphylococcal species, as shown that the sequences of beta-lactamase and *qacA* genes were identical in both *S. aureus* and *S. haemolyticus* indicating interspecies transfer of IS between these species (Baktharatchalam *et al.*, 2017).

S. haemolyticus is known for acquiring antibiotic resistance (MDR) which contributes to the establishment of more virulent clones (Cavanagh *et al.*, 2014). The important function of *S. haemolyticus* as reservoirs for antibiotic resistance and virulence traits is still an underestimated issue that requires more scientific attention. They may also have practical consequences for hospital hygiene by including resistant and virulent CoNS isolates into future infection control and surveillance measures (Pereira-Ribeiro *et al.*, 2022)

The efflux pump mechanism is documented in *S. haemolyticus* human clinical isolates, and it is associated with resistance to gentamicin, erythromycin, ciprofloxacin, chloramphenicol, and tetracycline (Sekyere and Mensah, 2018). The multidrug-resistant pump is mediated by several genes such as *qac G*, *qac H*, *qac J* genes (Correa *et al.*, 2008).

2.7 Bacteriocin

Bacteriocin is ribosomally synthesized proteins or short polypeptide that have antibacterial activity against closely related to the producing strain or other bacteria (Sugrue *et al.*,2020). Bacteriocin was first described in 1925 but the interest in their production function and possible applications in medical areas have been growing only recently (Lopetuso *et al.*,2019). In a microbial community, cells can be bacteriocinogenic (produce bacteriocin) sensitive or resistant to each bacteriocin, only a small percentage of bacteriocinogenic cells will be induced to produce and release bacteriocin (Bindiya *et al.*,2016). Some sensitive cells are immediately destroyed while others contain mutations that determine their resistance are persistent in the environment. Both Gram positive and Gram-Negative bacteria produce bacteriocins but lactic acid bacteria (LAB) are most important producers. There are numerous bacteriocins produced by wide range of bacteria and classified on basis of their size, mode of action, producer bacteria, spectrum of activity and molecular masses (Maryam *et al.*, 2017). Bacteriocins have possessed narrow spectrum of activity showing activity against specific bacteria but spectrum can be enhanced to other bacterial species also are natural antimicrobials as compared to other antibiotics. In addition enterococci bacteriocins are recognized for their wide spectrum antimicrobial activity including Gram positive foodborne pathogens. (Lauková *et al.*,2017).

Genetically bacteriocins are encoded by plasmid occurrence genes or chromosomal encoded genes which also contain genetic determinants of the producer's resistance to the produced bacteriocin. Genes that encode an active protein and genes encoding resistance to the protein, genes responsible for the export of bacteriocin from the cell and occasionally

genes coding for enzymes involved in posttranslational modification of bacteriocins undergo expression in parallel (Silva *et al.*, 2018).

2.8 Classification of Bacteriocins:

Bacteriocins of Gram-positive bacteria (G+ve) are abundant and diverse than those found in Gram negative bacteria (G-ve) bacteria, the bacteriocins from G+ve are similar to the antimicrobial peptides produced by eukaryotes; they are generally cationic, amphiphilic, membrane-permeable peptides and range in size between 2-6 Kd Dalton, Bacteriocins are classified into separate groups such as the lantibiotics (class I) the small (<10 kDa) heat stable; they are . Further subdivided in the pediocin-like and anti- *Listeria* bacteriocins (subclass IIa), the two peptide bacteriocins (subclass IIb), and the secdependent bacteriocins (subclass IIc); the large (>30 kDa) heat-labile non-lantibiotics (class III) and complex bacteriocins containing chemical moieties such as lipid and carbohydrate (class IV). (Kong *et al.*,2016) Bacteriocins from Gram positive bacteria (G+ve) bacteria are divided into four classes as in table (2-1) (Aslam *et al.*,2018)

Table (2-1): Classification of bacteriocins from Gram positive bacteria (G +ve) bacteria. (Aslam *et al.*,2018).

Class	Sub class	Characters	Example
Class I	Type A	Lantibiotics, small (< 5 kDa)	Nisin
	Type B	peptides, Elongated, Globular	Mutacin I
Class II	ClassIIa	Non-lantibiotics, small (<10 kDa) peptides	AcidocinA Lactacin F
	Class IIb	one-peptide,pediocin-like	
	Class IIc	bacteriocins	

	Class IId	Two-peptide bacteriocins Cyclic bacteriocins whose N- and C-termini are covalently linked Linear, non-pediocin-like, one-peptide bacteriocins	AcidocinB Carnobacteriocin A
Class III	-----	Large (>30 kDa) heat-labile proteins	Lacstrepsin
ClassIV	-----	Bacteriocins with non-protein moieties, i.e. glycoproteins and/or lipoproteins	Lactocin27

Bacteriocins from Gram negative bacteria (G-ve) bacteria are mostly produced by enterobacteriaceae, and are divided in two classes on the basis of molecular masses, high molecular mass bacteriocins include colicins and low molecular mass bacteriocins include microcins. (Merlich *et al.*,2019).

2.9 Mode of Action of Bacteriocin

Traditional bacteriocin exhibit five major mechanisms for inactivation of microbes. These mechanisms are: (Hegarty *et al.*,2016)

- 1) Inhibition of cell wall synthesis.
- 2) Inhibition of DNA transcription and replication.
- 3) Disruption in protein synthesis.
- 4) Inhibition of cell membrane function.
- 5) Folate synthesis inhibition.

Bacteriocins can be inactivate bacteria by using these reported antibiotic targets and these targets are:

2.9.1 Inhibit of Cell Wall Synthesis

Nisin A synthesized by *Lactococcus lactis*, nisin A is bacteriocin which inhibit cell wall synthesis either by forming pores in membrane leading to death of cells or by docking to lipid II in cell membrane which is precursor in cell wall synthesis. First mechanism is bactericidal at high concentration and second is bacteriostatic nisin exhibit an effective antimicrobial activity against gram positive bacteria and this makes it useful in pharmaceutical as well as food industry (García-Curieles *et al.*, 2021)

2.9.2 Inhibition of DNA Synthesis

There is structural difference between human and bacterial DNA gyrase and thus suitable site for antibiotic action (Heesterbeek *et al.*, 2019). Quinolones are antibiotics that inhibit DNA synthesis by preventing decatenation of DNA during replication, bacteriocin such as microcin B17 possess same mechanism. (Sahoo *et al.*, 2016)

2.9.3 Inhibition of Protein Synthesis.

Bacteriocins can inhibit protein synthesis by interfering with translation of proteins at various steps. These include colicin E3-E6 and cloacin DF13 exhibit rRNase activity at 16S (Ng *et al.*, 2010). Group E colicins possess endonuclease activity and they inhibit translation by acting on 3' end of the coding sequence and cleaving 16S rRNA. Some colicins like D and E5 are tRNases and they act by increasing exhaustion of tRNA resulting in limited protein synthesis. (Hashim *et al.*, 2019).

2.9.4 Voltage Gradient and Receptor-like Entity

It was evidenced that voltage independent pores are formed by Pediocin PA1 and lactococcin A (Moll *et al.*, 1999). Permeabilized vesicles are formed by Lactococcin A that are susceptible while liposomes are made from the same kind of cells that are not affected. It is also suggested that a

receptor-like entity on the cell surface is needed for inhibiting the cell (Negash and Tsehai,2020). The mechanisms of action of bacteriocin are shown in Figure (2-3).

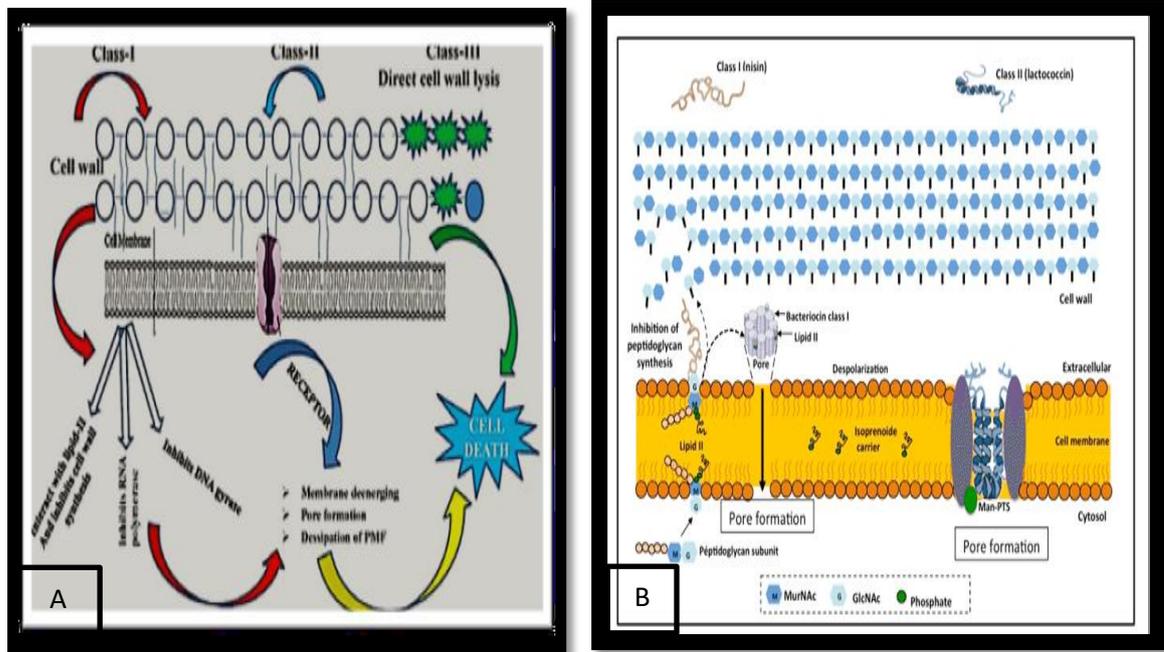


Figure (2-3A,B):Mechanisms of bacteriocin action including inhibits cell wall synthesis, cytoplasmic membrane pore formation, and inhibitts DNA gyrase (Ahmad *et al.*,2017) .

2.10 Characterization of Bacteriocin & Antibiotic

Multiple aspects discern bacteriocins and antibiotics are summarized in (Table 2-2):

Table (2-2): Comparison between antibiotic and bacteriocin

Characteristic	Bacteriocins	Antibiotics	References
Synthesis	Ribosomal ,or chromosomal (primary metabolite)	Enzymes (secondary metabolite)	Perez <i>et al.</i> , 2014
Engineering	Highly editable	Not editable	Perez <i>et al.</i> ,2014
Spectrum of activity	Specific, narrows, or broad	Narraw, or broad	Mathur <i>et al.</i> ,2017
side effects	None identified	Many	Tong <i>et al.</i> , 2014
Cost	Highly cost	Low cost	Hols <i>et al.</i> , 2019
Purification	Complicated, low yield	Possible, high yield	Hols <i>et al.</i> , 2019
Solubility	Low soluble	Variable (low to high)	Jenssen <i>et al.</i> , 2016
Plasma stability	Low stabilize	Dependent on drug	Jenssen <i>et al.</i> , 2016
Half Life	Low	Dependent on drug	Jenssen <i>et al.</i> , 2016
cytotoxicity	low	low to high	Perez <i>et al.</i> , 2014
Antibiofilm properties	strong	Resistance	Meade <i>et al.</i> , 2020
Chemical and thermal Stability	high for class I and II	low	Hols <i>et al.</i> , 2019
Degradation	proteolytic enzymes	Hydrolysis, thermal	Jenssen <i>et al.</i> , 2016

2.11 Biosynthesis of Bacteriocins

There are some factors affecting the production of bacteriocins, such as culture conditions and the type of microbial strain. Bacteriocins are post-

translationally modified and ribosomally synthesized peptides that are initially produced as inactive pre-peptides and then converted into an active form (Mokoena,2017).After transport and cleavage of produced pre peptides, they are converted into mature (active) bacteriocins. Depending on the type of bacteriocins, genes responsible for pre-peptides transport and modification are located close to the bacteriocin biosynthesis gene, and genes responsible for the immunity and active bacteriocin production are broadly located in operon clusters and might be harbored in plasmid, genome, or other mobile genetic factors (Kumariya *et al.*,2019). In addition to inducible expression these operons require auto-inducer peptides to induce bacteriocin production. The regulation of expression is generally fulfilled by a two-part regulatory system, but in some cases, this process is done by three-element ones. The production of bacteriocins is fulfilled in the exponential growth phase by retaining a line-to-line connection to the biomass production (Da Costa *et al.*,2019)

2.12 Optimization of Bacteriocin Production

The biosynthesis of bacteriocins can be influenced by various culture conditions,such as the composition of the medium, pH, temperature, and growth kinetics of the microorganisms(Sidooski *et al.*,2019).Fermentation studies on the class II bacteriocin production indicatethat it follows primary metabolite kinetics producing the bacteriocin during the growth phase and declines completely after entering the stationary phase (Abbasiliasi *et al.*,2017).The commercial availability of bacteriocins is still limited due to the low yield of the product. Moreover, bacteriocin-producing LAB needs complex nutrition to grow, and this not only increases the production cost but also gives rise to the difficulties related to their purification. The efficient use of these compounds requires various approaches to overcome the low yield and

the high production costs. In this regard, different studies have examined the effects of various media compositions and culture conditions on the yield of bacteriocins (Fahim *et al.*,2017) A multitude of fermentation factors including medium composition, temperature, and pH have a strong influence on bacteriocin production which is usually strain-specific. Consequently, scientists are motivated to develop high-yielding strains through the genetic engineering approach (Zhang *et al.*,2022).

2.13 Application of Bacteriocin

Bacteriocins are one of the important natural defense mechanism bacteria use to fight with pathogenic microorganisms in the same environment.in recent years a number of technologies have been employed to bolster the potential of bacteriocins in food preservation (Sharama *et al.*,2019). Bacteriocins from LAB have been used as food preservatives due to their heat stability and safety. Nisin is the most famous and best-studied bacteriocin, and it has high antibacterial activity against a wide range of Gram-positive bacteria. Nisin has received considerable attention in the food industry because it is the only purified bacteriocin approved for food use by the FDA (Umu *et al.*,2016). A wide variety of bacteriocins are capable in inhibiting the growth the foodborne and food spoiling pathogens have been recently described.The productivity of these compounds is still very low, so various techniques have been used to enhance their production. During the previous decades, many researchers have shifted their focus towards the bacteriocin application in the treatment of antibiotic-resistant disease-causing bacteria and infection. The therapeutic potential of bacteriocins has been enhanced by the use of engineered bacteriocin (Sharama *et al.*,2019).

However, several restrictions to the efficacy of nisin limit its range of practical applications. For instance, nisin has weak inhibition activity against Gram-negative bacteria, and its biological activity is reduced at an

elevated pH. Moreover, spontaneous nisin resistance may occur in target bacteria. Consequently, lots of researchers seek to find bacteriocins with different targeting spectra and biochemical properties (Umu *et al.*, 2016).

2.13.1 Medicine

As the antimicrobial efficiency of bacteriocins is increased when conjugated with nanoparticles they can be used to treat infections caused by MDR pathogens thereby avoiding further increase in antimicrobial resistance. There are several reports which show that antimicrobial activity (AMA) of bacteriocin nanoconjugates is better when compared to AMA of bacteriocin and nanoparticles alone. One such study showed the synergistic effect of bacteriocin isolated from *Lb. paracasei* and silver nanoparticles against MDR. MDR bacteria such as *S. aureus*, *P. aeruginosa*, *Proteus vulgaris*, *Streptococcus pyogenes*, *Klebsiella pneumonia*, *E. coli* and *Proteus mirabilis* were much more sensitive towards the bacteriocin conjugated with silver nanoparticles. The MIC (lg ml^{-1}) and minimum bactericidal concentration (MBC) (lg ml^{-1}) value of the nano-formulated bacteriocin was low when compared to the MIC and MBC value of bacteriocin and silver nanoparticle separately. The diameter of inhibition zone is produced by bacteriocin nanoconjugate against *S. aureus* was 14, 22, 0.2 mm. whereas for bacteriocin it was 3, 8, 0.2 mm and for silver nanoparticle it was 10, 17, 0.6 mm. The MIC value for bacteriocin nanoconjugate was 1, 56.3, 12 lg ml^{-1} , whereas for bacteriocin it was 12, 5, 25 lg ml^{-1} and for silver nanoparticle it was 3, 12, 12.5 lg ml^{-1} (Gomaa, 2019).

Antimicrobial peptides other than bacteriocins have also been tested for its antimicrobial efficiency when conjugated with nanoparticles. One such example is the combination of polymyxin B with silver nanoparticles which showed better activity when in synergy with low haemolytic activity against Gram-negative pathogens. Polymyxin B

conjugated with silver nanoparticles was reported to completely remove the endotoxins from the solution, thereby inhibiting biofilm formation on surgical blades (Baptista *et al.* 2018).

2.13.2. Meat Industry

Although some bacteriocins are active against both spoilage and pathogenic bacteria (Kumariya *et al.*, 2019), most of the bacteriocins are active to a greater extent against Gram-positive bacteria (Pisoschi *et al.*, 2018; Yildirim *et al.*, 2018). Most species from bacteria and archaea can produce at least one bacteriocin (Caulier *et al.*, 2019)

On the one hand, they have been traditionally recognized for accomplishing several relevant functions in meat products, such as (i) their contribution to the development of the typical colour and flavour of cured meats, (ii) their bacteriostatic or bactericidal effect against some pathogens such as *Clostridium botulinum* (Majou and Christieans, 2018), (iii) or the inhibition of oxidation processes, which overall result in an extension of the shelf life of meat products (Flores Llovera and Toldrà Vilardell, 2020).

2.13.3 Food Safety and quality are the major concern for food processing industries.

In today's world, people are getting more conscious about food safety parameters. In this regard, bacteriocin plays a major role in ensuring the safety and quality of food products. They are widely used in food preservation, agriculture and pharmaceutical industries. They have also been incorporated into food packaging material due to their both antibacterial and antifungal properties (Sharama *et al.*, 2019). Class II bacteriocins are a class of bacteriocins that are heat-resistant and do not undergo extensive posttranslational modification. In lactic acid bacteria (LAB), class II bacteriocins are widely distributed, and some of them

have been successfully applied as food preservatives or antibiotic alternatives (Zhang *et al.*,2022) .

2.14 Anticancer Properties of bacteriocin

Bacteriocins produced by Gram-negative bacteria, such as microcin) and colicins (A, D, E1, E2, E3) or by Gram-positive bacteria, including nisin (Ahmad *et al.*, 2017) The anticancer effects of the purified bacteriocins were tested against HepG2 cell line. The most promising enterocin (LNS18) showed the highest anticancer effects against HepG2 cells (with 75.24% cellular inhibition percentages), with IC50 value 15.643 μ M and without any significant cytotoxic effects on normal fibroblast cells (BJ ATCC® CRL-2522TM). The mode of anticancer action of enterocin LNS18 against HepG2 cells could be explained by its efficacy to induce cellular ROS, decrease HepG2CD markers and arrest cells inG0 phase (Al-Madboly *et al.*, 2020).The second class of bacteriocins termed non-lantibiotics includes non-modified peptides or those with some minor modifications like end-to-end circularization or disulfide bridge This class is characterized by expanded inhibitory spectrum (Tymoszezewska *et al.*, 2017) Cancer treatment is still one of the biggest challenges in the public health system globally, with a high mortality rate (Jafari *et al.*,2020). Enterocin 12a is a novel bacteriocin that has anticancer properties against human cell lines and negligible activity towards non-malignant cells. Therefore, it should be further evaluated for its anticancer potential in animal models (Sharma *et al.*,2021). Current therapeutic strategies, including surgery, radiotherapy, chemotherapy, or a combination of these treatments, will prolong a patient's life expectancy (Fernandes *et al.*,2017). Bacteriocins are cationic proteins of prokaryotic origin that have emerged as one of the most promising alternative antimicrobial agents with applications as food preservatives and therapeutic agents. Apart from their antimicrobial activities, bacteriocins

are also being explored for their anticancer potential (Sharma *et al.*, 2021). having extensively reviewed the anticancer activity of bacteriocins. It is important to note that the majority of studies relating to the anticancer properties of bacteriocins have been of an *invivo* nature and thus there is a need for *In vivo* validation. The exploration of bacteriocins as a novel anti-cancer therapy allows the opportunity for a better quality of life for cancer patients undergoing treatment (Lancet, 2017).

2-15 Cytotoxicity Against Eukaryotic cells of Bacteriocin

Cytotoxicity is a complex process affecting multiple parameters and pathways. These parameters are cellular organelles that undergo changes in their activity as a result of apoptosis and necrosis induced by drug. Nuclear morphology, cell permeability and mitochondrial function, resulting in the loss of mitochondrial membrane potential and release of cytochrome C from mitochondria are good examples for cytotoxicity parameters (Karanam and Arumugam, 2020). Bacteriocins have various modes of functions, including membrane permeability, cell wall damage, blocking of metabolic pathways, pore formation and reducing the risk of cross-resistance development (Hols *et al.*, 2019; Kumariya *et al.*, 2019; Soltani *et al.*, 2021). Bacteriocins showed minimal cytotoxicity to host cells (Baindara *et al.*, 2018). Bacteriocins combined with other antimicrobial agents or antibiotics with different mechanisms can improve their antibacterial activity and reduce the risk of drug resistance (Soltani *et al.*, 2021). OG716 was a derivative of mutacin1140 and did not show any side effects when taken orally by Golden Syrian hamsters (Pulse *et al.*, 2019). Several bacteriocins, such as nisin, colicin E1, E3, E7, K, enterocin DD14 (Caly *et al.*, 2017) enterocin AS-48 (Abengozar *et al.*, 2017) as well as semi-purified bacteriocins are produced by *Lactococcus lactis subsp. lactis* and *E. durans* (Cerqueira *et al.*, 2017)

showed absence or low levels of cytotoxicity at MIC concentrations against various eukaryotic cell lines. On the other hand, nisin, pediocin PA-1 and colicin E1, E3, E7, K have been shown to be cytotoxic at significantly higher concentrations against Vero cell lines. This was also apparent when a semi-purified bacteriocin produced by *Lactobacillus plantarum* ST8SH was demonstrated to be highly cytotoxic at the concentration of 25 $\mu\text{g mL}^{-1}$ but not at 5 $\mu\text{g mL}^{-1}$ (Favaro and Todorov 2017).

2-16 Nuc gene:

The staphylococcal nuclease is a thermostable nuclease encoded by the *nuc* gene, which hydrolyzes DNA and RNA in host cells, causing tissue destruction and spreading of Staphylococci, also promoting the escape of microorganisms when retained by neutrophil extracellular traps (NETs), allowing the bacteria to evade this host defense mechanism. For decades, the *nuc* gene has been considered the golden standard for *Staphylococcus aureus* identification and is still used presently. Moreover, the *Nuc* encoded staphylococcal thermonuclease is a biofilm inhibitor that degrades the environmental DNA (eDNA) associated with biofilm (Nara Cavalcanti *et al.*, 2021).

The *S. aureus* nuclease (*Nuc*) enzyme. This enzyme was originally identified in 1956 by Cunningham and was named “micrococcal nuclease” (also called “thermonuclease”), and in subsequent years, *Nuc* became a target of intense biochemical study at the enzymatic and structural level. Also, the biological role of *Nuc* was identifying, and it was demonstrated that this enzyme is a virulence factor in pneumonia and invasive murine models of disease (Megan *et al.*, 2014)

Like DNases produced by other Gram-positive species, *Nuc* is required for the evasion of neutrophil extracellular traps (NETs). *Nuc* also has a

role in the inhibition of biofilm formation through the cleavage of extracellular DNA (eDNA) and prevention of biofilm initiation (Kiedrowski *et al.*,2011) Most recently, the broad substrate specificity of *Nuc* has been exploited to track *S. aureus* infections *in vivo* using activatable probes (Hernandez *et al.*,2014).

2-17 *Ica* genes:

The ability to form biofilm is considered as one of the most important virulence factors, that has a role to play in various medical implants related chronic infections. The ability to form a biofilm is considered the most important virulence factor in CoNS foreign material-associated infections. (Becker *et al.*, 2020).

The intracellular adhesion (*ica*) genes are important for encoding of proteins mediating the synthesis of the polysaccharide intercellular adhesin (PIA), The N-acetylglucosamyltransferase encoded by *icaA* gene. The de-acetylation of mature PIA and the trans-membrane protein encoded by *icaB* (polysaccharide deacetylase), the trans-membrane protein encoded by *icaC* (transporter of PIA), Among *ica genes*, *ica A* and *icaD* , have been reported to play a significant role in biofilm formation(Abd *et al.*,2022).

The *icaADBC* genes, encoding PIA play important roles in biofilm formation among *S. aureus* and *S. epidermidis* isolates .Infections caused by isolates producing slime layer are difficult to treat. Many chronic infections due to *S. aureus*, especially through medical devices, are associated with biofilm formation (Ghasemian *et al.*,2015).

The biofilm formation is related to the production of an extracellular mucopolysaccharide, called “slime” composed of a high molecular weight polysaccharide, polysaccharide intercellular adhesin (PIA), which is a positively charged polymer of β -1,6-N-acetylglucosamine residues,

also known as PNAG. The intercellular adhesin (*ica*) operon formed by the *icaA*, *icaB*, *icaC* and *icaD* genes and the regulatory gene, *icaR*, mediates the PNAG production. These genes encode *icaA*, *icaB*, *icaC* and *icaD* proteins involved in the synthesis of this polymer. *icaA* is a trans-membrane glycosyltransferase and can synthesize short PNAG polymers in vitro using GDPN-acetyl-glucosamine as a substrate. *icaD* increases the efficiency of *icaA* biosynthesis. (Atkin *et al.*,2014).

The mechanism by which CoNS attach to prosthetic material and elaborate what is now termed biofilm This is a complex and multistep process. One important element in this process is the *ica* operon, a gene cluster encoding the production of polysaccharide intercellular adhesin (PIA), which mediates the intercellular adherence of bacteria and the accumulation of multilayer biofilm (De Silva *et al.*,2002).

2-18 Cytotoxins or hemolysins (*hla* gene)

The gene encoding *hla* is located in the core genome and is expressed as a water-soluble monomer (33.2 kD) that assembles to form a membrane-bound heptameric b-barrel pore (232.4 kD) on susceptible cells leading to cell death and lysis. The alpha-hemolysin or a-toxin (*hla*), is one of the major virulence determinants implicated in the pathogenesis of *S. aureus*, associated to severe skin and soft tissue infections (SSTI), necrotizing pneumonia and even sepsis. *hla* is the most prominent *S. aureus* cytotoxin that can act against a wide range of host cells including erythrocytes, epithelial cells, endothelial cells, T cells, monocytes and macrophages. (Tavares *et al.*,2014)

Hemolysins of staphylococci are categorized into four different types, including alpha (α), beta (β), gamma (γ), and delta (δ). The α -toxin is encoded by the *hla* and acts as a pore-forming cytotoxin (PFT) which actives against a wide array of human cells. Pathogenicity of this toxin is due to hemolytic, dermonecrotic, and neurotoxic effects. β -toxin which

encoded by the *hly* gene is known as Mg²⁺-dependent sphingomyelinase. (Nasaj *et al.*,2020).

Most species of CoNS including *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* produce hemolysins which are binds with the red blood cells resulting rapid release of internal molecules and lyses them with release of the free iron using by the bacteria (Al-Hilu and Al-Shujairi ,2020).

Few reports have described the presence of cytotoxin-encoding genes and their expression in CoNS (Even *et al.*;2010) Although there are reports of the presence of the *hla* and *hld* genes encoding α - and δ -hemolysin, respectively, in *S.epidermidis* (Okee *et al.*, 2012).

CoNS are capable of producing several toxins and enzymes characteristically associated with *Staphylococcus aureus* such as hemolysins, which are responsible for the invasion of host cells (Nasaj *et al.*,2020).

Chapter Three
Materials
and
Methods

3. Materials

3.1.1 Laboratory Apparatus

The apparatus that used in the study are listed in table 3-1.

Table (3-1): Apparatus used in this study.

No.	Aparatus	Company	Origin
1	Autoclave	Hirayama	Japan
2	Centrifuge	Fisher Scientific	USA
3	Cooling centrifuge	Beckman	England
4	Electronic Sensitive balance	Precisa	Switzerland
5	Electrophoresis System	Mupid	Japan
6	ELISA reader	Biotectk	USA
7	Haemocytometer	Sigma	USA
8	Incubator	Jrad	Jordan
9	Inverted Microscope	Olympus	Japan
10	Laminar Air Flow	Kand K	Korea
11	Magnetic stirrer	Kand K	Korea
12	Micro spin Centrifuge	My Fugene	China
13	Microscope	Nikon	Germany
14	Microwave Oven	Gosonic	China
15	Nano drop 2000	Bio drop	USA
16	Oven	Memmert	Germany
17	pH meter	Hanna	Italy
18	Refrigerator	BEKO	Turkey
19	Spectrophotometer	Aurora instrument Ltd	U.K
20	Thermal Cyclor	BioRad	USA
21	U.V-Visible Spectrophotometer	Shimadzu	Japana
22	VITEK 2 compact	Biomerieux	France
23	Vortex	Stuart Scientific	England
24	Water bath	Hettich	Germany

3.1.2 Biological and Chemicals Materials and Kits

The chemical and biological materials and kits that used in the study listed in table (3-2).

Table (3-2): Biological and Chemicals Materials and Kits used in the study.

No	Materials and kits	Company	Origin
1	Absolute Ethanol	Hemadia	India
2	Agarose	Promega	USA
3	Ammonium sulfate (NH ₄) ₂ .SO ₄	BDH	U.K
4	DEAE-cellulos	Sigma	USA
5	EDTA , Dimethyl , sulfoxide (DMSO), Trypsin, Phosphate Buffer Saline, Sodium bicarbonate,Fetal bovine Serum,RPMI-1640Media	Sigma	USA
6	Ethanol 70%	Merck	Germany
7	Ethidium Bromide Solution (10mg/ml)	Promega	USA
8	Glycerol	Romil	England
9	Gram Stain	Sigma	Germany
10	Hydrogen peroxide (H ₂ O ₂)	Solvochem	U.K
11	McFarland Standards	Biomerieux	France
12	Meat Extract ,Tryptophan, Yeast extract ,Albumin, Gelatin, Glucose, Sucrose, and Esculin	BDH	(England)
13	MTT Kit	Intron Biotech	Korea
14	NaOH, Tris-HCl, HCl, Sodium dodosul sulphat (SDS), acrylamide, Na ₂ CO ₃ , APS, Bis-acrylamide,sodium Potassium tartarate, NaCl. Na ₂ HPO ₄ , NaH ₂ PO ₄ , Bromcresol purple, silica gel, glucose	BDH	U.K
15	Nuclease Free Water	Promega	USA
16	protein standard(Broad range 10- 250 kDa)	Biolabs	England
17	TBE 40X, Quantiflor dsDNA System	Promega	USA

3.1.3 Cultured Media

The culture media which used in the study are shown in the table.

Table (3-3): The culture media used in the study

No.	Medium	Company	Origin
1	Blood agar base ,Agar	Oxoid	England
2	Brian heart infustion broth (BHI)	HiMedia	India
3	DNase agar	HiMedia	India
4	Mannitol salt base, agar	HiMedia	India
5	Muller-Hinton agar (MHA), Muller-Hinton Broth (MHB)	HiMedia	India
6	Nutrient broth (NB)	HiMedia	India
7	RPMI	Prepared in Lab.	

3.1.4 Molecular Score Resources Commercial Kits:

The commercial kits were used in the study are showed in table(3-4).

Table (3-4): List of Molecular Materials Used in Study

No.	Element	Company	Orgin
A	DNA ladder 100bp-1500bp	Promega	USA
B	DNA ladder 50bp-1500bp	SMO Bio Techology	Taiwan
C	FavorPrep Blood/ Cultured Cells Genomic DNA Extraction Mini Kit	Favorgen	Taiwan
D	PCR preMix 20 µl reaction	Promega	USA

Tabe (3-5): Genomic DNA Extraction Mini Kit FavorPrep (Fivogen)from Blood/ Cultured Cells

1	RBC Lysis Buffer (Lysozyme)
2	FATG Buffer
3	FABG Buffer
4	W 1 Buffer
5	Wash Buffer * (concentrate)
6	Elution Buffer
7	FABG Mini Column
8	Collection Tube

Table (3-6): PCR preMix 20 µl reaction (Promega)

Components	20 µl reaction
Each: dNTPs (dATP, dGTP, dCTP, dTTP)	250µM
Kcl	30 mM
Mgcl2	2 mM
Top DNA polymerase (Taq)	1 U
Tris-Hcl (pH 8.0)	10 mM

Table (3-7): DNA Ladder 100bp (Promega)

Materials	
1-	Ladder consist of 11 double-stranded DNA with size (100-1500bp).
2-	Loading dye has a composition: [15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA].

Table(3-8): Details of Primers Sequencing in Study of *nuc* Gene, *icaADB* Gene and *hla-haem* Gene.

<i>nuc</i>	Primer sequence (5' - 3')	Product Size (bp)	Ref.
Forward	5'- GCTGTTTTAGTGGTAGGCGT -3'	354 bp	Manoharan <i>et al.</i> ,2021
Reverse	5'- CACACATAAGCAAGTGTC CG-3'		
<i>icaADB</i>	Primer sequence (5' - 3')	Product Size (bp)	Ref.
Forward	5'-TTATCAATGCCGCAGTTGTC-3'	546bp	Skiba-Kurek <i>et al.</i> ,2021
Reverse	5'-GTTTAAACGCGAGTGCGCTAT-3'		
Reverse	5'- CATTGTAAGCACCTCAC-3'		
<i>hla_haem</i>	Primer sequence (5' - 3')	Product Size (bp)	Ref.
Forward	5'-TGGGCCATAAACTTCAATCGC-3'	72bp	Pinheiro <i>et al.</i> ,2015
Reverse	5'-ACGCCACCTACATGCAGATTT-3'		

3.1.5 Reagents, Buffers and Solutions:

3.1.5.1 Bradford stain co-reagent (Bradford ,1976)

This reagent is composed of

Commassie Brilliant Blue G-250.....0.1g
Phosphoric acid 85%100 ml
Ethanol 95%.....50 ml

The components were mixed together and kept in a refrigerator at 4°C.

3.1.5.2 Stock solution of bovine serum albumin (Bradford, 1976)

- Stock solution of Bovine serum albumin was obtained by dissolving 0.1gm of it in 100ml of D.W.
- Certain concentrations of Bovine serum albumin were prepared (10 mg/ml ,20 mg/ml, 100 mg/ml).

3.1.6 Cell Lines

3.1.6.1 PC3 Cell Line:

This is hypotriploid human cell line the model chromosome No.62

3.1.6.2 HdFn Cell Line:

Primary dermal fibroplast its isolated from Clinical sample.

3.1.7 Media, Reagents and Solutions and Solvents

3.1.7.1 Media, Reagents and Solutions Used in Tissue Culture Technique:

Media, reagents and solutions used for cell culture were prepared according to Freshney (Ascar *et al.*,2019)

A- Antibiotic solution:**1. Streptomycin (1 g/vial):**

It was prepared by dissolving the vial contents in 5ml of sterile distilled water to prepare a stock solution of $200,000\mu\text{g ml}^{-1}$. The stock was stored at -18°C .

2. Benzyl Penicillin :

It was prepared by dissolving the contents of one vial which has 10^6 IU ml^{-1} . The stock was stored at -18°C .

B. Sodium Bicarbonate Solution:

The solution was prepared by dissolving 4.4g of NaHCO_3 100ml distilled water. The solution was sterilized by autoclaving and kept at 4°C until use.

C. Trypsin Solution:

It was prepared by dissolving 1 g of trypsin powder in 100ml PBS and sterilized by filtration. The solution was dispensed into 10ml aliquots and stored at -20°C .

D. EDTA Solution:

It was prepared by dissolving 1 g of ethylene-diamine-tetra Acetic Acid (EDTA) in 100ml of PBS and sterilized in autoclave for 10 minutes. The solution was dispensed in 10 ml aliquots and stored at 4°C .

E. Trypsin-EDTA Solution:

It was prepared by mixing 20ml of trypsin solution, 10ml EDTA solution and 370ml PBS. The mixture was stored at 4°C

F. Roswell Park Memorial Institute – 1640 Medium (RPMI):

A ready to use package (100ml) RPMI was used throughout this study. The medium was already supplied with 4-(2-hydroxyethyl)-1piperazine-ethane sulfonic acid (HEPES) and L-glutamine as illustrated by manufacturer.

The medium was completed by adding the following ingredients:

Penicillin G	10 ³ IU
Streptomycin	0.001 g
Sodium Bicarbonate	1%
Fetal Bovine Serum	10 %

G. Serum Free Medium:

Serum free medium is RPMI-1460 excluded from fetal calf serum.

3.2 Method

3.2.1 preparing of Media

3.2.1.1 Ready to use media:

The media were prepared according to instructions of the manufacturer. They were brought to boil in water to dissolve all constituents completely then the pH was adjusted to 7.2 and sterilized by autoclaving at 121°C (15Ib/In2) for 15min. They were incubated at 37°C for (18- 24) hr to sterilization.

- Nutrient broth.
- Brain heart infusion broth.
- Mannitol salt Agar

3.2.1.2 Laboratory prepared media:

1- Blood agar:

These media were prepared by adding 5% of fresh human blood(Baghdad Blood Bank) to blood agar base which was sterilized by autoclaving and cooled to (45- 50)°C added . These media were used for cultivation of bacteria and testing the ability of bacteria to hemolysis the blood and the type of hemolysis (Forbes *et al.*, 2007).

2- Modified of Brain Heart Infusion Broth (BHIB)

The brain heart infusion were modified by adding each of (Meat Extract, Tryptophan, Yeast extract, Albumin, Gelatin, Glucose, Sucrose, and Esculin) separately with 1% concentration that sterilized by filtration, and incubation at 37°C for 72hr, in pH 7. After incubation the activity estimated by UV. Spectrophotometer and by disc diffusion method then selection the glucose and yeast extract because performed the best activity the other component. modify method to (Melanie *et al.*, 2016; Namasivayam *et al.*,2014)

3.2.2 Solutions and Buffers:

1- Phosphate Buffer Saline PBS (pH=7.2). The solution was prepared by two methods:

A. Dissolved phosphate buffer saline tablet in 100 ml of D.W the pH was adjusted to 7.2 using pH meter then sterilized by autoclave according to the manufacturer instructions and kept at 4°C (Bradford ,1976).

B: Dissolved 68.85g of NaCl in 1000 ml of deionised water and adding 15.0381g of Na₂H₂SO₄ dissolves well by using magnetic stirrer hot plate after that adding 2.451g of KH₂PO₄ and adjust the pH to (7.2-7.4)

(if required) with HCl, and then add deionised water to complete the volume to 1 liter sterilize them by autoclave at 121°C (15 lb/inch²) for 15min

2- 1 M NaCl: Dissolved 5.844g of NaCl in 100 ml D.W.to purification bacteriocin.

3- 0.1 M NaCl: Dissolved 0.0584 g of NaCl in 10ml D.W. to extraction

4- 1 N of NaOH: This solution is prepared by dissolve 5 g of sodium hydroxide in 250 ml of distilled water.

5- 1 N of HCl: is prepared by adding 10.8ml of HCl to 239.2 ml of D.W. according to the following law:

6- Preparation of kit Solution

- Phosphate buffer solution: Dissolved Phosphate buffer solution tablet in 200 ml D.W.
- Bovin Serum Albumin: Taked 0.3g of Bovin Serum Albumin in 30 ml PBS.

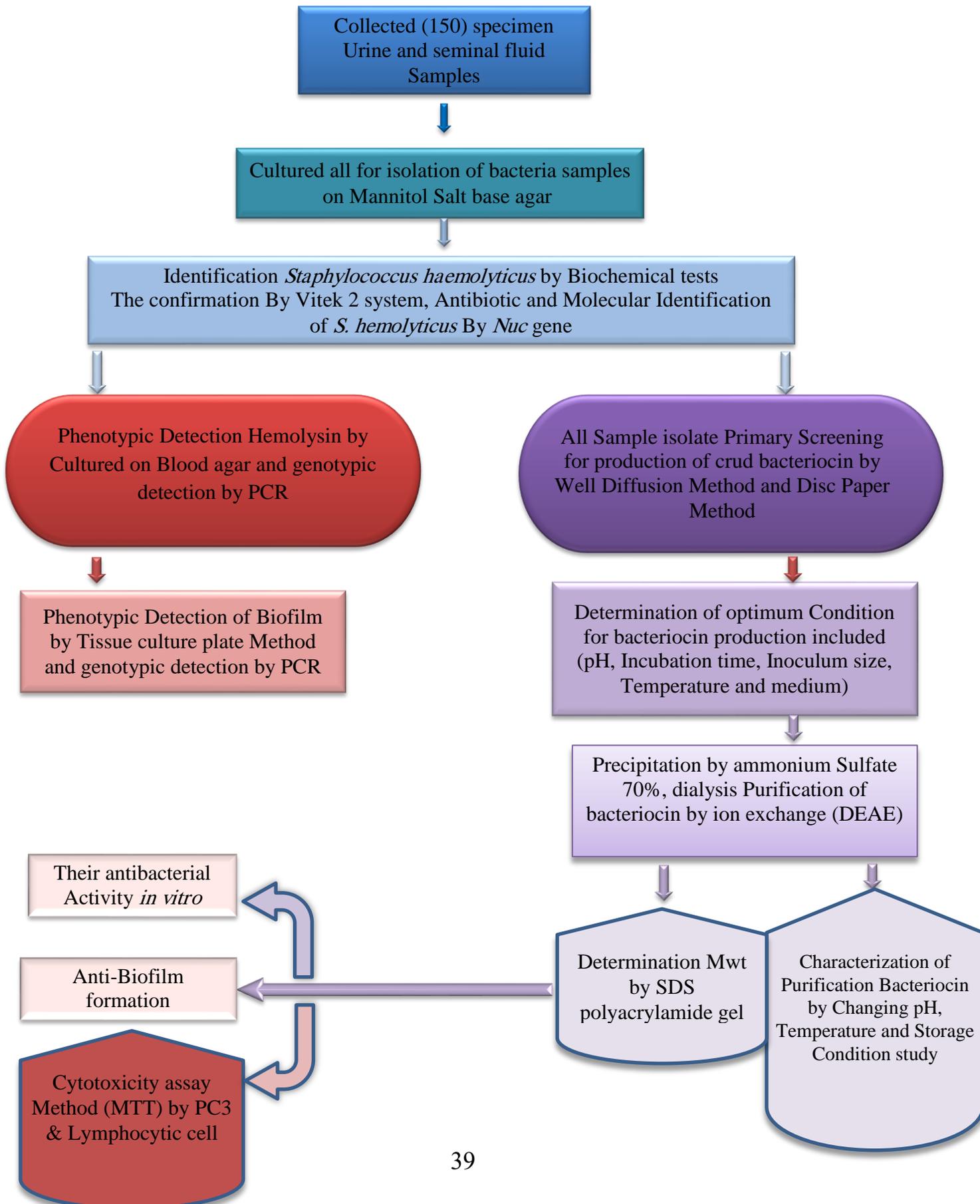
7- Normal Saline Solution: This solution was prepared by dissolving 0.85 g NaCl in 100ml D.W and adjusting the pH to 7.0 then sterilized by autoclaving at 121°C (15 lb/inch²) for 15min after (Benson,2002).

8-Solutions used for Protein Determination (Bradford,1976)

Reparation of protein reagent Coomassie Brilliant Blue G-250(100 mg) was dissolved in 50ml 95% ethanol. To this solution 100ml 85% (w \V) phosphoric acid was added. The resulting solution was diluted to a final volum of 1 liter. Brilliant Blue G-250,4.7%(w\v) ethanol, and 8.5% (w \v) phosphoric acid.

3.2.3 Plan of Study

The following (3-1) Showed Plan of the Study.



3.2.4 Specimen *S. haemolyticus* Isolation and Identification Collection

All sample (150) One hundred and fifty Samples urine and seminal fluid were collected at the period between October to December 2022 in Baghdad.as seventy five Urine from patient Urinary tract infection(U1-U75)and seventy five Seminal fluid from patient Genital tract infection (S1-S75) . infection and cultured on Mannitol salt agar and Blood agar media then incubated for 24-48 hr at 37°C under aerobic conditions, suspected colonies were identified morphologically and biochemically and then Isolates were subsequently confirmed as *S. haemolyticus* by using the Vitek 2 compact system (bioMe`rieux France), according to the manufacturer's instructions.

3.2.5 Biochemical Identification

Bacterial isolates were subjected to biochemical tests. These tests facilitated the identification and characterization of the isolates. The biochemical tests used in this study were as following (Forbes *et al.*, 2007).

Gram Stain

Gram Stain is according to the diagnostic procedures which recommended (Forbes *et al.*, 2007). It was used to study cells morphology and their arrangement, to differentiate between Gram-negative and Gram +positive bacteria.

DNase Agar

The isolates of *S.haemolyticus* were cultured by streaking on DNase agar. The result is positive result on DNase agar by changing the color from blue to pink or rose color white. Weckman and Catlin (1957) suggested that DNase activity could be used to identify pathogenic staphylococci.

Catalase Test

This test was used to detect the ability of tested bacteria to produce catalase enzyme. One drop of catalase 3% was added on slide and then one to two colonies of bacteria were added with drop. The test result is positive if bubbles appear within 15 second (Brown *et al.*, 2014).

Oxidase Test

A piece of filter paper placed in a clean Petri dish and 2-3 drops of freshly prepared Oxidase reagent (1% N,N,N,N-tetramethyle-p-phenylenediamine dihydrochloride) were added to the filter paper. A colony from tested organisms was transferred to the filter paper and rubbed onto the reagent with an applicator stick. Formation of blue purple color within 10-15 second indicates a positive result (Harley and Prescott, 2007).

Coagulase Test

fresh human plasma diluted 1:5 is mixed with an equal volume of (BHIB) culture then incubated at 37°C. A tube of plasma mixed with sterile broth is included as a control. Formation of clots in 1-4 hr. means the test is positive. (Forbes *et al.*, 2007)

3.2.6 Identification of Bacteria by Vitek 2 System

The Procedure was done according to (Bassem *et al.*, 2019):

1. Normal saline (3 ml) were placed in Kahn tube and inoculated with a loopful of isolated colony and mixed by vortex.
2. The Kahn tube was checked for standardization to McFarland standard (1.5×10^8 CFU/ml) by using Dens Check machine (the density must be 0.6 – 0.8) and spectrophotometer 620 nm.
3. In Kahn tube ID card was inserted into the cassette and identification number of a sample entered via barcode.

4. In the filler module the cassette then placed when the card was filled transferred the cassette to the reader and incubator module.
5. The incubation temperature the optical reading of the cards and monitors were controlled by the instruments and data transfer to the computer for analysis. Then the system automatically ejected the cards into a waste container when the test cycle was completed. work the vitek was done in the laboratory of the AL-Nokhba-Baghdad-Iraq.

3.2.7 Preservation and Maintenance of Bacteria

3.2.7.1 Short-Term Preserving

Bacterial isolates were maintained by streaking on a slant of maitiol salt agar for clinical sample. This media was prepared in plain tube containing 3ml of medium. The isolate was streaking on a slant medium and incubated at 37°C under aerobic for 24 hr After that the slants were taken and wrapped with a parafilm and then stored at 4°C(Devay *et al.*,1963).

3.2.7.2 Long Term Storage

1- Preservation by Glycerol

Bacteria were stored for one year in Brain Heart Infusion Broth (BHIB) containing 15% glycerol. This was done by adding 15ml to 85ml of BHIB and sterile in 121° C for 15 min (Ausubel *et al.*, 1992).

2- Preservation by Silica gel

Silica gel sterilized with dry heat for 90 min at 180°C and stored in tightly sealed containers to avoid moisture absorption. Prepare a bacterial cell suspension in sterilized 10% skim milk then, spread enough suspension over the silica so as to wet 0.5 - 0.75 % of the particles. Then stored for 1 week at room temperature after that check for viability by shaking out a few particles on a suitable culture medium if the cultures are viable stored

in a tightly sealed container at 4°C by this method (Dhingra and Sinclair, 1985).

3.2.8 Hemolysin Production:

Hemolysin production bacteria were tested by using 5% blood agar, these isolates were inoculated on blood agar and incubated for 24 hr at 37°C. Hemolysin production was identified by clear zone around the colony which indicate erythrocyte lysis (Forbes *et al.*, 2007).

3.2.9 Biofilm Activity of *S. haemolyticus*

Briefly, 18 hr-old cultures of respective strains were inoculated in a sterile BHIB at a final cell concentration of $\sim 10^8$ CFU/ml. A volume of 180 μ l of brain heart infusion broth contained 1% glucose were added to sterile 96-well polystyrene microtiter plates. A volume of 20 μ l of bacterial suspension was added to three wells of sterile 96-well polystyrene microtiter plates. A total of six wells with bacteria-free brain heart infusion broth were considered as a negative control. Prepared plates were incubated at 37°C for 24hr to allow the setups to form biofilms. After allowing the biofilms to form crystal violet assay was carried out to quantify the biofilms. The assay was carried out by carefully discarding the cultures, followed by washing using 1 \times PBS. The attached biofilms were fixed with 150 μ l of methanol for 15 min, and the excess was discarded. Subsequently, the plates were left to dry for an additional 10 min and stained with 250 μ l of 0.2% (w/v) crystal violet for 15 min. The excess crystal violet was flushed out using distilled water, and plates were left to dry for 30 min. The adhered CV to the biofilms was extracted by 95% ethanol (v/v) and incubated for 15 min before absorbance reading at OD 630 nm (Stepanović *et al.*, 2007) and the results were as follows: $OD \leq OD_c$ = no biofilm producer; $OD_c < OD \leq 2 OD_c$ = weak biofilm producer; $2 OD_c < OD \leq 4 OD_c$ = moderate biofilm

producer and $4 \text{ ODc} < \text{OD} =$ strong biofilm producer. The cut-off value for the negative control is ODc , which is defined as three standard deviations (SD), above the mean OD of the negative control: $\text{ODc} = \text{average OD of negative control} + (3 \times \text{SD of negative control})$.

3.2.10 Detection of Bacteriocins produced by *S. haemolyticus*.

To screen *S. haemolyticus* isolates for their ability to produce bacteriocins primary and secondary screening techniques which done with 2 replicates. All Isolates of *S. haemolyticus* was screened for their inhibitory activity towards a series of indicator strains using by Agar Well Diffusion Assay (AWD) method and filter paper disc (FPD) method to select the higher bacteriocin producing isolate . The indicator strains included gram positive and Gram-negative bacteria were isolated from clinical sample. as the following:

3.2.10.1 Primary screening Agar by Well Diffusion Assay (AWD) Method

The AWD method was used to evaluate the production of bacteriocin of isolates as follows: Tubes contained 10 ml of BHIB were inoculated with McFarland standard (1.5×10^8 CFU/ml) of an overnight culture of the producer isolate. Then tubes were incubated for 24 hr and 37°C under aerobic conditions After incubation the cultured broth were centrifuged at 6000 rpm for 15 min and the cell-free supernatant (CFS) was collected. Then CFS was filtered with $0.22 \mu\text{m}$ Millipore filter paper under sterile conditions. The CFS of each isolate was assayed for the presence of bacteriocin using AWD method as follows: 0.1 ml of an overnight growth 24 hr culture of the indicator bacterium (1.5×10^8 CFU/ml) was spread on surface of MHA in the plate circular well of 5mm in diameter was cut

by using a cork borer after that 100 µl of CFS were put in wells then plate was incubated under O₂ for 24 hr. (Hashim *et al.*,2017)

3.2.10.2 Secondary screening by Filter paper disc method(FPD):

Staphylococcus haemolyticus were inoculated in BHIB broth and incubated aerobically at 37°C for 24-48 hr and then CFS were obtained by centrifuging at 6000 rpm for 15 min. The CFS were neutralized to pH 7 with 1N NaOH and sterilized by filtration through 0.22 µm membranes. In the filter paper disc method sterile filter paper discs measuring 5 mm diameter saturated with 100 µl of CFS were placed on the seeded MHA agar plates (Balouiri *et al.*, 2016) the plates were left in laboratory temperature for 2 hours and then incubated aerobically at 37°C for 18-24 hr. The inhibition zones formed around the paper discs and measured in mm and recorded. According to the results of secondary screening the most efficient isolate in the bacteriocin production of *Staphylococcus haemolyticus*.

3.2.11 Determination of the Optimal Conditions for Bacteriocin Production.

In order to determine the medium and culture conditions that support the maximal production of bacteriocin several optimization experiments were performed by using the bacteriocin production isolate that was selected from the screening section. (Kong *et al.*,2016)

3.2.11.1 Determination of the Optimal production Medium

Four culture media were tested in order to select the one that can support the maximum production of bacteriocin. These media were varied in their contents of carbon and nitrogen sources included: Nutrient broth (NB), Moller Hinton broth (MHB), Brian heart infusion broth (BHIB), modified

BHIB with 1% glucose. Active culture of producer isolate was inoculated 1.5×10^8 CFU/ml into above selective media and incubation was carried at 37°C under aerobic conditions for 24hr then the activity of CFS was measured by FPD method against indicator isolates.(Kong *et al.*,2016).

- **Effect of Carbon(Sugars)Source:**

Different sugars (glucose, sucrose, and Esculin) and added to medium after filtration by millifilter pore(0.22mm) were used, 1% of each one of them was added to Four culture media were tested in order to select the one that can support the maximum production of bacteriocin without sugars, separately. All media were inoculated with 1% of 18-24 hr old culture of the bacterial isolate and incubated at 37°C for 24 hr. Protein concentration and activity of *S.haemyticus* were determined.The optimal concentration of the best carbon source was determined by adding different concentrations of it 1% to broth without glucose separately, then inoculated with 1% of 18-hr old culture of the selected isolate and incubated at 37°C for 18-24 hrmodify method to (Melanine *et al.*, 2016)

- **Effect of Nitrogen Source:**

To determine the best nitrogen source and its concentration, five organic nitrogen sources (Gelatin, Albumin, Tryptophan, Meat extract and yeast extract) with same concentrations were used. These concentrations were added to BHIB without nitrogen sources, separately. All tubes were inoculated with 1% of 18-24hr. Old culture of the bacterial isolate was incubated at 37°C for 18-24 hr. Protein concentration and activity of *S.haemyticus* were determined modify method to (Namasivayam *et al.*,2014).

3.2.11.2 Determination of the Optimal production Temperature

To study the inhibited effect of incubation temperature, the bacterial isolates were grown in optimal medium for production at different incubation temperatures (25,30, 37, 40and 45) °C Producer strain was inoculated (1.5×10^8 CFU/ml) to 10 ml of incubated for 24 hr under aerobic conditions (Kong *et al.*,2016).

3.2.11.3 Determination of the Optimal Incubation period

The effect of incubation period was tested in modified BHIB medium at different incubation time (18,24, 48 and 72) hr. 10 ml of concentration (1.5×10^8 CFU/ml) of selected isolate were incubated at 37°C under aerobic conditions (Kong *et al.*,2016) .

3.2.11.4 Determination of the Optimal production pH

The effect of pH on the growth and on production of bacteriocin of isolates was tested. The BHIB was preparing in 10 ml tubes at different values of pH as in following (5,6,7,8 and 9) with 1 N HCl or 1 N of NaOH after autoclaved. Tubes after that were inoculated with selected isolates and then incubated at 37°C under aerobic conditions for 24 hr After change the culture of pH the bacterial growth and the bacteriocin]roduction were measured. (Barbosa *et al.*,2021).

3.2.11.5 Determination of Optimal Inoculum

The effect of inoculum volume of producer isolate was examined in(9×10^8 CFU/ml), (6×10^8 CFU/ml), (3×10^8 CFU/ml), and (1.5×10^8 CFU/ml) McFarland concentrations under aerobic conditions for 24hr at 37°C.(Kong *et al.*,2016).

3.2.12 Estimation of Protein Concentration

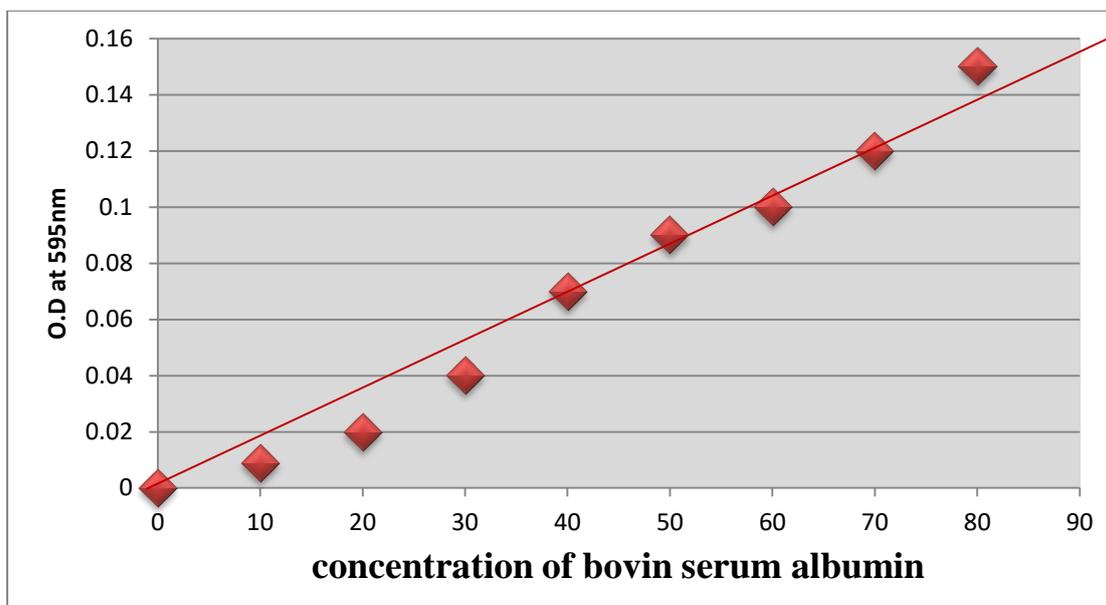
3.2.12.1 Preparation of Standard Curve for bovine serum albumin

- Stock solution of Bovine serum albumin was prepared (3.1.5.2)
- Serial concentrations of Bovine serum albumin (10-100mg/ml) were prepared from the stock solution by dilution with D.W.

Bradford reagent 4.9ml was added to each tube, waiting for ten minutes at a dark condition then measured the O.D at 595nm. The O.D of each concentration was measured by spectrophotometer as 595nm as in table (3-9)

(Table 3-9) Dilution, Concentration and O.D Measurement of Bovine Serum albumin

Number of isolates	Bovine serum albumin solution	D.W. (ml)	Final volume(ml)	Concentration(mg/ml)	O.D at 595-600nm
1	0.0	10	10	0	0.
2	0.1	9.9	10	10	0.009
3	0.2	9.8	10	20	0.02
4	0.3	9.7	10	30	0.04
5	0.4	9.6	10	40	0.07
6	0.5	9.5	10	50	0.09
7	0.6	9.4	10	60	0.1
8	0.7	9.3	10	70	0.12
9	0.8	9.2	10	80	0.15
10	0.9	9.1	10	90	0.16
11	1	9	10	100	0.18



(Figure 3-2) Standard curve of Bovine Serum Albumin

3.2.12.2 Protein Assay of Solution (Bradford,1976)

A volume of 4.9 ml of Bradford reagent (3.1.5.1.) was added to plastic test tubes and 0.1 ml of the Protein solution was added to the reagent and incubated at 30°C for 10 minutes the optical density was measured at 595nm the protein concentration was estimated depending on the standard curve of Bovine Serum Albumin. (Bradford ,1976) Protein concentration (mg/ml) = O.D (595nm)/ (slope* 1000)

3.2.13 Partial Purification of Bacteriocin

Partial purification of bacteriocin was prepared via precipitation with ammonium sulfate at different Concentrations. A CFS of the production isolate was prepared from 500ml of an overnight growth culture by centrifugation at 10000 rpm for 30 min at 4°C. 720 ml of CFS were Precipitation by adding solid ammonium sulfate at saturation level (70) % after neutralized the pH by using 1N NaOH to CFS and stay for 1 hr incubation to confirm as bacteriocin. In the 70 % Concentrations, 209.52 g of ammonium sulfate is added gradually to the CFS and placed in an ice bath with continuous mixing for 1hr at stirrer. After overnight with

continuous mixing at 4° C. The mixture was centrifuged at 10000 rpm for 20 min at 4°C. precipitate obtained was collected and dissolved in phosphate buffer saline (0.1 M, pH 7).The remaining solution was assayed to next Concentrations 70% by adding 221.76 g of ammonium sulfate and also the precipitate obtained was collected and dissolved in 10 ml of buffer and bacteriocin activity was measured by each saturation Concentrations by FPD method. (Cherukuri *et al.*,2019)

3.2.14 Dialysis

Dialysis membrane (SIGMA) of molecular weight cut off (MwCO) 8000-14000 Da of suitable length was boiled in 2% (w/v) sodium bicarbonate (NaHCO₃) and 0.05% EDTA (Ethyle diamine tetra acetic acid) solution for 5 min., cooled and again boiled for 10 minutes in sterile distilled water. One end (bottom end) of dialysis membrane was sealed and through other opening precipitate dissolved in 10 ml of buffer (pH 7) was added and again securely sealed. Membrane was placed in beaker containing 1 L of the same buffer and placed on magnetic stirrer to allow gentle stirring of dialysis membrane. Dialysis was carried at 4°C over night with frequent changes of buffer to remove ammonium sulphate from bacteriocin which may hinder further purification experiments. After completion of dialysis an aliquot was assayed for protein concentration at A₆₀₀. (Cherukuri *et al.*,2019).

3.2.15 Ion-exchange Chromatography (DEAE-Cellulose)

According to Whitaker (1972) this method was prepared by using 20g of DEAE-Cellulose powder and was suspended in 1000 ml DW and left to settle The supernatant was discarded and this step was repeated several times until supernatant became clear. DEAE-Cellulose was activated with

0.25N HCl for 30min then filtrated by Buchner funnel containing Whatman No.1 filter paper and washed with DW twice. After that DEAE Cellulose was activated with 0.25N NaOH. The filtration and washing processes were repeated twice. The activated DEAE-Cellulose was equilibrated with (10 mM Tris buffer pH 7.5) and packaged in a column with dimension (2 × 40) cm. The concentrated proiten from previous steps was added to the ion-exchanger column. Flow rate was organized to be 0.8 ml / min for washed and eluted samples which was collected 5 ml per each fraction. Phosphate buffer (10 mM, pH 7.5) was prepared for washing Lip and the protein was eluted by the same buffer with NaCl (0.15 – 0.75) M. The absorbance for each fraction was measured at 280 nm. Active fractions were collected for measuring protein concentration and specific activity (Duong-Ly and Gabelli,2014).

3.2.16 Tricine–SDS-Polyacrylamide gel electrophoresis

Purified bacteriocin were separated on a one-dimensional tricine–SDS-polyacrylamide gel electrophoresis as described by (Cui *et al.*,2020) with some modification by adding spacer gel. Each tricine–SDS-PAGE gel was prepared with the following composition: the separating gel (16%) consisted of (1.6 ml of glycerol, 2.7 ml of 30% acrylamide, 2.14 ml of 3 M Tris HCl (pH 6.8), 0.06 ml of 10% SDS , 0.06 ml of 10% of Ammonium persulfate (APS) and 0.003 ml of TEMED) was overlaid by the spacer gel (10%) consisted of (1.2 ml of ddH₂O, 0.8 ml of 30% of acrylamide, 1 ml of 3 M Tris HCl, 0.03 ml of 10% SDS, 0.03 ml of 10% APS, 0.003 ml of TEMED) and the stacking gel (4%) consisted of (1.4 ml of ddH₂O, 0.3 ml of 30% acrylamide , 0.3 ml of 1 M Tris HCl, 0.02 ml of 10% SDS, 0.02 ml of 10% APS, 0.002 ml of TEMED) was poured on top of the spacer gel. 20 µl aliquot of bacteriocin sample was mixed

with 20 µl twofold concentrated sample buffer and heated for 5 min at 60 °C. Poly molecular mass standards were included in each gel.

Electrophoresis was run at constant voltage (30 V in the stacking gel and at 150 V during the rest of separation). At the end of electrophoresis, the gel was dyed for 30 min and then put into the decolorizing solution. The decolorizing solution was changed every 1 hr until clear electrophoretic bands appeared. Finally the electrophoretic gel was photographed and analysed.

3.2.17 Characterization of Bacteriocin

3.2.17.1 Thermal Stability of Bacteriocin

In order to test the thermo stability of bacteriocin samples were exposed to different temperatures (40, 60 and 80)°C for 20 min. The residual activity was then determined by protein concentration (Zhou *et al.*,2014).

3.2.17.2 pH Stability of Bacteriocin

Bacteriocin preparation was treated with either 1 N HCl or 1 N NaOH to achieve the desired pH values between (5, 6,7,8 and 9). The pH adjusted pure extracts were then incubated for 30 min. After incubation, aliquots were neutralized and activity was measured by protein concentration modify method to (Naz *et al.*,2015).

3.2.17.3 Effect of Storage Stability of Bacteriocin

The stability of crude bacteriocin over storage at different temperatures for a period of 30 days and three month was analysed by storing at two different temperatures: -20°C, 4°C and respectively. The activity was checked every 10 days for one month by FPD (Dheeraj *et al.*,2012)

3.2.18 Antimicrobial and Spectrum Activity of Bacteriocin *invitro*

Antimicrobial activity of bacteriocin was measured by FPD method on MHA against gram positive such as (*Streptococcus spp*, *Staphylococcus aureus*, *E. faecalis*, *Bacillus subtilis*) and gram negative strain such as (*E. coli*, , *Klebsiella puenmonie*, *Serratia spp*, *P aeruginosa* ,*P mirabilis*, and, *C. freundii*) as indicator strains. The diameter of inhibition zone determined after incubation for 18hr at 37°C. All isolates were taken from the microbiology laboratory in the biology department- College of Science for Women-Babylon University ready and diagnosed using the Vitek 2 compact system

3.2.19 Study the Activity of Bacteriocin as Anti-Biofilm Formation.

Bioflim inhibition assay with the extracted *S.haemolyticus* were performed in pre-coating .Briefly ,in pre coating experiments (modified from , flatbottomed polystyrene 96-well microtiter plates formation.

Individual wells of the sterile microtiter plate were filled with 180 µl of BHIB with 1% glucose and inoculated with of overnight grown culture. To the mixture, 10 µl of bacteriocin was added from the stock so that the final concentration of bacteriocin was achieved between 1.30 and 1.20 µg/ml. The microtiter plates were incubated for 24 hr at 37°C. After incubation, content of each well was gently removed and washed with 0.2 ml of phosphate buffer saline (PBS, pH 7.2) three times, to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate wall were fixed with methanol (95%) and stained with crystal violet dye (0.1 %, w/v). Excess stain was rinsed off by thorough washing with sterilized Millipore water and plates were kept for drying. After drying, 200 µl of 95 % (v/v) ethanol was added to the wells. The

absorbance at 630 nm was measured on an ELISA reader, and values obtained were considered as an index of bacteria adhering to the surface of well wall for developing biofilms. (Barapater *et al.*, 2016) The percentage of biofilm inhibition was calculated using the equation:

$$\% \text{ biofilm inhibition} = \frac{\text{OD of untreated isolate} - \text{OD of treated isolate}}{\text{OD of untreated isolate}} * 100$$

Experiment was performed in triplicate, the data were then averaged, and the standard deviation was calculated. The fungal cell-free filtrate (10 µl).

3.2.20 The Cytotoxic Effect of Bacteriocin Purified from *S.haemolyticus*

This *in vitro* method was performed to investigate the possible cytotoxic effect of different compounds isolated from *S.hemolyticus* pure Bacteriocin extract on tumor cell lines (PC3) and normal cell line (Hdfn).

3.2.20.1 Cell Line Maintenance (Shamsee *et al.*, 2019).

When the cells in the vessel formed confluent monolayer, the following protocol was performed:

- A-** The growth medium was aspirated and the cell sheet washed with PBS.
- B-** Two to three ml trypsin/versine solution was added to the cell. The vessel was turned over to cover the monolayer completely with gentle rocking. The vessel allowed incubation at 37°C for 1 to 2 min, until the cells were detached from the vessel.
- C-** Fresh complete RPMI medium (15-20 ml) was added and cells were dispersed from the wedding surface into growth medium by pipetting.
- D-** Cells were redistributed at required concentration in to culture vessels, flasks or plates whatever needed and incubated at 37°C in 5% CO₂ incubator. Cell concentration was achieved by counting the cells using the haemocytometer and applying the formula:

Total Cell Count/ml: cell count x dilution factor (sample volume) x 10^4

3.2.20.2 MTT Protocol

The cytotoxic effect of Bacteriocin isolated from *S.haemolyticus* performed by using MTT ready to use kit:

A- Kit contents:

- MTT solution 1 ml x 10 vials.
- Solubilization solution 50 ml x 2 bottle.

B- Protocol:

- Tumor cells (1×10^4 – 1×10^6 cells/ml) were grown in 96 flat well micro-titer plates, in a final volume of 200 μ l complete culture medium RPMI-1640 per each well. The microplate was covered by sterilized parafilm and shaken gently.
- The plates were incubated at 37°C, 5% CO₂ for 24hr.
- After incubation, the medium was removed and two fold serial dilutions of the desired compound (12.5, 25, 50, 100, 200 μ g/ml) were added to the wells.
- Triplicates were used per each concentration as well as the controls (cells treated with serum free medium). Plates were incubated at 37°C, 5% CO₂ for selected exposure time (24 hr).
- After exposure, 10 μ l of the MTT solution was added to each well. Plates were further incubated at 37°C, 5% CO₂ for 4 hr.
- The media were carefully removed and 100 μ l of solubilization solution was added per each well for 5 min.

The absorbance was determined by using an ELISA reader at a wavelength of 575 nm. The data of optical density was subjected to statistical analysis in order to calculate the viability rate. (Al-Saffar *et al.*,2017)

3.2.21 Isolation of Human Lymphocytes

Lymphocytes were obtained from the blood of healthy, nonsmoking male donor, who did not exhibit any symptoms of infectious disease at the time of blood sample collection. Following dilution with equal volume phosphate buffer saline (PBS), blood was poured on 3 ml Ficoll-Paque and centrifuged at 2000gm for 20 min. Plasma layer was removed slowly and the layer containing lymphocytes (white color layer or buffy coat) was transferred to a new 15 ml tube. Transferred cells were diluted with 10ml PBS and centrifuged at 1000gm; the supernatant was removed. After the washing step, lymphocytes were suspended in erythrocyte lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA, 183 pH 7.4) and incubated for 5 min at 37 °C. The cells were then washed twice with PBS (Assadian *et al.*, 2017).

3.2.22 Dissolving of Primers

All primer pairs used in this study were dissolved using nuclease free water, Firstly, the primer stock tube is prepared and then the working solution would be prepared from primer stock tube. According to the instruction provided by primer manufacturer (Macrogene-Korea), the nuclease free water was added to get 100 picomole/microliter concentration of primer stock solution. The working solution is prepared from stock by dilution with nuclease free water to get 10 picomole/microliter.

3.2.23 Genomic DNA Extraction from *S. haemolyticus* Isolated:

DNA extraction was performed according to the protocols recommended by manufacturer (Favorgen / Taiwan) as follow:

1. Transferred the appropriate number of bacterial cell (up to 1×10^9) to a 1.5ml microcentrifuge tube and centrifuged at speed 14,000 rpm or 18,000 x g for 1 min. Discard the supernatant.
2. Two hundred microliter (200 μ l) of lysozyme buffer (20 mg/ml lysozyme; 20 mM Tris-HCl; 2 mM EDTA; 1% Triton X-100, pH 8.0; prepare fresh lysozyme buffer immediately prior to use) was added and resuspended the pellet by vortex or pipetting.
3. Incubated for 10 minutes at room temperature. During incubation, invert the tube every 2-3 min.
4. Two-hundred of FABG Buffer was added to the sample mixture. And mixed well by vortexing.
5. Incubated the sample mixture at room temperature for 10 min or until the sample mixture is clear. During incubation, invert the tube every 3 min.
6. Preheated required Elution Buffer in a 70°C water bath.
7. Added 200 μ l of ethanol (96~100%) to the sample and vortex for 10 sec. Pipetted the sample to mix well if there is any precipitate formed.
8. FABG Column was placed to a Collection Tube and transferred the sample mixture carefully to FABG Column. Centrifuged at speed 14,000 rpm x g for 1 min. then Discarded the Collection Tube and place the FABG Column to a new Collection Tube.
9. Added 400 μ l of W1 Buffer to the FABG Column and centrifuged for 30 sec at speed 14,000 rpm x g. then Discarded the flow-through and place the FABG Column back to the Collection Tube.
10. six hundred microliter (600 μ l) of Wash Buffer was added to the FABG Column and centrifuged for 30 sec at speed 14,000 rpm x g. Discarded the flow-through and place the FABG Column back to the Collection Tube.

11. Centrifuged for an additional 3 min. at speed 14,000 rpm x g to dry the column.
12. The dry FABG Column was placed to a new 1.5 ml microcentrifuge tube.
13. One hundred microliter (100 μ l) of Preheated Elution Buffer was added to the membrane center of FABG Column.
14. Incubated the FABG Column at 37°C for 10 min in an incubator.
15. Centrifuged for 1 minute at full speed 14,000 rpm x g to elute the DNA .
16. DNA extracted was Stored at 4°C or -20°C.

3.2.24 The Estimation of DNA Concentration and Purity.

The DNA concentration of samples was estimated by using the Nano drop by putting 2.5 μ l of the extracted DNA in the machine to detect concentration in ng/ μ l and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The accepted 260/280 ration for purifying DNA was between~1.8 (Sambrook and Russell, 2001).

3.2.25 The Reaction Mixture

Amplification of DNA was carried out in a final volume of 25 μ l reaction mixture for detection of genes included study as mentioned in table (3-10)

Table (3-10): Contents of the Reaction Mixture (Promega).

No.	Contents of reaction mixture	Volume	Company
1.	PCR PreMix	12.5µl	promaga
2.	Upstream primer (generic antisense)	1.5 µl	Macrogene
3.	Downstream (sense) primer (G/A)	1.5 µl	Macrogene
4.	DNA template	5 µl	promaga
5.	Nuclease free Water (Promega)	4.5 µl	promaga
6.	Total volume	25 µl	

3.2.26 Thermal Cycling Conditions

The PCR reaction as shown in table (3-11).

Table (3-11): Thermal cycling conditions for *nuc*, *icaADB*, and *hla_haem* genes.

<i>Nuc</i> gene			
Step Type	Temperature	Time	Cycling
Initial Denaturation	94°C	4 min.	1 Cycle
Denaturation	94°C	15 Sec.	30 Cycles
Annealing	58°C	30 Sec.	
Extension	72°C	30 Sec.	
Final Extension	72°C	5 min.	1 Cycle
Hold	4°C	α	1 Cycle
<i>icaADB</i> gene			
Step Type	Temperature	Time	Cycling
Initial Denaturation	94°C	3 min.	1 Cycle
Denaturation	94°C	1 min.	30 Cycles
Annealing	58°C	1 min.	
Extension	72°C	1 min.	
Final Extension	72°C	5 min.	1 Cycle
Hold	4°C	α	1 Cycle
<i>hla_haem</i> gene			
Step Type	Temperature	Time	Cycling
Initial Denaturation	94 °C	4 min.	1 cycle
Denaturation	94 °C	1 min.	35 cycles
Annealing	60 °C	1 min.	
Extension	72 °C	1 min.	
Final Extension	72 °C for	5 min.	1 cycle
Hold	4 °C	∞	1 cycle

3.2.27 Detection of Amplified Products by Agarose Gel Electrophoresis

The amplified products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. Agarose gel was prepared by dissolving 2 gm of agarose powder in 100 ml of 1X TBE buffer (pH:8) in boiling water bath, allowed to cool to 50°C and 0.5mg/ml of ethidium bromide stain at the concentration of (10mg/ml) added to agarose (Sambrook and Russell, 2001). The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. Then the comb was removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 5µl of the amplified product was loaded into the wells in agarose gel, and in one well we put the 5µl of DNA ladder. The electric current was allowed at 100 volt for 45min (Sambrook and Russell, 2001). E-graph gel documentation system was used for the observation of DNA bands and captures the pictures of the gel.

3.2.28 Statistical Analysis:

A one-way analysis of variance ANOVA (Tukey) and Student's *t*-test were performed to test whether group variance was significant or not. Statistical significance was defined as * $p < 0.05$ or ** $p < 0.01$. All samples analyzed statistically were ran in triplicates. Data were expressed as mean \pm standard deviation and statistical significances were carried out using GraphPad Prism version 9 (GraphPad Software Inc., La Jolla, CA). and Chi-square test was used to significant compare between percentage and Least Significant Difference –LSD test was used to significant compare between means in this study.

Chapter Four
Result
and
Discussion

4. Results and Discussion

4.1. Isolation and Identification of *S. haemolyticus*

4.1.1. Isolation of *S. haemolyticus*

In this study, a total of One hundred and fifty (150) urine and seminal fluid samples were collected at the period between October to December 2022 in Baghdad from male patients of various age groups .as seventy-five Urine from patient Urinary tract infection(U1-U75) and seventy-five Seminal fluid from patient Genital tract infection (S1-S75). All samples cultured on Mannitol salt agar and Blood agar media then incubated for 24-48 hr at 37°C under aerobic conditions, one hundred sample give positive result on culture media while fifty (50) samples give negative growth on culture media. The (100) isolates were identified depending on the morphological features (colony size, shape, color, nature of pigments, translucency, edge, elevation, and texture) on culture media and biochemical tests. sixty 60(40%) specimens Isolates were subsequently confirmed as *S. haemolyticus* by using the Vitek 2 compact system (bioMérieux France), and forty (40) isolated were excluded because they were not diagnosed as *S. haemolyticus*

The result of *S. haemolyticus* on Mannitol Salt Agar (29) isolates Non fermenter pink colonies and (31) isolates yellow fermenter colonies.

Colonies on mannitol salt agar were small (smaller than the typical yellow pin head colonies of *S. aureus*) and showed variable reaction on mannitol salt agar. Some strains showed yellow fermenter colonies (Shittu *et al.*,2006) and some other strains showed whitish pink non fermenter colonies (Alahmadi *et al.*,2021), developed bacterial growth on Mannitol salt agar media as a selective and differential medium. As shown in Figure (4-1) and table (4-1).

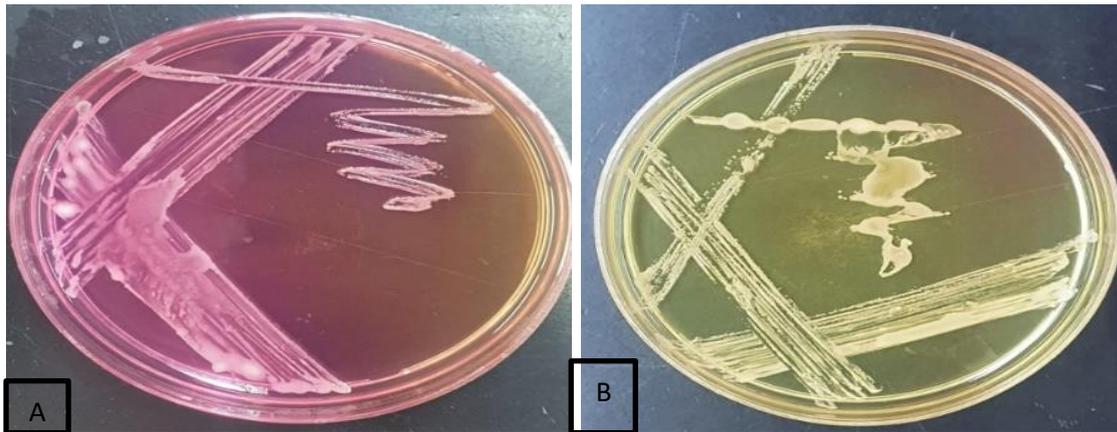


Figure (4-1): *S. haemolyticus* on Mnantio Salt Agar at 37°C for 24 hr.

A) Non fermenter pink colonies and B) yellow fermenter colonies.

Table (4-1): Prevalence of *Staphylococcus* spp and *S. haemolyticus* isolates clinical Samples

Source of Samples	No. of Samples	No (<i>Staphylococcus</i> spp) (%)	No. of <i>S. haemolyticus</i> (%)	Chi-square value
Seminal fluid	75(50%)	22(29.3%)	34(45.3%)	0.042*
Urine	75(50%)	18(24%)	26(34.6%)	0.151
Total	150(100%)	40(26.6%)	60(40%)	0.0005**
Chi-square value		0.460	0.182	

* (P<0.05) ** (P<0.01)

4.1.2. Biochemical Characterization

The results of biochemical test displayed that the isolates were Gram positive bacteria, positive catalase, and Negative to coagulase, oxidase, spore formation and DNase see Appendix(I) and growth carbohydrate such as glucose, sucrose and mannitol as mentioned in (Table 4-2). This results agreed with (Cunha *et al.*, 2004; Paul *et al.*, 2009).

(Table 4-2): Biochemical Tests for *S. haemolyticus*.

Test	Result
Coagulase	-
Catalase	+
Oxidase	-
DNAase	-
Gram stain	+

+Positive, - Negative

4.1.3. Confirmation of Identification *S. haemolyticus* using Vitek 2 System

The System Compact Vitek-2 was used to confirm the diagnosis of bacterial isolates isolated from clinical samples. This system provides 64 biochemical tests needed to diagnose bacterial isolates. Vitek 2 system gives confirmation of positive results for *S. haemolyticus* as a selected organism with a probability (98-99)%. The VITEK-2 system was used according to the manufacturer's instructions; ID-Gram Positive cards (ID-GPC cards; bioMérieux) were used for identification gives confirmation of positive results for all strains with probability (98%-99%) (Ligozzi *et al.*, 2002) See Appendix (II,III).

4.1.4 Antibiotic Sensitivity Testing (AST)

The antibiotic sensitivity test was done for all 60 isolates by Vitek 2-compact system by using card contains 16 different antibiotics See Appendix (IV, V) and the antibiotic resistance to *S.haemolyticus* according to CLSI 2021 as shown in the figure (4-2).

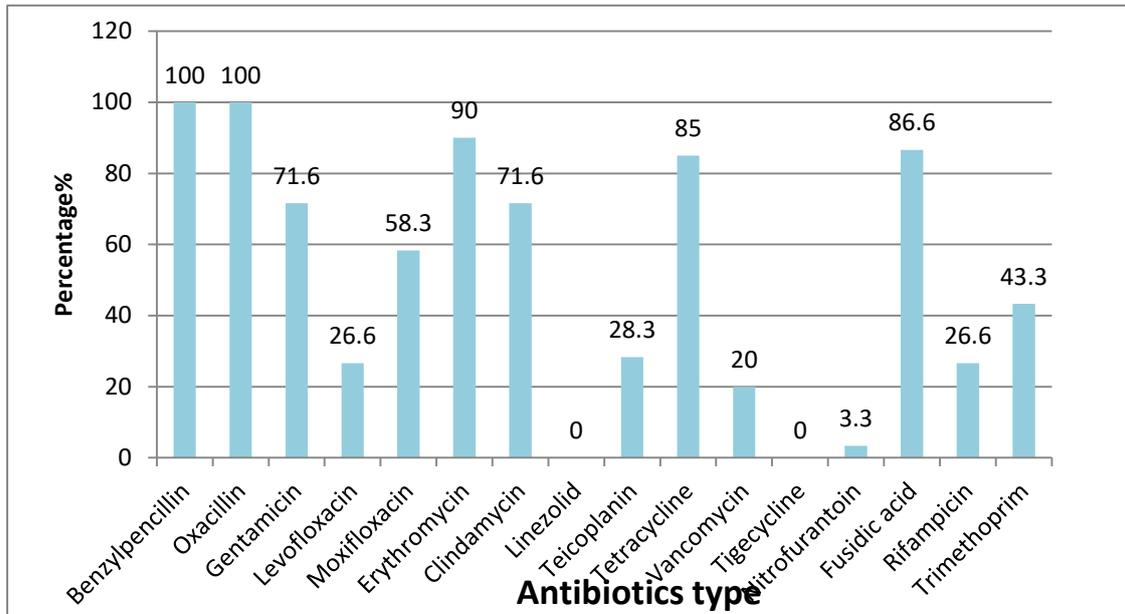


Figure (4-2): *S. haemolyticus* antibiotic resistance pattern towards different antibiotics

The results demonstrated that all the 60 (100%) isolates showed a high level of resistance toward oxacillin and benzylpenicillin, respectively followed by Erythromycin (90%), Fusidic acid (86.6%) and Tetracycline (85%). Resistance to Gentamicin and Clindamycin were present in (71.6%) isolates. A moderate degree of resistance was observed among isolates to Moxifloxacin (58.3%) and Trimethoprim/Sulfamethoxazole (43.3%). A lower degree of resistance was seen to Teicoplanin (28.3%), Levofloxacin and Rifampicin (26.6%) followed by Vancomycin (20%) and Nitrofurantoin (3%). All (100%) isolates showed high level of sensitivity toward Tigecycline and Linezolid.

The results are approximately parallel with a study by (Naqid *et al.*, 2020) who demonstrated that (100%) and (95%) of isolates were resistant to oxacillin and benzylpenicillin, respectively. Another study done by (Al-Naqshbandi *et al.*, 2019) who demonstrated that (82.05%) and (94.87%) of isolates were resistant to oxacillin and benzylpenicillin, respectively.

4.2 Molecular Identification of *Staphylococcus haemolyticus*

Molecular methods based on nucleic acid amplification and hybridization aim to circumvent these problems. In such methods, the pathogen is simultaneously detected and identified, which results in more rapid diagnoses than those obtained by conventional culturing methods and obviates the need for additional culture tests.

Two sets of specific primers were synthesized for this study and these primers were targeting the *nuc* gene as shown already in material and methods. Previously, it has been revealed that rapid diagnosis of *S.haemolyticus* depends on the amplification of the *nuc* gene.

As a result of this study, All isolates of *Staphylococcus haemolyticus* yielded positive results with *nuc* gene -specific primers. As show in figure (4-3).

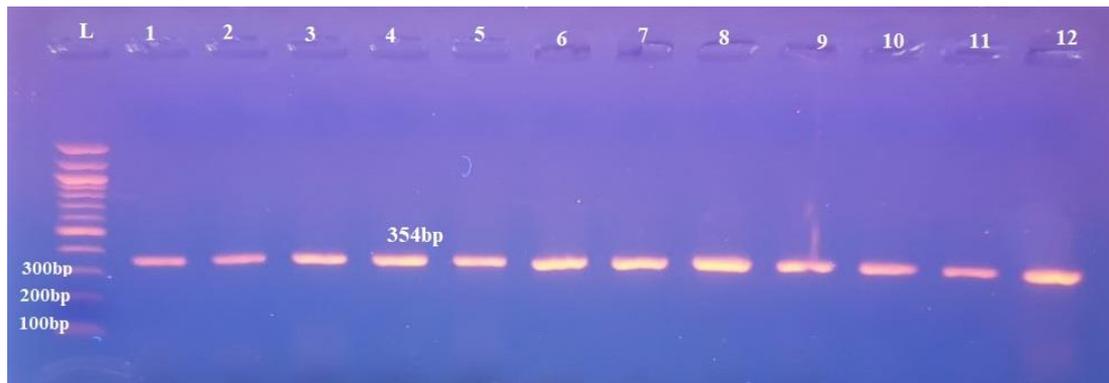


Figure (4-3) Results of the amplification of *nuc* gene of *Staphylococcus haemolyticus* were fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide at 100v/m Amp for 45min. , L: Marker DNA ladder (100bp); Lanes 1-12 resemble PCR Positive products for *nuc* gene amplicon (354bp).

Manoharan *et al.*, (2021) concluded the *nuc*-PCR designed in study was found to be the best method for the identification of *S. haemolyticus* from a group of CoNS and had the highest sensitivity (100%) and specificity (100%).

Aksakal *et al.*, (2022) performed that the diagnosis by PCR with specific gene was thought to be for benefit the community by contributing to the rapid and effective diagnosis and treatment of infections caused by coagulase-positive and negative Staphylococci.

Current result which agreed with the result of another study by Khodabux *et al.*, (2022) they revealed the *nuc* gene was present in all the isolates, confirming the identification as *Staphylococcus haemolyticus*.

4.3. Detection the Ability of Isolates to Hemolyze Blood

4.3.1 Phenotype Detection the Ability of Isolates to Blood Hemolysis

The bacteria isolates (60) were cultured on blood agar to test and determine the ability to hemolyze blood and the type of hemolysis. that showed yellow-gray colonies are (4-3) mm in diameter on the zones of β -hemolysis as shown in figure (4-4)

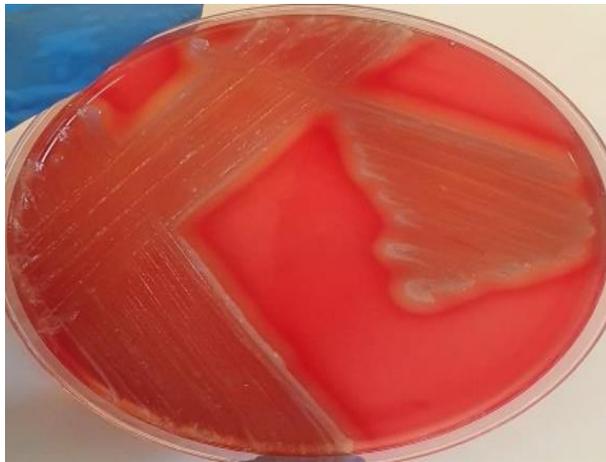


Figure (4-4): *S. haemolyticus* on blood Agar at 37°C for 24 hr. shown yellow colonies with β -hemolysis.

This results agree with study (Hebert and Hancock,1985; Pinheiro *et al.*,2015).

4.3.2 Detection of Hemolysin gene.

The objective of the present study was to characterize the presence of the cytotoxin-encoding genes *hla_haem* using species-specific primers in *S. haemolyticus* Clinical isolates.

The results showed that all isolates were positive for the *hla_haem* gene, whereas the product of PCR detects by using gel electrophoresis as shown in Figures (4-5).

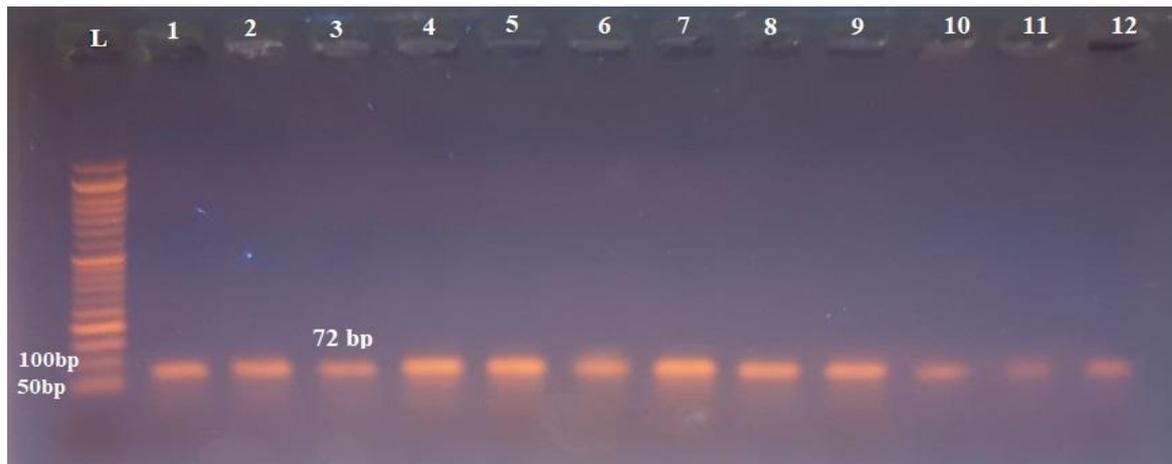


Figure (4-5): Agarose gel electrophoresis of *hla_haem* gene of *S. haemolyticus*, L: Marker DNA ladder (100bp); lane 1-12 positive for *hla_haem* amplicon (72 bp) run on 2 % agarose, TBE 1X, 100 Volt for 45 min, stained with ethidium bromide.

Study by Nasaj *et al.*, (2020) exposed out of (2020) CoNS strains, about 39.5% of *S. haemolyticus* with hemolytic activity.

Also, Pinheiro Showed *S. haemolyticus* revealed a high frequency of *hla* genes in nosocomial isolates of these species. The findings demonstrate an important role of these cytotoxin genes in the establishment of these species and possibly in the development of infections caused by CoNS (Pinheiro *et al.*; 2015).

But, in contrary with the findings of Azih and Enabulele (Akinkunmi and Lamikanra 2010) Among CoNS species, 94.6% of the *S. haemolyticus* were determined to be positive to the *hla* gene., On the other hand the survey carried out by Pinheiro *et al.*, who demonstrated the emergence of the *hla* gene (91.7%) in *S. hemolyticus* strains (Pinheiro *et al.*; 2015).

4.4. Biofilm Formation of *S. haemolyticus*:

4.4.1 Phenotype detection of the biofilm production (micro-titer plate).

Biofilm formation of the MDR *S. haemolyticus* isolates was assessed for its ability to produce biofilms using quantitative method by microtiter plates (MTP). The results demonstrated that isolates of *S. haemolyticus* biofilm production are (n=3) 3(5%) non producer (n=18) 18(30 %) isolates produce weak biofilm (n=30) 30(50 %) isolate as moderate biofilm and(n=9) 9(15%) isolates are strong biofilm producers as shown in Figure(4-6).and Appendix(VI,VII).

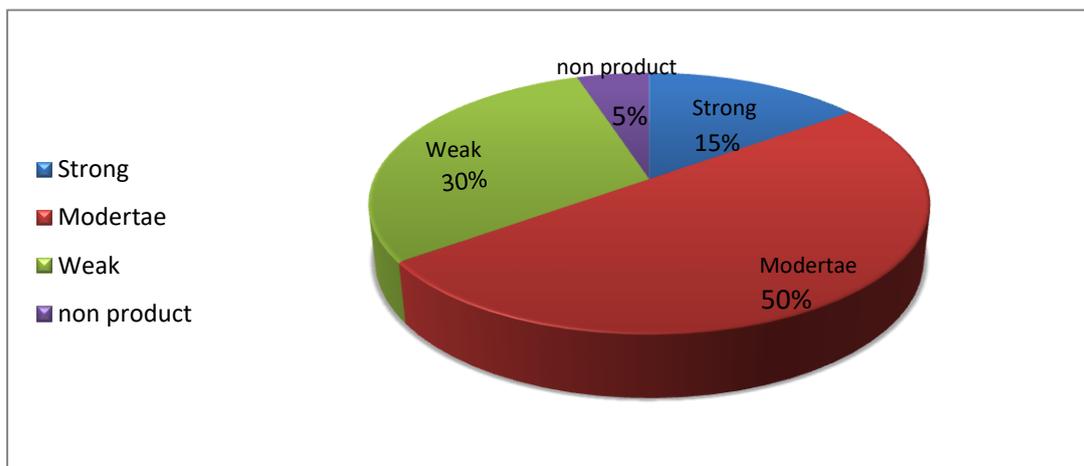


Figure (4-6): Percentage of biofilm values of *S. haemolyticus* isolates by Microtiter plate method.

Almost all *S. haemolyticus* isolates were biofilm producers, these results are compatible with the local study by Al-mousawi and Al-kaabi (2018) which conclude the ability of bacteria for biofilm formation with strong form. Also in line a local study reported by Alhusain *et al.*, (2020) which revealed that all the isolates of *S. haemolyticus* were capable to yield biofilm, one of which displayed a high accumulation of biofilm (O.D 0.7).

This study is also quite similar to a study by Fredheim *et al* (2009). who found that (74%) of the *S. haemolyticus* isolates analyzed could produce biofilms. In contrast another local study by Khudheir *et al.*, (2022) showed that strong biofilm were 10(52.6%), weak biofilm were 2(10.6%) and non-biofilm producer were 7(36.8%). These results disagreed with current study.

Differences in biofilm formation related to the presence of associated genes and even between phenotypic methods may be due to the influence of different culture media, pH, temperature, and osmotic pressure. Possible differences between *in vitro* and *in vivo* biofilm formation that may be related to factors such as stress and molecules released by the host immune system (Pinheiro *et al.*,2016; Rossi *et al.*,2020).

4.4.2 Molecular Detection of Biofilm Formation:

Due to the clinical and therapeutic problem of multidrug resistance of *Staphylococcus haemolyticus* strains isolated from clinical samples, it is necessary to conduct clinical analyses to determine their ability to produce a biofilm which may pose an unusual mechanism of resistance

Molecular detection of the *icaADB* gene, which encodes for biofilm production *S. haemolyticus* isolates was carried out.

The results of the detection of the gene of (546 bp) in size by PCR technology and using the heat cycler showed that 57 / 60 (95%) % isolates possess the *icaADB* gene, as shown in the figure (4-7).

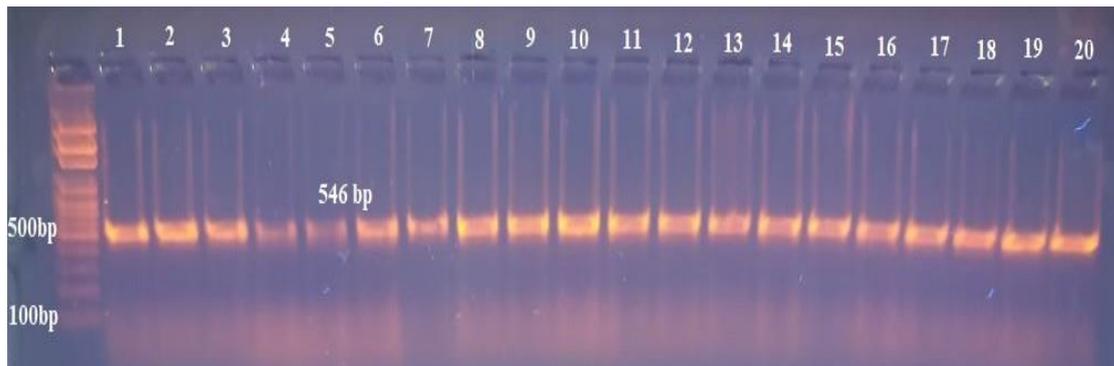


Figure (4-7) : Agarose gel electrophoresis of *icaADB* gene of *Staphylococcus haemolyticus*, L: Marker DNA ladder (100bp); lane 1-20 positive for *icaADB* amplicon (546bp) run on 1.5 % agarose, TBE 1X, 100 volt for 45 min, stained with ethidium bromide.

The resistance of biofilms to toxic substances is likely to be caused by their complex structure, especially by the exopolysaccharide content of the extracellular matrix. EPS form a gel-like substance that acts as a physical barrier against the penetration of antimicrobial agents into bacterial cells (Yin *et al.*, 2019). Biofilms prevent penetration of phagocytes, antibodies, and antibiotics into bacterial cells which increase their resistance to drug and disinfectants. (Shrestha *et al.*,2018).

4.5 Screening for Production of Crude Bacteriocin in *S. haemolyticus*

The bacteriocins produced by these strains showed strong antibacterial activity According to their widest inhibition zone which reached 39and 36 mm on the basic indicator isolates among the *S.haemolyticus* isolates from Seminal fluid(S) and Urine (U)respectively. *S. haemolyticus* was chosen as a good crude bacteriocin producer among all isolates The bacterial productivity of bacteriocin was tested using the filter paper disc method (FPD) and the well diffusion method (AWD).

4-5-1 The Agar Well Diffusion (AWD) method:

These results indicated the secretion of antibacterial compounds into the extracellular environment during bacteria growth, as shown by the clear zone. This method revealed that only 5% less activity of the *S. haemolyticus*. It was observed that *S. haemolyticus* was difficult to be spreaded in the solid medium, and this gives inhibition diameters ranging from (10-13)mm the inhibition zone for each isolation (U and S) respectively. as shown in figure (4-8).



Figure (4-8): Screening of Crud Bacteriocin activity against *S. haemolyticus* on MHA at 37°C for 72 hr. in PH=7 by WDA method, appearance core zone this inhibition zone as clear region.

The present results disagree with Rahimifard and Moghni (2016) who found that the well diffusion assay as the best method to identify the antagonism of microorganisms and other study found the Well diffusion assay as best method comparat with (FPD) (Barman *et al.*, 2018).

4.5.2. The Filter Paper Disc (FPD) Method:

Moddification from conventional methods are required for the secondary screening process.

Consequently, the technique was employed to isolates with bacteriocin production potential alongside bacteriocin activity assessment. This

approach identified approximately 95% of bacteriocin production and screening, proving to be the most effective method. The best the inhibition diameters of both isolates increased to inhibition diameters rang from (36 -39) mm isolate from (U and S)respectively show in figure (4-9)

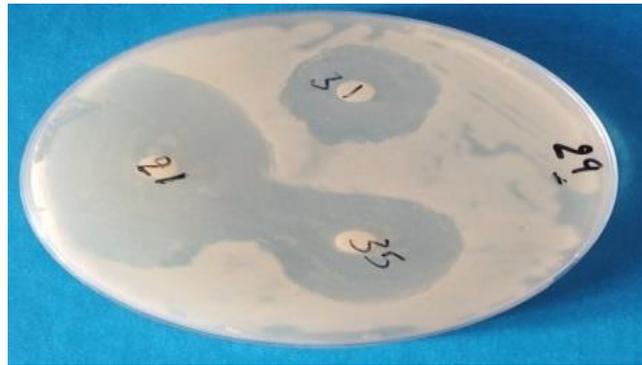


Figure (4-9): Screening of Crud Bacteriocin activity against *S.haemolyticus* on MHA at 37°C for 72 hr. in PH=7 by FPD method. appearance core zone this inhibition zone as clear region.

While other study by Abbasiliasi *et al.*,(2016) showed that the antimicrobial activity disappear under different laboratory conditions and this indicates that existence of the indicator strains in culture may induced bacteriocin production. Crude MRSAcin produced by MRSA S12 were may be active against a wide range of microorganism bacteria and fungi (Ahmed,2018).Recent local study showed the antimicrobial effects crude Salivarcin production *S.salivarius* were may be active against a wide range of microorganism(Mansure,2022).While other study The screening for the production of BLIS was performed with the staphylococci in agar diffusion assays by the spot-on-lawn method indicator bacteria of different genera and species were used. Gram-positive and Gram-negative bacteria of 8 different genera (*Staphylococcus*, *Enterococcus*, *Micrococcus*, *Streptococcus*, *Clostridium*, *Listeria*, *Escherichia*, and *Pseudomonas*) (Fernández-Fernández *et al.*,2022).

4.6. Determination of some Optimum Conditions for Bacteriocin Production

4.6.1. Optimum Production Medium:

The four media were used in this experiment in order to investigate the best medium that can support maximum bacteriocin production. Results showed that the optimum production medium for production of crude bacteriocin was the best medium to support bacteriocin production with an activity in BHIB modified by adding 1% glucose, under aerobic condition at 37°C for 72 hr. with recorded higher inhibition zones reached (39 and 36) mm respectively for both isolations, as shown in Figure (4-10).

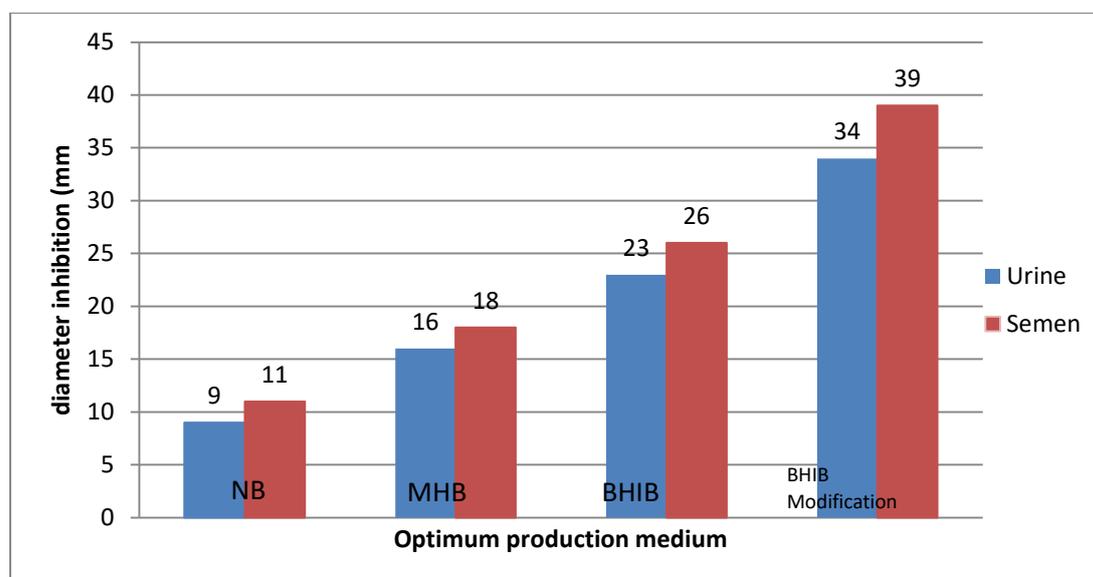


Figure (4-10) The production of bacteriocin by *S.haemolyticus* in different media.at 37°C for 72hr .in pH=7 and 1.5×10^8 CFU/ml .

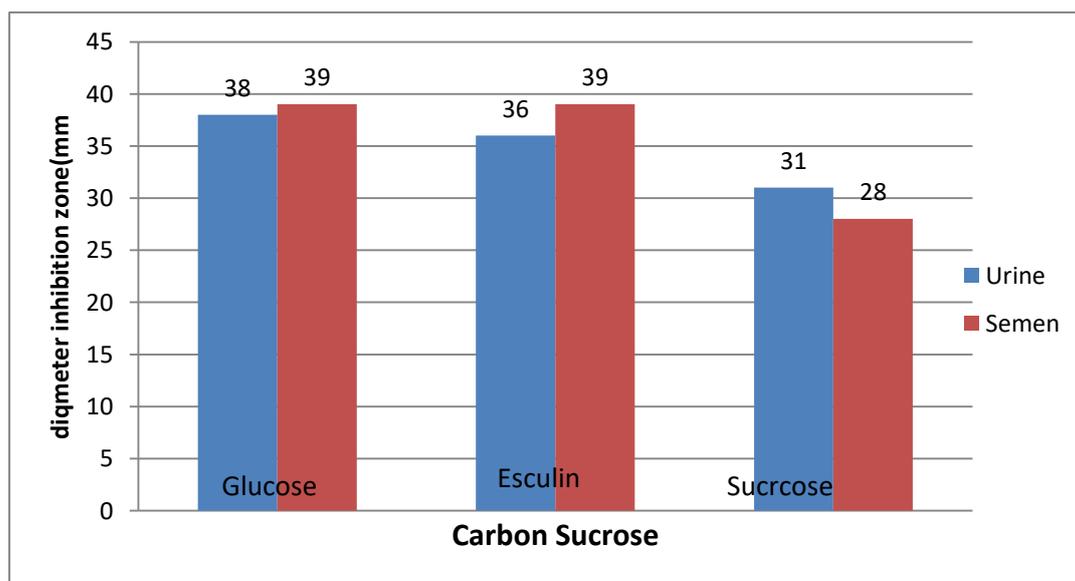
while the lowest Bacteriocin production was in NB medium with inhibition zone of 11 and 9 mm. This result may due to the differences in medium components the BHIB gives the higher activity. Therefore, this medium was used in the next experiments as well as in the preparation.

The present results show an increase in bacteriocin production when glucose is added to the culture medium. Biomass amounts increase when

the initial glucose concentration was between 0.5–2 % during fermentation of *E. faecium* DPC1146 (Rams *et al.*,2013). The other study Tryptic soya broth was selected as the best medium to support bacteriocin production (Saeed *et al.*,2020) While other result (Al- Saeedi and Luti,2018) used the same culture media used for optimization of bacteriocin from *S. salivarius* and show that Tryptic soy broth with 2% yeast extract and 0.1% calcium carbonate was the best medium for production compared THB and BHIB.

- **Optimal Carbon Source:**

Figure (4-11) shows the different carbon sources of sugars related to their consumption by bacteria during metabolic processes growth of *S.haemolyticus* in the presence of 1%glucose that recorded optimum Carbon Source that gave the greatest inhibition zone in S about (39 and 38 mm) for both isolation respectively, compared with other Carbon Source and without any source .Glucose was the best carbon source for both S and U production with highly when compare without carbon source (Sucrose ,Esculin and Glucose), while the minimum effect was seen with Sucrose.



Figure(4-11)Effect of Carbon source on bacteriocin production after added to BHIB media.at 37°C for 72hr.in pH=7and 1.5×10^8 CFU/ml.

Similar results have been reported for carbon such sugar sources on bacteriocin secreted by *Lactococcus lactis* (Mélanie *et al.*, 2016). Based on these results glucose showed the optimal carbon source, On other hand complex media and on wastes have demonstrated by Olfat *et al.*, (2011) that bacteriocin production depends on the medium composition, also reported by (Vamanu and Vamanu, 2010) who found the nutrient sources mainly those of galactose and raffinose that had significant influence on bacteriocin production. Response surface methodology was used to optimize the culture conditions based on various carbon/nitrogen sources in different temperatures (30, 32,35, and 37) °C and incubation periods (Noktehsanj Avval *et al.*,2023).

- **Determination of the Optimal Concentration of Nitrogen Source:**

Evaluation of the nitrogen source on the growth of the different each isolation was done in the preliminary study. Although it was known that bacterial growth in the fermentation medium, were considered superior complex nitrogen sources and were therefore blended in different proportions for use as the final growth medium.

It has shown that the result of optimal concentration of nitrogen source for production of bacteria study by added of each the yeast extracts, meat extract, Gelatin, Tryptopon and Albumin to the BHIB for determine the ability of bacteriocin production. These material were highest inhibition zone compared to carbon source in S best U, but yeast extract is more inhibition zone than other material. yeast extracts gave higher inhibition zone than other material.and that shown in Figure (4-12) and show in table (4-3) also Appendix (VIII).

The result agree with Ribeiro *et al.* (2021) Various blends of the different nitrogen sources were used in formulating the growth media, and these formulations were inspired based .

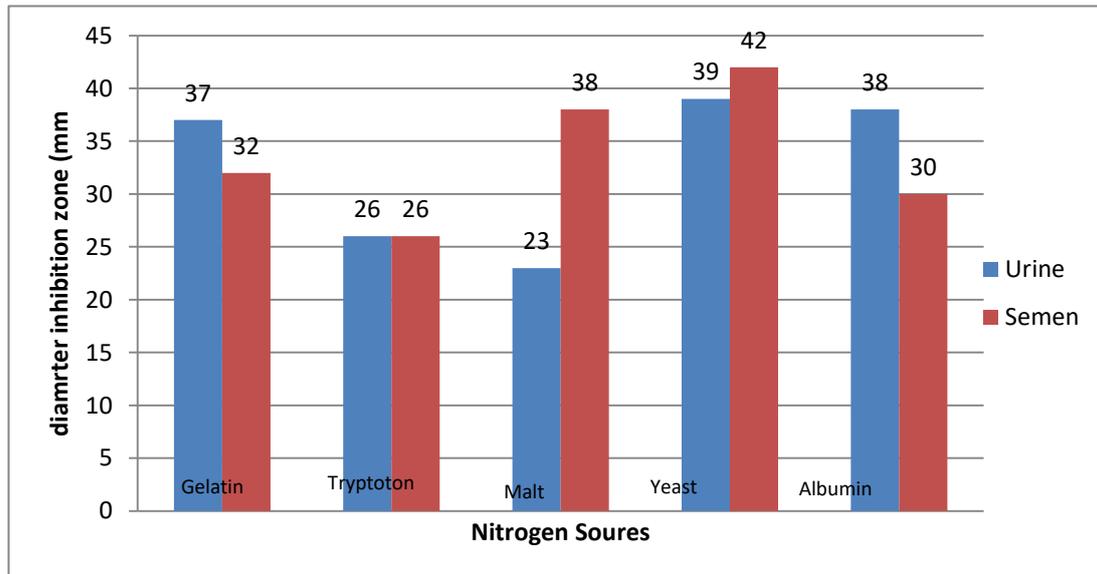


Figure (4-12): Effect of Nitrogen source on bacteriocin production by *S. haemolyticus*, after added to BHIB media.at 37°C for 72hr. in pH=7 and 1.5×10^8 CFU/ml.

The bacteriocin production curve was plotted within 58 hours showing that the maximum production and activity happened at 35°C/48 hr in containing peptone, yeast extract, tween 80 and glucose. (Noktehsanj Avval *et al.*,2023).

Table (4-3) activity of bacteriocin produced by *S. haemolyticus* after added Nitrogen &Carbon Sucrose on MHA at 37°C for 72hr. in pH=7 by FPD method.

Carbon & Nitrogen Source	Mean of Zone of inhibition (mm) isolation from S.	Mean of Zone of inhibition (mm) isolation from U.
Glucose	39	38
Esculin	39	36
Sucrose	28	28
yeast extracts	42	39
meat extract	38	23
Gelatin	32	38
Tryptoton	26	26
Albumin	30	38
	LSD=1.730895 P<0.05	LSD=1.730895 P<0.05

4.6.2. Effect of Incubation Temperature

Temperature also played an important role in bacteria growth, particularly, influenced found that the most important factor influencing the lag phase of isolation was temperature, although its influence on the final cell yield was low. Moreover, the bacteriocins activity of the strains was increased continuously during the exponential growth phase and the highest activity was reached by the end of this phase.

Temperature plays a critical role in bacterial growth as well as bacteriocin production. It was observed from the experiment that maximum production has been achieved at 37 °C with inhibition zones diameters against tested bacteria of 39 and 32mm both strain respectively, The optimum temperature for bacteriocin production was 37°C (Figure 4-13).

Temperature plays an important role in bacteriocin production An increase in temperature led to an increase in the BLIS antimicrobial activity of the LC/PA bacteria, while a reverse interaction was observed for temperature and BLIS antimicrobial activity as there was a reduction in the BLIS antimicrobial activity with increasing temperature. While

bacteriocin product at 45°C give same diameter inhibition zone of 13 mm respectively S. and U. in other study have produce extracellular proteases at optimum temperatures of 25°C to 55°C (Noktehsanj Avval *et al.*,2023). other study while the temperature for *S. epidermidis* Y73 growth were 37°C (Saeed *et al.*,2020)

The variation of at higher and lower temperature reduction in the bacteriocin production referred to slow growth that lead to retardation of bacteriocin production. This result disagrees with (Tulini *et al.*, 2011) who studied bacteriocin produced *E. faecium* and showed that a reduction of the bacteriocin production at 25°C was observed when compared to the control incubated at 37°C. Some of bacteriocin produced by streptococci spp and *E. faecalis* incubated at 37°C such as steptocin produced by *sanguis* (Schlegel,1974).

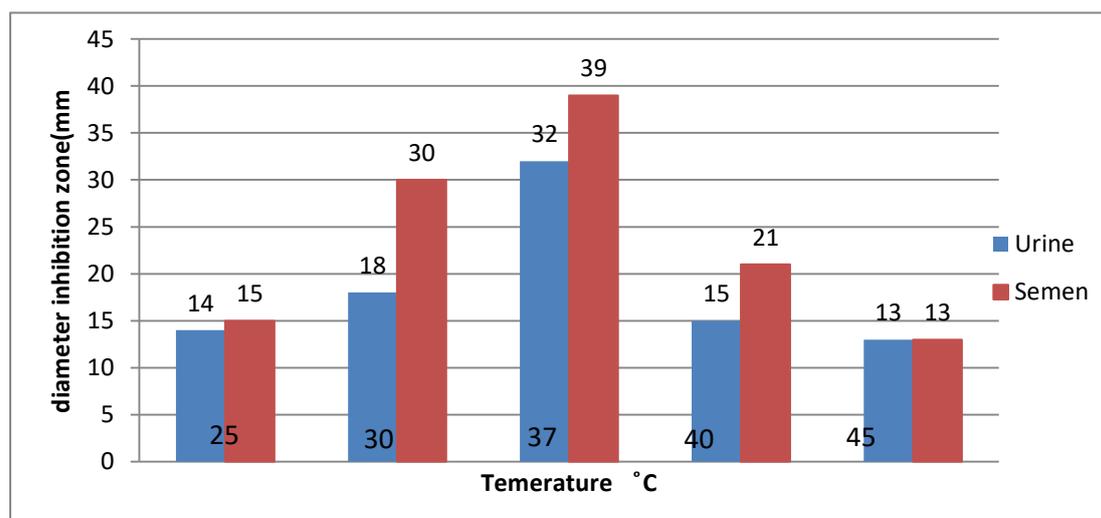


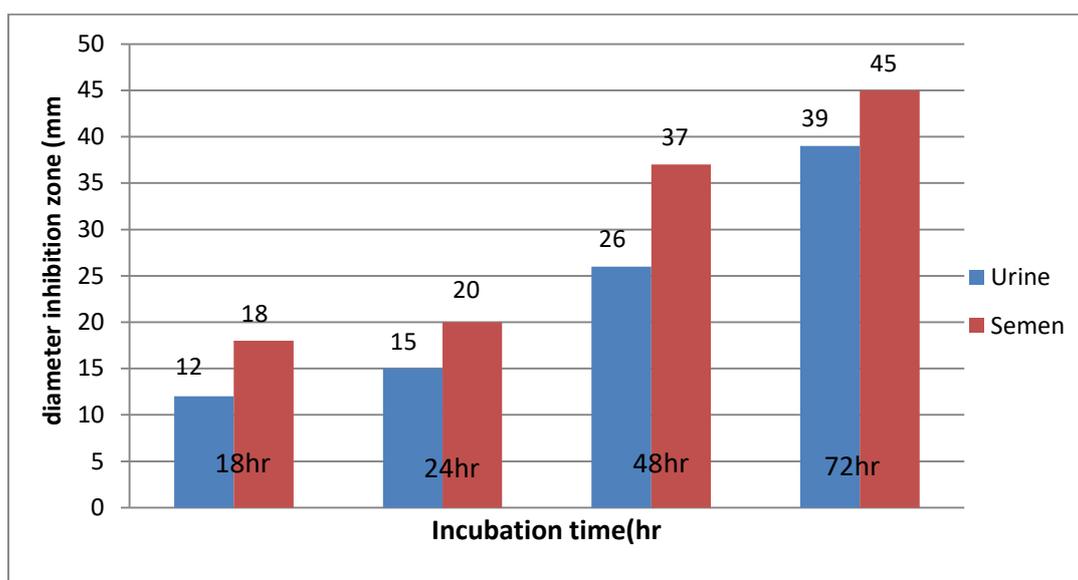
Figure (4-13): Effect of temperature on bacteriocin production. On BHIB media. for 72hr.in pH=7 and 1.5×10^8 CFU/ml.

4.6.3. Effect of Incubation Periods:

The optimum time production of bacteriocin was determined by the incubation period required to produce bacteriocin antimicrobial compounds. The optimum time was based on the protein content of each

72hr sampling point using the Bradford method with a wavelength of 595 nm.

The optimum time production of bacteriocin of incubation time plays a vital role in bacteriocin production. The evolution of the bacteriocin production from the isolate *S. haemolyticus* was followed up at different incubation periods the results Bacteriocin production was evolution at different incubation periods shown maximum production was observed after incubation for 72hr. the highest inhibition zone appeared for seminal isolate 39mm and 45mm for urine isolates, while the inhibition zone about 26mm and 37mm for seminal and urine respectively with 48hr. incubation period (Figure 4-14).



Figure(4-14):Effect of incubation time on bacteriocin production. on BHIB media,at 37°C.in pH=7and 1.5×10^8 CFU/ml.

The activity decreased after 24 and 18 hr and the lowest inhibition zone was recorded to be 15,20,12 and 18mm respectively. This implies that bacteriocin production relies on the cell density. Which is consistent with bacteriocins generated by LB bacteria (Lv *et al.*,2017) as they depends on the number of viable cells at growth's end. The bacteriocin activity

reduction contradicts Phumisantiphong *et al.*, (2017) who found that the highest activity from *E.faeacalis* 48 was after 24hr .

4.6.4 Effect of Medium PH

The optimal pH for *S. haemolyticus* growth were investigated. In fact, a relatively wide range of pH was used to mimic the pH variation in human fluid and to cover all the potential possibilities, since the normal pH value of the stratum corneum is 4.1–7. Agree with (Proksch, 2018). In addition, physiological gaps that include axillae, groin, toe, and anus exhibit pH values between 6.1 and 7.4. Therefore, using such a range is justified by the natural variety of the human pH.

The maximum production of bacteriocin and bacterial growth of *S. haemolyticus* was found at pH values between 6 to 8. The inhibition zones about (30,38 and 21mm) at pH of 6.7 and 8 respectively against tested bacteria from Seminal fluid but inhibition zone with bacteria isolate from urine were(17,21and16mm) at PH of 6,7 and8 respectively. Results showed that at pH 7 was the best value for Bacteriocin production (Figure 4-15). Other study for enterocin the pH 5.5 gives the highest inhibition zone of 15 mm (García-Curiel *et al.*, 2021). The lowest activity recorded at basic pH with no inhibition zone obtain by streptocin bacteriocin production from *S. sanguinis* which was more sensitive to acidity compared alkalinity with enterococci (Tymoszezewska *et al.*,2020). while the optimal pH for *S. epidermidis* Y73 growth were 7 (Saeed *et al.*,2020). According to MD Sidek *et al.*,(2018) the bacterial density of *Enterococcus* and *Streptococcus* obtained from cultured at pH 6 was higher than cultured at pH (8-9). and other study maximum bacteriocin activity was observed at pH 6 and 7 for 30min and disappeared completely at pH 4and 9. (Al- Saeedi and Luti,2018) *Lactococcus lactis*

subsp.lactis. Maximum extracellular bacteriocin production by the isolates takes place in MRS medium (pH 5.0–7.0) at 28°C and it showed strong correlation with bacterial growth (Barman *et al.*,2018).

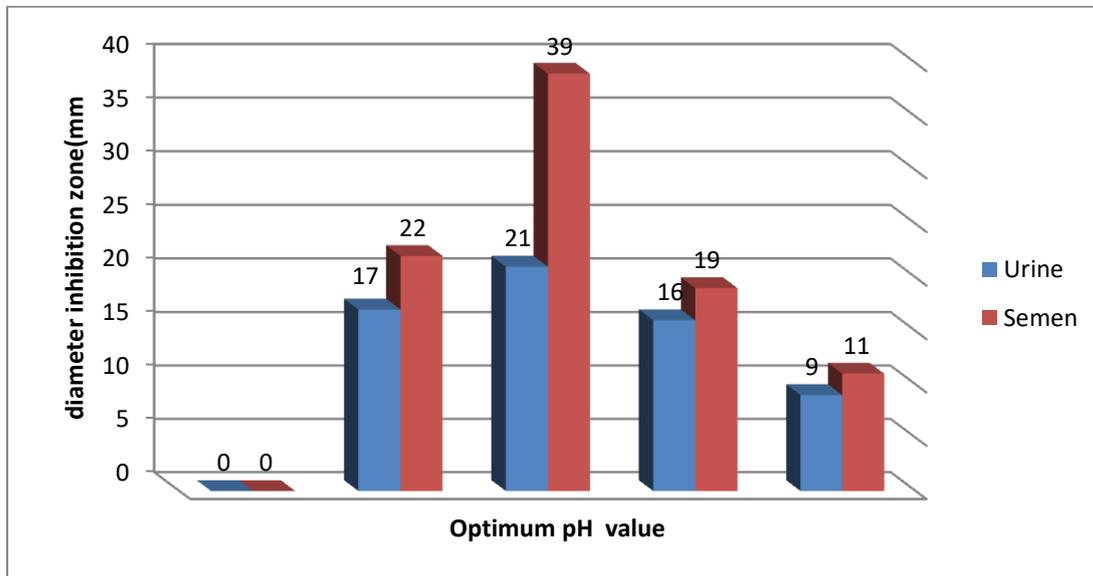


Figure (4-15): Effect of PH on bacteriocin production. on BHIB media, at 37°C for 72hr. and 1.5×10^8 CFU/ml.

4.6.5. Optimum inoculum volume

The current result showed that the optimum inoculum volume was 1.5×10^8 CFU/ml where the greatest zones of inhibition reached 38 and 25 mm for bacteriocin production and the size of inoculum in both strains S. and U. (Figure 4- 16).

There is a gradual increase in the production of bacteriocin with decrease in cells number in the inoculum that added to cultivation media. This agrees with another study results which showed that the bacteriocin production concentration protein the lag and exponential phases of growth (Abbasiliasi *et al.*,2016). Similarly, other study showed maximal activity of *Lactococcus lactis* subsp. with lower concentration of inoculum volume (Wang *et al.*,2019)

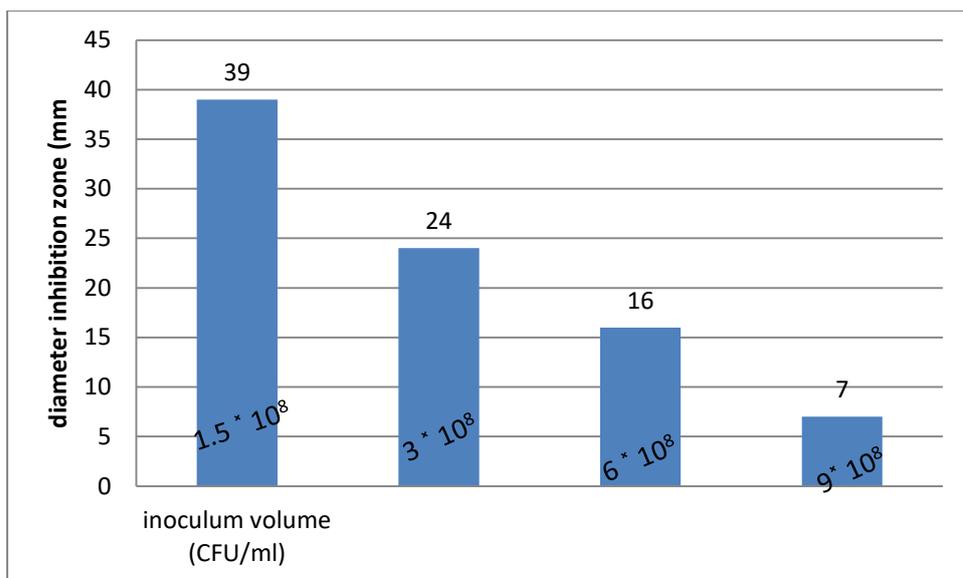


Figure (4-16) Effect of Initial inoculum size on bacteriocin production both S and U sample On BHIB media.at 37°C for 72hr, and in pH=7.

4.7. Ammonium sulfate precipitation and desalting by dialysis:

The partial purification was achieved by ammonium sulfate precipitation where it showed maximum activity with 70% saturation and very less activity was observed with 40%–60% saturation. The purification of bacteriocin is important from the perspective of developing a better understanding of the functioning of the bacteriocin (Mahdi *et al.*, 2017). Purification of Bacteriocin from *S. haemolyticus* was achieved by several steps as follows: Firstly, the crude extracellular Bacteriocin was subjected to ammonium sulfate precipitation with a 70 % saturation ratio. It was found that this ratio gave proteins concentration is increased to reach (1.30 and 1.20) mg/ml for both isolated respectively compared with that of the crude extract (0.99 and 0.89) mg/ml. See (Appendix XI). Salting out using ammonium sulfate is one of the classical methods in

protein biochemistry. Formerly it was widely used for the fractionation of proteins; is rather used as an inexpensive way of concentrating a protein extract such as bacteriocins (Mahdi *et al.*, 2017). This technique is useful to quickly remove large amounts of contaminant proteins; as the first step in many purifications, it is also often employed during the later stages of purification to concentrate protein from dilute solution following procedures such as gel filtration (Mahdi *et al* 2018).

4.8. Purification of Bacteriocin:

Further purification was carried out by using DEAE cellulose column chromatography. It was found that in every step from crude to final active fraction, the protein content was decreased but antimicrobial activity was gradually increased. The specific activity has increased from (1.30 and 1.20) mg/ml respectively. The concentrated bacteriocin was successively subjected to ion exchange chromatography (IEC) on the DEAE-cellulose column. The elution profile with 280nm absorption reveal the maximum bacteriocin activity in the fraction (27,45, and 62) for seminal isolate, while fraction for urine isolate (23,42 and 63) see figure (4-17). The active fractions were pooled and used for further study. The Ion Exchange Chromatography with DEAE cellulose enhanced the proteins concentration of the bacteriocin to 1.30,1.20 mg/ml at zones of inhibition of 43 and 41.5mm isolated from bacteria study seminal fluid and urine respectively (Table 4-4) and figure (4-18) Bacteriocins from many sources were purified successfully by employing DEAE-cellulose resin such as bacteriocin from *S. haemolyticus* activity.

Table (4-4): The Purification steps of Bacteriocin from *S.haemolyticus*

Purification step		Culture filtrate	(NH ₄) ₂ SO ₄ precipitate	DEAE-cellulose
Volume(ml)	S	500	100	60
	U	500	100	60
Zone of inhibition at (mm)	S	39	41	43
	U	38	40	41.5
Protein centration (mg/ml)	S	0.99	1.07	1.30
	U	0.89	0.99	1.20

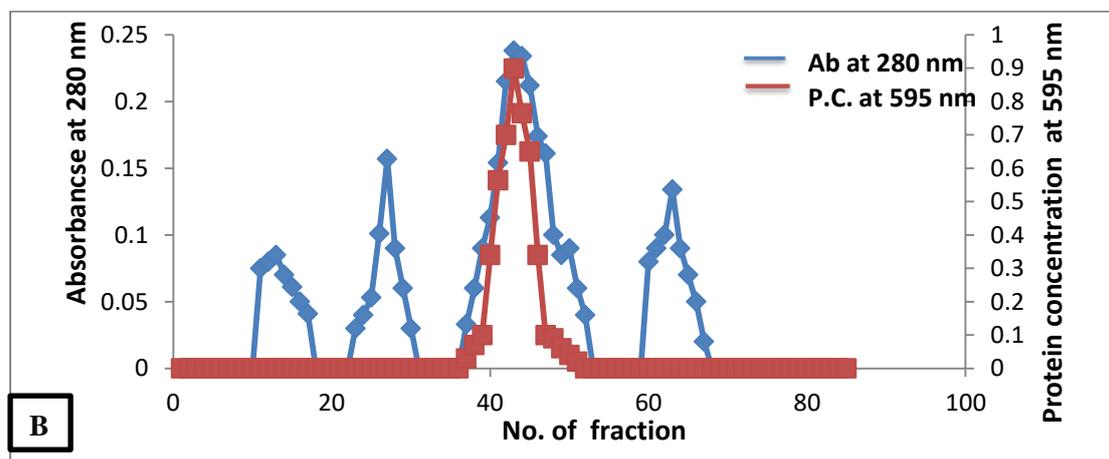
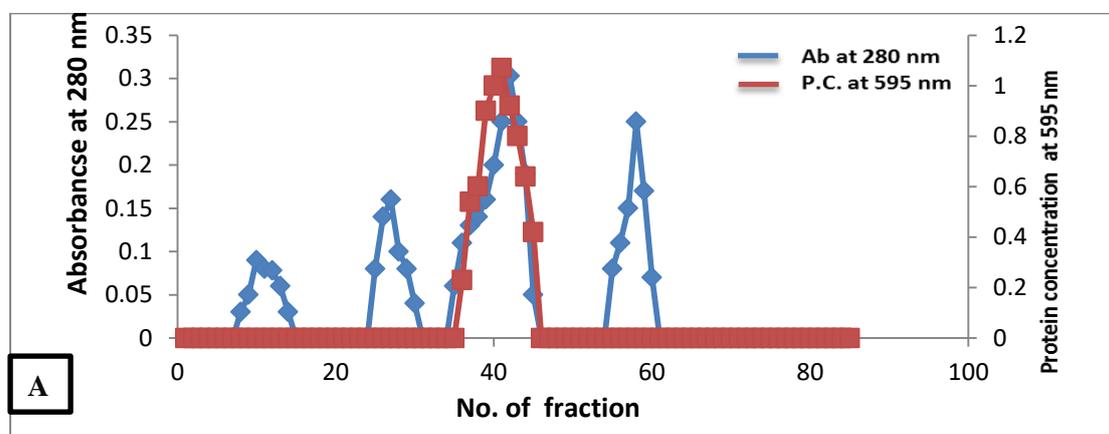


Figure (4-17): Ion exchange chromatography for Bacteriocin using DEAE-cellulose column (2×40cm) equilibrated with Tris-HCl buffer (10 mM , pH 7), eluted with Tris –HCl buffer with NaCl (0.5 -4.7 M) in flow rate 0.8 ml /min., 5ml for each fraction. A) Seminal isolates, B)Urine isolates

Ion exchange (DEAE-Cellulose) features used in this step due to high resolution power, easily prepared, high capacity, possibility of reactivate

and used many times as well as the simplicity of the principle of separation, which mainly depends on net charges of protein (Kateete *et al.*, 2010). The result showed that there is a decrease in the volume of purified bacteriocin when treat DEAE-cellulose when compared with (NH₄)₂SO₄ and culture filtration. The maximum activity was MAR-pyocin served in the fractions was 14 and specific activity for these fractions was 1200AU/mg protein with 1.2 purification folds and 17% yield (Mahdi *et al.*, 2018).

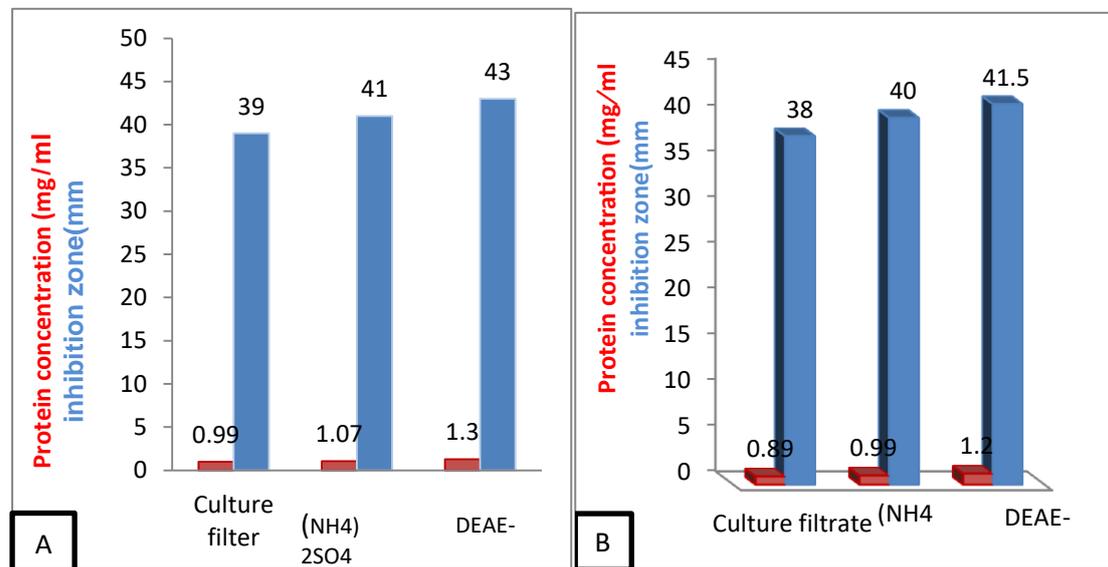


Figure (4-18): The Bacteriocin purification from *S. haemolyticus*.

A) Seminal isolates B) Urine isolates

During the purification procedures each step resulted in a considerable Loss of protein concentration while specific activity increase (Ogunbanwo *et al.*, 2003) a good resolution of different sizes of proteins could be obtained by using this technique. If some criteria follow a volume of matrix to volume of samples, low flow rate appropriate column diameter with high length quality of sample application and absence of any denaturing agents in elution buffer (KP *et al.*, 2016).

4.9. Molecular Mass Determination

The purified Bacteriocin sample collected from different steps of purification were analyzed by Tricine-SDS-PAGE. A single target band appeared (Figure 4-19) indicating that the resultant bacteriocin is a single active peptide in nature. After calculating the gel length and the migration distance of the sample compared with protein standard (Biolabs, England., Broad range 10- 250 kDa) the molecular weight of the *S.haemolyticus* was about 29 kDa.

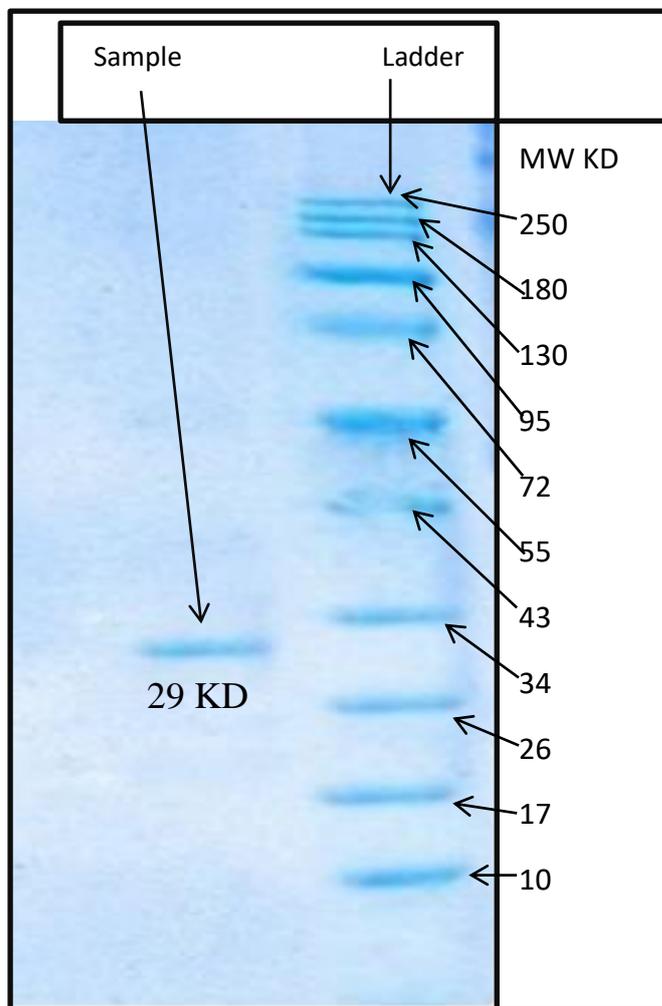


Figure (4-19): Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS PAGE) of the purified bacteriocin with molecular weight 29KDa from *S.haemolyticus* (S23) by stain Commassie Brilliant Blue G-250

The other study reports that the molecular weight of bacteriocin a broad-spectrum, cell membrane-permeabilizing enterocin with a molecular weight of 65kDa was purified and characterized from the culture supernatant of vaginal *Enterococcus faecium*12a (Sharma *et al.*,2021), Staphyloccin 188, the elut size exclusion gel chromatography were subjected SDS-PAGE The molecular weight ranging from 29 to 35KDa. (Abo-Amer,2007). While other study showed that the produced bacteriocin has a molecular weight ranging from 47-54 KDa. Estimation of the concentration and physical and chemical properties of bacteriocin were also investigated (Saeed *et al.*,2020) and other study The molecular mass of bacteriocin PA166 was found to be 49.38 kDa by SDS-PAGE and liquid chromatography–mass spectrometry (MS)/MS (Wang *et al.*,2022)

4.10. Characterization of purified bacteriocin:

Purified bacteriocin was then subjected to various physicochemical analysis like the effect of temperature, pH, and storge on the stability of bacteriocin.

4.10.1 Temperature stability

The results showed the effect of temperature, purified bacteriocin was showed resistant to a wide temperature range, whereas at (40–80)°C, a steady decrease in antimicrobial activity was observed and activity was severely decreased at 100°C, The effect of temperature on the antimicrobial activity of purified bacteriocin (PB) produced from *S. haemolyticus* against test bacteria, showed Pure bacteriocins were thermostability at different temperatures (40, 60 and 80)°C and it remained active without losing its activity after being treated with 20 min. The largest activity of bacteriocin from (S) compared bacteriocin isolated of (U) was remained active in the range (40- 60)°C and decreased at 80°C.

Heat stability of bacteriocin could be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions and stable cross-linkage. Temperatures stability is important if the bacteriocins are to be used as a food preservative because many procedures of food preparation involve a heating step show in Figure (4-20).

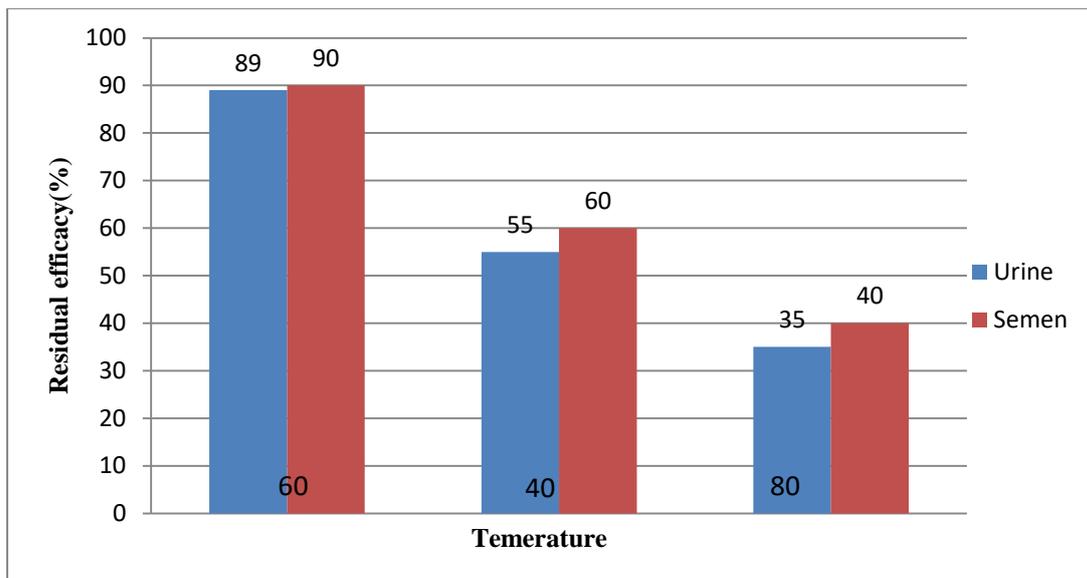


Figure (4-20): Thermal stability effect of bacteriocin at pH=7 on modification BHIB, for 72hr. and 1.5×10^8 CFU/ml.

The good thermal stability of the bacteriocin produced by *E. faecium* SH1. This is in agreement with the bacteriocins from *E. faecium* JCM 5804 T (Park *et al.*, 2003). However, the result disagrees with bacteriocin from *E. faecium* HY11240 that was stable at 100°C but the activity decreased by two fold after 121°C for 20 min (Abanoz and Kunduhoglu, 2018). While bacteriocin produced by *E. faecium* Cl remained stable after heating to 40°C and 60°C for 20 min but the activity of the bacteriocin was reduced after heating to 80°C and 100°C (Goh and Philip, 2015). The result disagrees with Skilton and Tagg (1992) who showed that the streptococin san-K11 produced by *S. sanguinis* was heat stable until at 100°C for 10 min but in current study the activity of

staphylococin was lost at this temperature. These differences may be due to the difference in the period of exposure of the bacteriocin to heat as in the previous study it was 10 min but in the current study 20 min.

4.10.2. pH stability

The purified bacteriocin was found to be stable in a pH range of 4–9, but the activity was severely decreased at pH 4 and 5, while the activity was completely lost at pH 3. Results in Figure (4-21) show that the activity of (PB) was stable at pH values of 7-9. While at the pH values of 5-6 activity was decreased to the half against both of the indicator strain and completely lost at pH of 4.

The results showed that the bacteriocin produced by *S. haemolyticus* had good alkali and acid resistance. This bacteriocin remained relatively stable at pH (5–9) bacteriocins are generally stable at acid or neutral pH indicating that the substances are well adapted to the environment of the bacteria producing them.

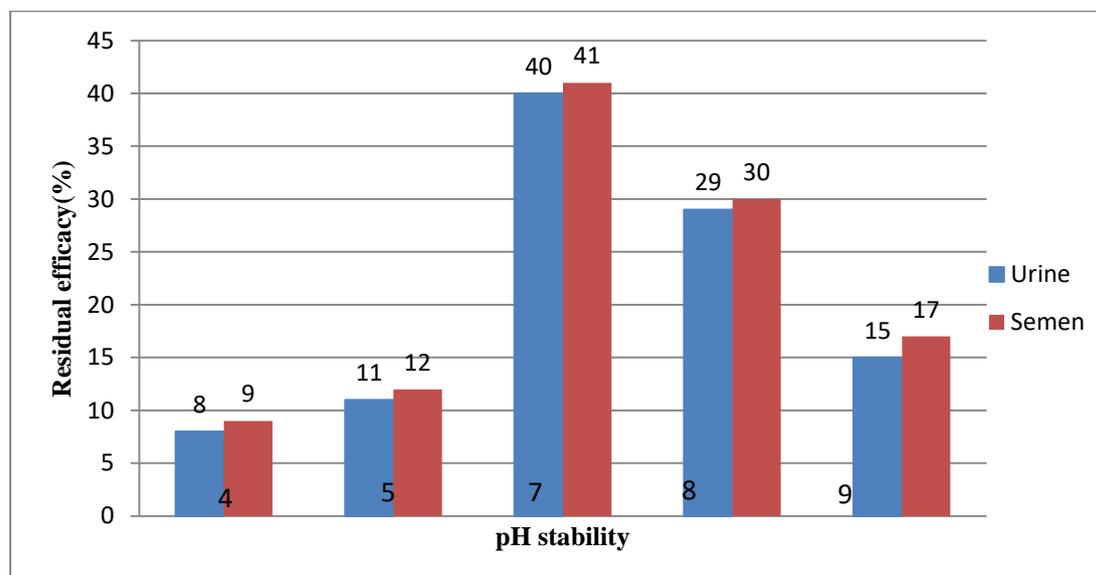


Figure (4-21) pH stability effect of bacteriocin at 37°C on modification BHIB, for 72hr, and 1.5×10^8 CFU/ml.

Cherukuri *et al.*, (2019) reported that bacteriocin produced from *E. faecium* KY11240 lost their activity at pH 2 and 10 but the activity at pH range 5 to 7 was stable and decreased at pH of 3, 4, and 9. However, results by Saelim *et al.*, study (2015) Showed that bacteriocin BacCl produced by *E. faecium* was stable at low pH from 2-6 and activity was lost at pH values more than 6.

4.10.3. Effect of Storage:

Purified *S.haemolyticus* still active during freezing for at least 1 months of storage, with the highest inhibition of zone(30,25) and (27 ,21) mm respectively from seminal fluid and urine at -20°C for three months and -20°C for 30 day respectively, while decreasing zone of inhibition reached (9.5 and 7) mm at 4°C for 30 day. The results as shown in Figure (4-22).

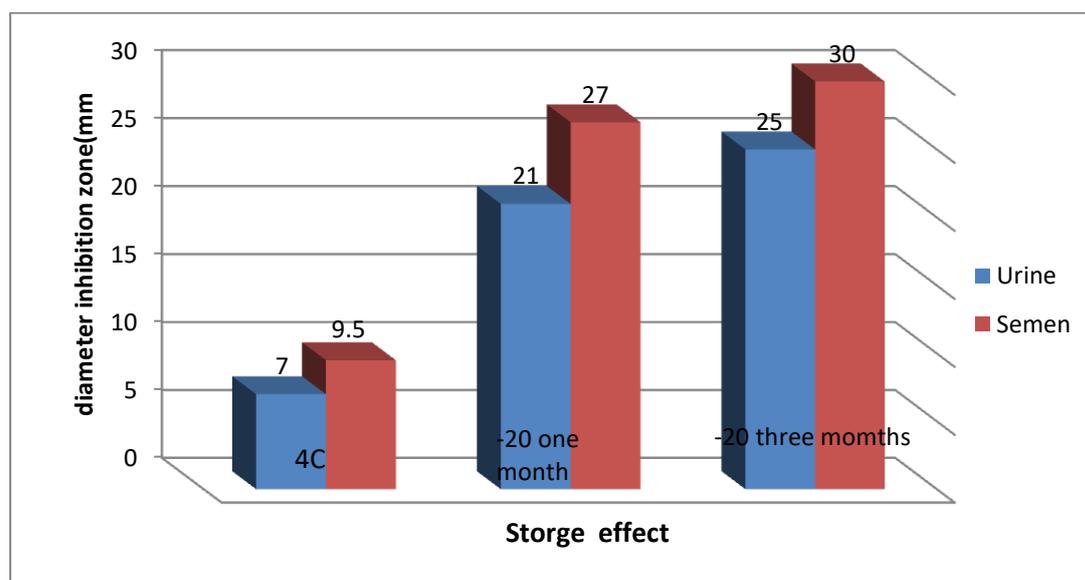


Figure (4-22): Effect of storage of bacteriocin at 37°C, pH=7 on modification BHIB for 72hr and 1.5×10^8 CFU/ml.

Similar results observations have been reported by Dheeraj *et al.*, (2012) whose evaluation of antibacterial activity from *Lactobacillus casei* after different storage periods of (7, 15 and 30) days at 40°C showed that there were no change in activity after 30 days. Further incubation for along

time led to a decrease in the activity. Come in accordance with Corsetti *et al.*, (2004) who found that the bacteriocin activity produced by *Lactobacillus spp.* was stable during freezing for at least 3 months of storage. Storage of the active substance produced by *L. acidophilus* at 40°C for more than 2 months (Deraz, 2015). Finally, the achievement of current results allow to conclude that *S. haemolyticus* bacteriocin has a broad spectrum of antibacterial activity against the examined pathogenic bacteria (*Bacillus subtilis*, *Staph. aureus* and *E. coli* ect.) after storage (-20 and 4)°C for 30 and 60 day.

4.11. Comparison of Antimicrobial Activity between Crude and Purified Bacteriocin by FDA.

The crude and purified bacteriocin whether as alone recorded activity by FPD against different pathogenic bacteria (Gram positive and Gram negative), The highest inhibition zone recorded against gram negative bacteria (*P. aeruginosa*) when tested with bacteriocin reached (20.3 and 34) mm for crude and purified bacteriocin respectively isolate from S and (18.3 and 31) mm for crude and purified bacteriocin respectively isolate from U, while effect against gram positive bacteria (*S. aureus*) reached (17 and 31) mm respectively isolate from S, and (15 and 30.3) mm respectively isolate from Urine.

Also other study obtained by Abanoz and Kunduhoglu, (2018) where the bacteriocin produced by *E. faecalis* KT11 exhibited antimicrobial activity against *S. aureus*, *Bacillus subtilis*, and *Listeria monocytogenes*. These differences may due to difference in the source of producer strain.

The ecological role and evolution of bacteriocinogens are less clear but a synthesis of bacteriocins may have both invasive and defensive functions in microbial communities.

Venigalla *et al.*, (2017) who found that *S. aureus* did not show inhibitory response for purified bacteriocin produced by *Lactobacillus plantarum* JX138220, while larger zones of inhibition by bacteriocin were observed against *P. aeruginosa*. The present results show more activity than obtained by Nguyen and Van Duy (2016) who found that purification bacteriocins extract of the bacterium *Lactobacillus casei* test has inhibitory *Staphylococcus sp* activity which has inhibition diameter between (6-11) mm by WDA. Gillor *et al.*, (2009) who found an initial purified antibacterial from *Brevibacillus laterosporus* against *Staphylococcus aureus* and clinical isolates of methicillin-resistant from all samples that were inhibited measuring in range of (11 – 14 mm). Also Kadhim (2014) found that the two isolates of *P. fluorescens* produced crude bacteriocin with a wide range effect on growth of G+ve bacteria with a range of inhibition zone (10-13) mm by WDA. show in Table (4-5) and Addendixes(X).

Table (4-5): Antimicrobial activity for Crude and purified bacteriocin against different spp bacteria on MHA for 72hr. at 37°C in PH=7.

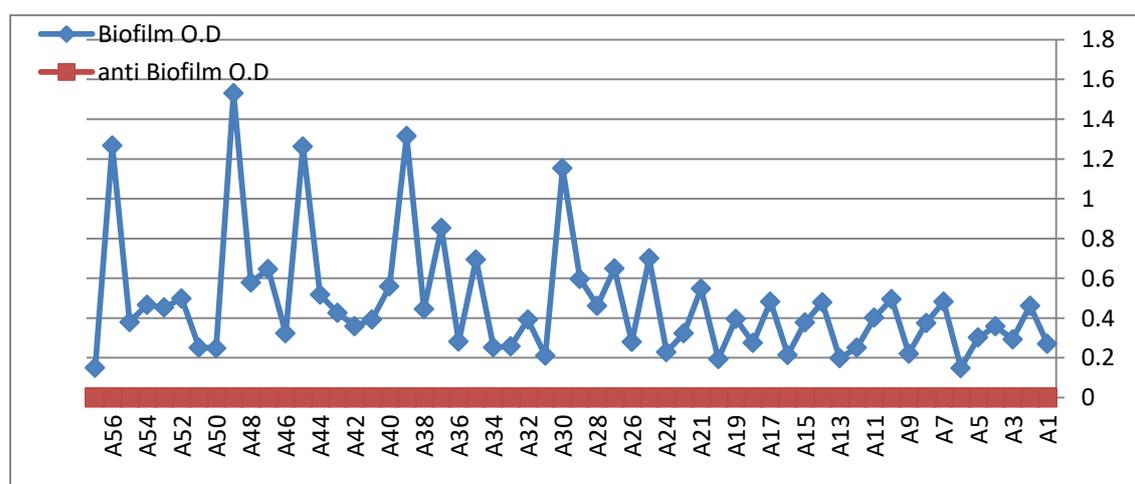
Type of Bacteria		Mean of Zone of inhibition (mm) from Crud Bacteriocin		Mean of Zone of inhibition (mm) from Purified Bacteriocin	
		(S)	(U)	(S)	(U)
Gram Positive Bacteria	<i>Staphylococcus.aureus</i>	17	15	31	30.3
	<i>Streptococcus pneumonia</i>	21.3	16.7	23	20.3
	<i>Bacillus subtilis</i>	2	2	18.3	15.7
	<i>Eenterococcus faecealis</i>	10	10.3	18.7	16
Gram Negative Bacteria	<i>Klebsiella puenmonie</i>	2	1.5	31.7	30
	<i>Citreobacter freundii</i>	20.3	21	21.7	23
	<i>Serratia spp</i>	18.7	17.7	21	22.3
	<i>Proteus mirabilis</i>	12.7	13	21	21
	<i>Pseudomonas aeruginosa</i>	20.3	18.3	34	31
	<i>E.coil</i>	17	14.7	21.7	20.7
		LSD=1.49 1298 P<0.05	LSD=1.39 2032 P<0.05	LSD=2.13 1814 P<0.05	LSD=1. 916868 P<0.05

4.12. Study the Activity of Bacteriocin as Anti Biofilm Formation:

Biofilm play pivotal roles in bacterial drug resistance. Here, the inhibitory effect of bacteriocin on the formation of *S. haemolyticus* biofilms was analyzed by crystal violet staining and absorbance value detection.

This study shows that bacteriocin have strong antibacterial activity against *S. haemolyticus*. In addition, crystal violet analyses and observations demonstrated that bacteriocin inhibited biofilm formation and disrupted mature biofilms of *S. haemolyticus* in a dose-dependent manner. All samples were revealed without ability or inhibition in formation Biofilm with zero(0) absorbance value detection. like in figure (4-23) and appendixes(XI).

This result Agrees with study of the fusaric acid derivatives inhibit the expression of biofilm formation-related effector and virulence genes of *S. haemolyticus*. These findings provide new potential drug candidates for hospital-acquired infections caused by *S. haemolyticus*, the antibiofilm function of *qy17* was achieved by down regulating transcription factors (*sigB*), transpeptidase genes (*srtA*), and bacterial surface proteins (*ebp*, *fbp*) and upregulating biofilm-related genes and the density sensing system (*agrB*) (Wang *et al.*,2022) .



Figure(4:23) Bacteriocin activity against Biofilm production *S. haemolyticus*, the peaks(blue line) reveals product Biofilm while, (red line) reveals no product Biofilm formation.

4.13. Cytotoxicity Test of Purification of Bacteriocin Using Cancer Cell Lines:

However, *invivo* studies need to be performed to confirm these findings and expand on the role of bacteriocin migration across cell barriers. insufficient data on the safety and toxicity of bacteriocins represent a barrier against the more widespread use of bacteriocins Here, it is focused on the most recent trends relating to the application of bacteriocins, their toxicity and impacts.

The MTT assay was applied to determine the cytotoxic activity of the bacteriocin on cell lines assay that was used to determine *in vitro* the cytotoxic effect of Purified of Bacteriocin. Both of cancer cell line (PC3) and normal (HdFn) cells were treated with concentrations of 200-100-50-25-12.5 $\mu\text{g}/\text{mL}^{-1}$ study the inhibitory percentage against the cancer (PC3) and normal (HdFn) cells. The cells viability was decreased with increasing the concentration of the Bacteriocin. The IC₅₀ values of the PC3 and HdFn cells were 61.82-122.5 $\mu\text{g}/\text{ml}$ respectively for seminal samples while 3.763-168.2 $\mu\text{g}/\text{mL}$ for urine sample , respectively, as shown in Figure (4-24) The cells viability was decreased with increasing the concentration of the Bacteriocin. cells viability was increasing the concentration of the bacteriocin and displayed dose-dependent sequence of progressive cytotoxicity beginning at lower concentration of (S and U) to its maximum inhibition (20 and 25) % repectively inhibition of HdFn cells while (45 and 35) % inhibition of UBC-40 cells respectively .

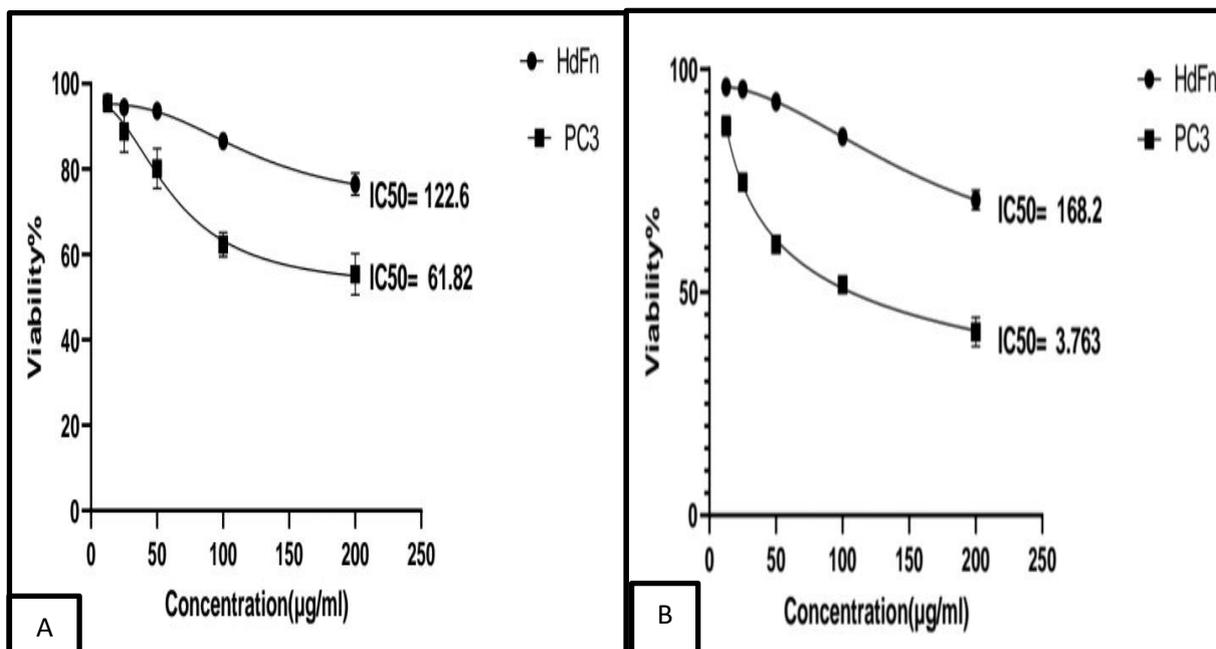


Figure (4-24) : Cell survival curve (mean±SD%) of UBC-40 cells and HdFn after treatment with Bacteriocin A)S B)U. using MTT *in vitro* assay at 37°C, 5% CO₂ for 24 hr. **: $p \leq 0.01$, NS: Non-Significant, SD: Standard Deviation, $n = 3$.

as shown in table (4-6) it can be noticed that the inhibition rate of UBC-40cells was significantly ($p \leq 0.05$) increased by increasing bacteriocin concentration comparing with pattern of normal HdFn cells inhibition, especially at concentrations (12.5,25, 50, 100,and 200) µg/ml The maximum rate of inhibition (76.42 ± 2.6 %) in semen (70.717 ± 2.14) in urine was observed at 200 µg /ml table (4-6), however the IC₅₀ calculated from bacteriocin treatment against UBC-40cells was found to be higher than that calculated for HdFn cells, 122.6 and 168.2 µg mL⁻¹ respectively.

Table (4-6 A): Comparison study between the cytotoxic effect of Bacteriocin on PC3 and HdFn.

Concentration $\mu\text{g mL}^{-1}$	Mean viability (%) \pm SD	
	HdFn	PC3
200	76.42 \pm 2.6	55.3 \pm 4.8
100	86.57 \pm 1.91	62.30 \pm 2.85
50	93.59 \pm 0.96	80.131 \pm 4.6
25	94.4 \pm 0.90	88.812 \pm 4.8
12.5	95.71 \pm 0.23	95.48 \pm 0.61

Table (4-6 B) U: Comparisons between PC3 and HdFn concerning bacteriocin toxicity. (X: Bacteriocin concentration)

Concentration $\mu\text{g mL}^{-1}$	Mean viability (%) \pm SD	
	HdFn	PC3
200	70.717 \pm 2.14	41.165 \pm 3.28
100	84.838 \pm 1.6	51.7 \pm 0.83
50	92.6 \pm 1.79	60.7 \pm 1.37
25	95.56 \pm 0.46	74.73 \pm 1.4
12.5	96.065 \pm 0.20	87.26 \pm 2.16

** $: p \leq 0.01$, NS: Non-Significant, SD: Standard Deviation

Kaur and Kaur (2015) have extensively reviewed the anticancer activity of bacteriocins. It is important to note that the majority of studies relating to the anticancer properties of bacteriocins have been of an *in vitro* nature and, thus, there is a need for *in vivo* validation.

Other result has shown that crude antimicrobial substance of *B. subtilis* exhibited 6% cytotoxicity on Caco-2 cells and in another study

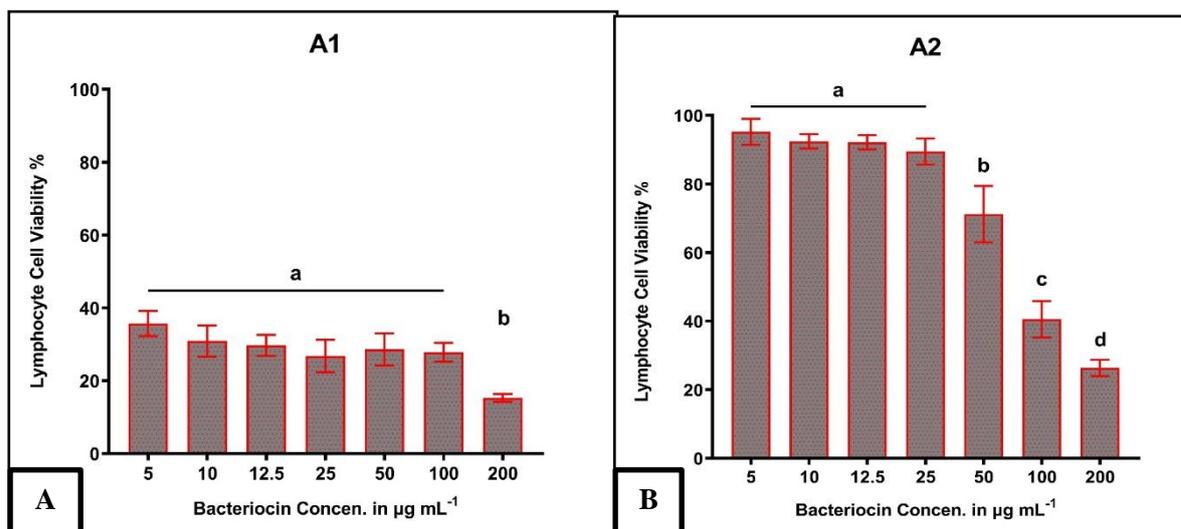
bacteriocin from *Bacillus* sp. showed 48% and 91% cell inhibition on HEK293 and HT29 cells, respectively (Poormontaseri *et al.* , 2017). of these results agree with that of Lavery and Gilmore (2014) where the extracted bacteriocins have been reported for their variable effect to cytotoxic on mammalian cell line. This vaiation in the cytotoic effects could be due to different preparation varying duration of exposure, and many other factors lead to variations in the cytotoxicity but the actual mechanism involved in the difference in toxicity is still not completely elucidated. While Enterocin has also shown to cause an apoptotic response in cancer cells and this leds to strongly believe Pediocin PA-1 interacts with TLRs in order to cause cell death. If this is the case, it would explain the difference in cytotoxicity towards HeLa over HT29 cells. enterocin 12a selectively inhibited the proliferation of various human cancer cell lines in a dose-dependent manner but not that of normal human peripheral blood mononuclear cells. (Sharma *et al.*,2021). On other hand, the IC50 value of commercial nisin was 105 μ M in Vero cell line and nisin A was shown to exert cytotoxicity against epithelial cell lines HT29 and Caco-2 with IC50 values of 89.9 and 115 μ M, respectively. Nisin has been reported to be cytotoxic in Vero, MCF-7 and HepG2 cell lines with IC50 values of 13.48, 105.46 and 112.25 μ M, respectively by (Maher and McClean 2006; Paiva *et al.*,2012).

4.14. The Cytotoxic Effect of Bacterocin on lymphocytes

The results of the cytotoxicity assays revealed that no significant reduction in viability of human lymphocytes treated with Bacterocin .The cytotoxic effect of Bacterocin on lymphocytes viability was evaluated after the cell cultures were exposed to bacterocin for about 24 hr, results of MTT assay showed that bacterocin A1 isolation from (S) had remarkable reduction on viability of human lymphocytes treated with the

bacteriocin exhibited (62 –85) % cell viability concentration between (5-200) $\mu\text{g/ml}$ as shown in Figure (4-25 A) .

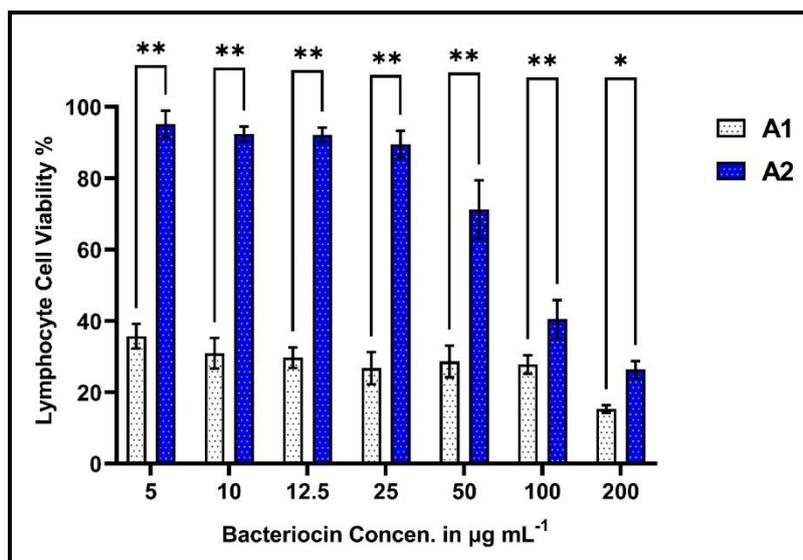
While Bacterocin A2 isolation from (U) at same concentration. It was observed that almost remarkable reduction little compare A1 isolation on viability exhibited range reached (30–70) % cell viability shown in Figure (4-25 B)



** $p < 0.01$, NS: Non- Significant, SD: Standard Deviation.

Figure (4-25): Mean (\pm SD) Cell viability of human lymphocytes after treatment with Bacterocin isolation A1) S A2) U (5, 10, 12.5 , 25, 50,100 and 200 $\mu\text{g mL}^{-1}$) compared with untreated cells: Standard Deviation, ($n = 3$). A) Seminal isolates B) Urine isolates.

The *in vitro* potency of the bacteriocin A1 and A2 is well documented show Where it was observed that there are clear significant differences between A1 andA2 at different concentrations, this may be attributed to the source of the isolation shown Fig (4-26).



** : $p < 0.01$, NS: Non- Significant, SD: Standard Deviation.

Figure (4- 26): (A) Flow Comparison Bacteriocin isolation A1) Seminal isolates A2) Urine isolates after treatment on Cell viability of human lymphocytes and were compared with untreated cells (negative control) for 24 hr. at 37°C ($n = 3$). (B) Statistical Bacteriocin distribution mean percentage (\pm SD); differences for significance

According to (Baños *et al.*,2019) The *in vitro* cytotoxicity assays are indicative of the intrinsic ability of a compound to cause cell death, defining concentration ranges for safe and rational administration. Overall, these results prove that AS-48 is safe against fresh human blood at concentrations >10-fold higher than those needed to inhibit the least sensitive species bacteriocin produced from *E. faecalis* this lytic effect is lower than that shown by the enterocin S37 (74.2% using 10 µg/ml)

Other study these *in vitro* experiments demonstrated that P34 and P40 peptides from *Bacillus* exhibits a broad bactericidal activity in blood with no significant haemolytic effect against human erythrocytes, suggesting high selectivity for bacteria human cells. its pro-inflammatory effects and ability to induce *invivo* lymphocyte proliferation in mice, and absence of lymphocyte proliferation in local lymph nodes after topical exposure to

different concentrations of AS-48(Martín-Escolano *et al.*, 2019). These findings showed that bacterocin purification could be used as a good alternative to the current physical and chemical methods associated with environmental toxicity.

Chapter Five
Conclusion
and
Recommendations

Conclusion and Recommendations

5.1 Conclusion:

- 1-The *staphylococcus haemolyticus* isolation from Urine and Seminal fluid and Almost all *S. haemolyticus* isolates were multidrug resistance.
- 2-Noval Bacteriocin extracted from *staphylococcus haemolyticus* and by Filter paper disc method (FPD) was the best method for screening on bacteriocin production compared with agar well diffusion .
- 3-The best conditions for production of bacteriocins was after culturing in the BHIB in PH=7 at 37°C for 72 hr. under aerobic condition.
- 4-The best saturation level of ammonium sulfate for bacteriocin was at 70%.and The Pure Bacteriocin production have a molecular weight by SDS PAGE (29) KDa and purified Bacteriocin are stable at wide range temperature until 80°C, PH between (6-9) and storage for 30 day under -20°C.
- 5-The purified bacteriocin was further analyzed and evaluated *in vitro* affected antibacterial activity against both Gram-positive and Gram-Negative pathogenic bacteria and *in vivo* mammalian cell on tumor cell lines (PC3) and normal cell line (Hdfn) cytotoxicity and the possible mode of action.
- 6-Most of the *Staphylococcus haemolyticus* showed their ability to produce biofilm because they possess the gene responsible for biofilm production. and *Staphylococcus haemolyticus* showed their ability to hemolysis red blood cells because they possess the hemolysin gene.

5.2 Recommendations

- 1- Study the gene expression of the Novel bacteriocin extracted from *Staphylococcus haemolyticus*.
- 2- Application studies mice model for purified bacteriocin against pathogenic bacteria.
- 3- Future research should concentrate on the possibility of using the purified bacteriocin in Clinical application like UTI infection.
- 4- Possibility of using a new bacteriocin as a bioactive compound to benefit human health.

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Appendixes

Appendixes

Appendixes(I)

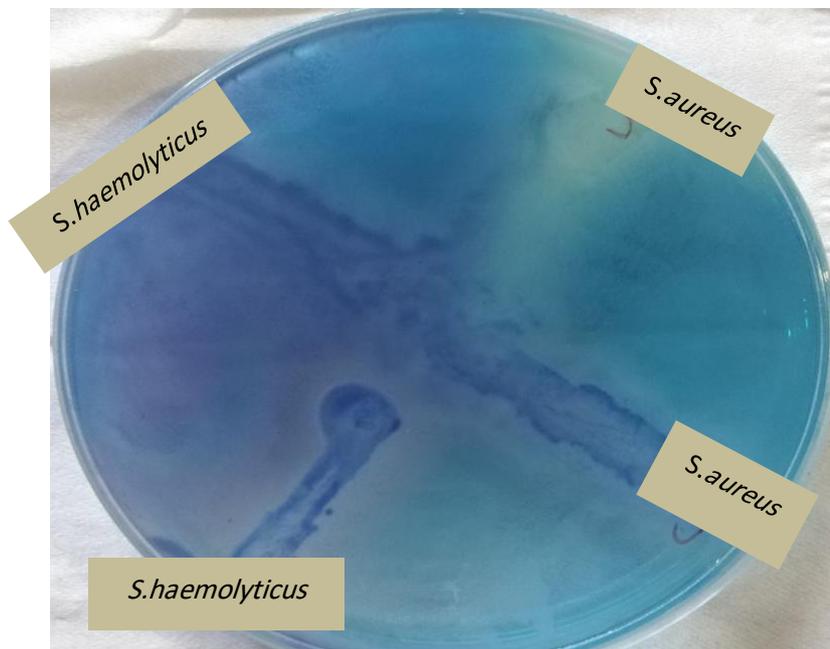


Figure (1):*S. haemolyticus* on DNase Agar at 37°C for 24 hr non appearans inhibition zone around bacteria growth.compart with *S. aureus*.

Appendixes

Appendixes(II)



bioMérieux Customer:

Microbiology Chart Report

Printed May 12, 2022 5:52:38 PM AST

Patient Name: Ahsan Shaker Mohmed, .

Patient ID: A4593M_Ahsan

Location: Wathiq

Physician: Maryam

Lab ID: A4593M_Ahsan

Isolate Number: 1

Organism Quantity:

Selected Organism : Staphylococcus haemolyticus

Source: Urine

Collected:

Comments:	

Susceptibility Information			Analysis Time: 8.27 hours		Status: Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	≥ 0.5	R	Teicoplanin	≥ 32	R
Oxacillin	≥ 4	R	Vancomycin	≥ 32	R
Gentamicin	≤ 0.5	S	Tetracycline	≥ 16	R
Tobramycin	≤ 1	S	Nitrofurantoin	32	S
Levofloxacin	≥ 8	R	Fusidic Acid	≥ 32	R
Moxifloxacin	≥ 8	R	Rifampicin	16	R
Erythromycin	≥ 8	R	Trimethoprim/ Sulfamethoxazole	≥ 320	R
Clindamycin	≥ 8	R			

Appendixes

Appendixes(III)

bioMérieux Customer:

Microbiology Chart Report

Printed July 28, 2022 2:15:06 AM AST

Patient Name: Ali Abd Alamear Jabar, .

Patient ID: A5103M_Ali Abd A

Location: Madina

Physician: Maryam

Lab ID: A5103M_Ali Abd Alam

Isolate Number: 1

Organism Quantity:

Selected Organism : Staphylococcus haemolyticus

Source: Semen

Collected:

Comments:	

Susceptibility Information	Analysis Time: 9.02 hours	Status: Final
-----------------------------------	----------------------------------	----------------------

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	>= 0.5	R	Teicoplanin	16	I
Oxacillin	>= 4	R	Vancomycin	16	I
Gentamicin	<= 0.5	S	Tetracycline	8	*R
Tobramycin	<= 1	S	Nitrofurantoin	32	S
Levofloxacin	>= 8	R	Fusidic Acid	>= 32	R
Moxifloxacin	>= 8	R	Rifampicin	<= 0.5	S
Erythromycin	>= 8	R	Trimethoprim/ Sulfamethoxazole	40	S
Clindamycin	<= 0.25	S			

*= AES modified **= User modified

Appendixes

Appendixes(IV)

bioMérieux Customer:

Microbiology Chart Report

Printed October 8, 2022 5:32:40 PM
AST

Patient Name:

Patient ID:

Location:

Physician:

Lab ID: Mustafam

Isolate Number: 1

Organism Quantity:

Selected Organism : Staphylococcus haemolyticus

Source:

Susceptibility Information						Analysis Time: 14.87 hours	Status: Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation		
Benzylpenicillin	>= 0.5	R	+Cefotaxime		R		
+Amoxicillin		R	+Cefepime		R		
+Amoxicillin/Clavulanic Acid		R	+Meropenem		R		
+Ampicillin/Sulbactam		R	Gentamicin	1	*R		
+Ticarcillin		R	+Pipemidic Acid		R		
+Azlocillin		R	Ciprofloxacin	4	R		
+Piperacillin		R	Moxifloxacin	1	I		
+Piperacillin/Tazobactam		R	+Norfloxacin		R		
+Cloxacillin		R	+Ofloxacin		R		
+Methicillin		R	+Azithromycin		R		
Oxacillin	>= 4	R	Erythromycin	>= 8	R		
+Cefaclor		R	+Roxithromycine		R		
+Cefadroxil		R	Clindamycin	1	*R		
+Cefalexin		R	Linezolid	1	S		
+Cefazolin		R	Teicoplanin	8	S		
+Cefotetan		R	Vancomycin	>= 32	R		
+Cefoxitin		R	Tigecycline	<= 0.12	S		
+Cefdinir		R	Fusidic Acid	16	R		
+Cefditoren		R	Rifampicin	1	S		
+Cefixime		R	Trimethoprim/ Sulfamethoxazole	<= 10	S		
+Cefmenoxim		R					

Appendixes

Appendixes(V)

**Table (1) : Antibiotics susceptibility test for *S. haemolyticus* isolates
by Vitek-2 system.**

Antibiotic	R (%)	I (%)	S (%)
Benzylpenicillin	60 (100)	0	0
Oxacillin	60 (100)	0	0
Gentamicin	43 (71.6)	0	17 (28.3)
Levofloxacin	16 (26.6)	2 (3.3)	42 (70.06)
Moxifloxacin	35 (58.3)	2 (3.3)	23 (38.3)
Erythromycin	54 (90)	2 (3.3)	4 (6.6)
Clindamycin	43 (71.6)	0	17 (28.3)
Linezolid	0	0	60 (100)
Teicoplanin	17 (28.3)	16 (26.6)	27 (45.03)
Tetracycline	51 (85)	0	9 (15)
Vancomycin	12 (20)	13 (21.6)	35 (58.3)
Tigecycline	0	0	60 (100)
Nitrofurantoin	2 (3.3)	0	58 (96.6)
Fusidic acid	52 (86.6)	0	8 (13.3)
Rifampicin	16 (26.6)	0	44 (73.3)
Trimethoprim/Sulfamethoxazole	26 (43.3)	0	34 (56.6)

Appendixes

Appendixes(VI)

Table (2): Biofilm Formation of isolates of *S. haemolyticus*. *(+/- SD + : high ; - : low)

Isolates code	O.D	± SD*	Biofilm response	Isolates code	O.D	± SD*	Biofilm response
A1	0.27	±0.04	Weak	A30	1.153	±0.11	Strong
A2	0.462	±0.15	Moderate	A31	0.211	±0.01	Weak
A3	0.293	±0.07	Weak	A32	0.392	±0.006	Moderate
A4	0.358	±0.17	Moderate	A33	0.257	±0.08	Weak
A5	0.302	±0.04	Weak	A34	0.253	±0.09	Weak
A6	0.149	±0.01	Non-producer	A35	0.695	±0.26	Strong
A7	0.482	±0.31	Moderate	A36	0.281	±0.04	Weak
A8	0.375	±0.23	Moderate	A37	0.854	±0.03	Strong
A9	0.221	±0.05	Weak	A38	0.445	±0.15	Moderate
A10	0.496	±0.2	Moderate	A39	1.315	±0.17	Strong
A11	0.402	±0.05	Moderate	A40	0.559	±0.27	Strong
A12	0.251	±0.06	Weak	A41	0.393	±0.17	Moderate
A13	0.199	±0.01	Weak	A42	0.359	±0.03	Moderate
A14	0.479	±0.09	Moderate	A43	0.427	±0.12	Moderate
A15	0.378	±0.12	Moderate	A44	0.517	±0.18	Moderate
A16	0.215	±0.03	Moderate	A45	1.263	±0.63	Strong
A17	0.483	±0.43	Moderate	A46	0.324	±0.02	Moderate
A18	0.275	±0.18	Weak	A47	0.646	±0.05	Moderate
A19	0.396	±0.25	Moderate	A48	0.579	±0.12	Moderate
A20	0.194	±0.01	Weak	A49	1.531	±0.48	Strong
A21	0.548	±0.14	Moderate	A50	0.248	±0.03	Weak
A23	0.323	±0.07	Weak	A51	0.252	±0.07	Weak
A24	0.229	±0.07	Weak	A52	0.499	±0.08	Moderate
A25	0.7	±0.14	Strong	A53	0.453	±0.09	Moderate
A26	0.28	±0.07	Weak	A54	0.467	±0.08	Moderate
A27	0.649	±0.13	Moderate	A55	0.38	±0.11	Moderate
A28	0.462	±0.02	Moderate	A56	1.268	±0.11	Strong
A29	0.597	±0.23	Moderate	Control	0.150	±0.005	

Appendixes

Appendixes(VII)

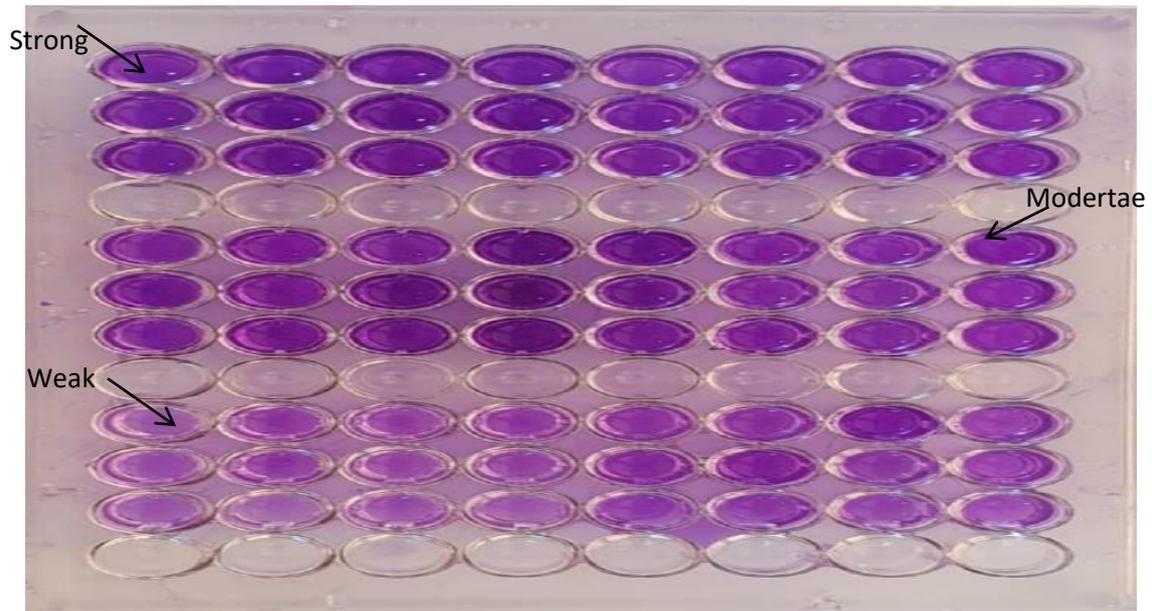
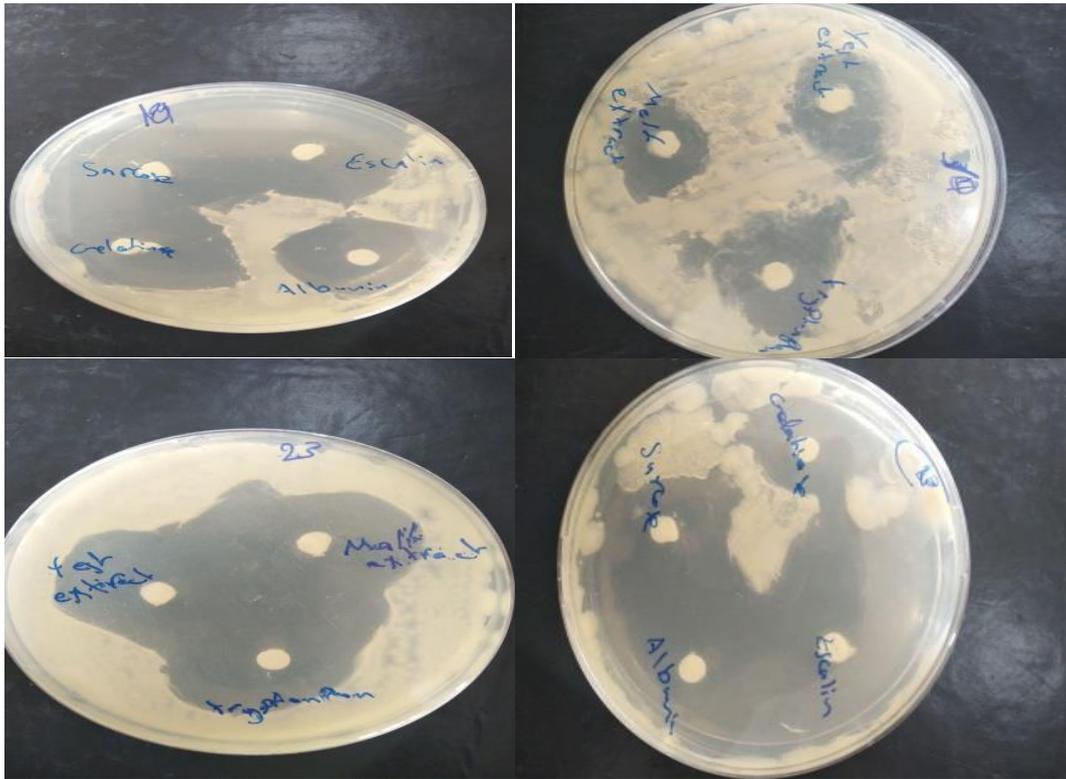


Figure (2): Detection of biofilm formation of *S. haemolyticus* isolates by microtiter plate method using crystal violet stain.

Appendixes

Appendixes(VIII)



Figure(3):activity of bacteriocin after added Nitrogen&Carbon Surcose on MHA at 37°Cfor 72hr in pH=7by FPD method.

Appendixes

Appendixes(IX)

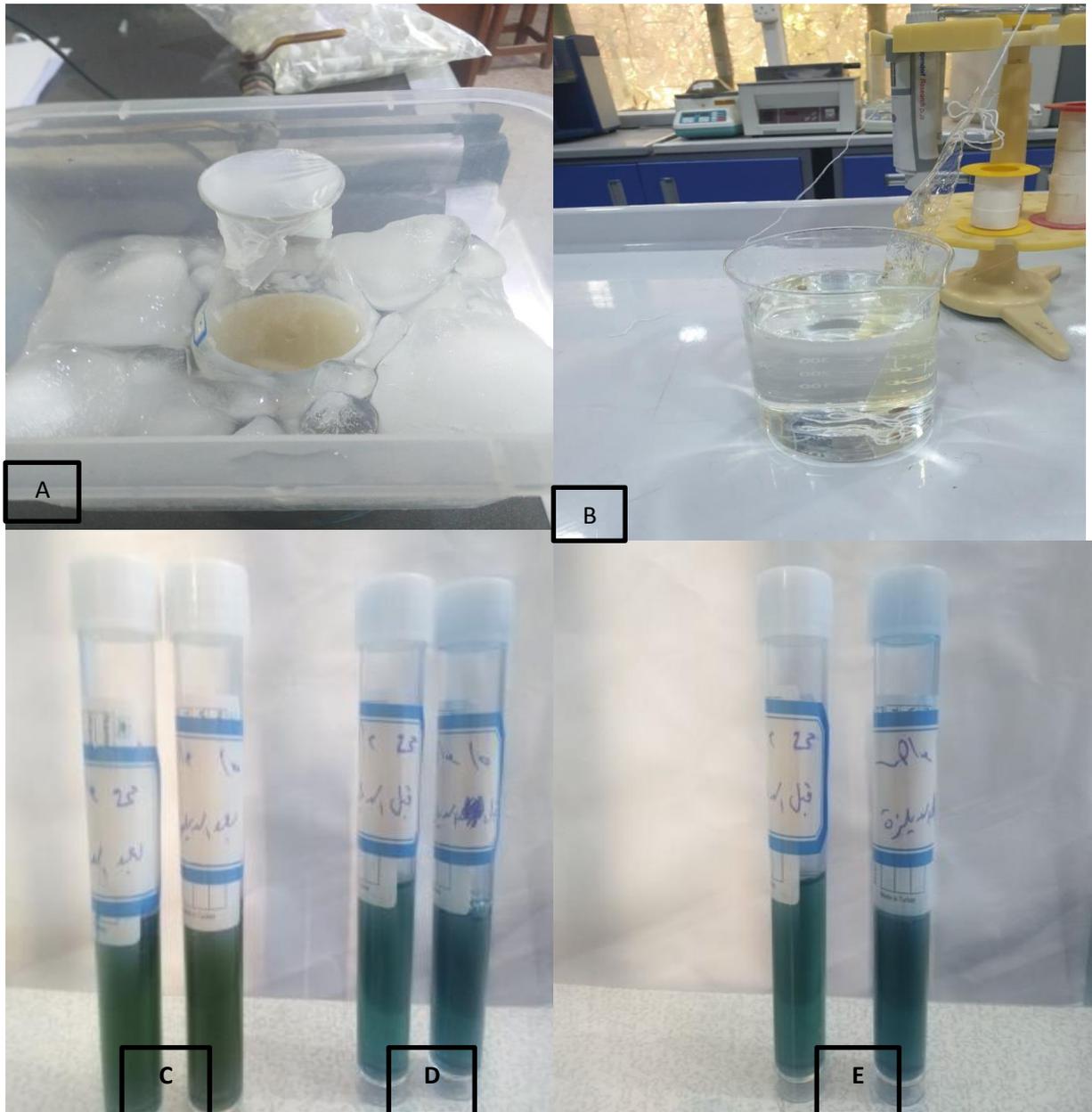


Figure (4);Step of Bacteriocin Purification

A- $(\text{NH}_4)_2\text{SO}_4$ Precipitate

B-Dialysis

c-protein concentration after $(\text{NH}_4)_2\text{SO}_4$

D-protein concentration after dialysis

E-protein concentration after DEAE- cellulose

Appendixes

Appendixes(X)

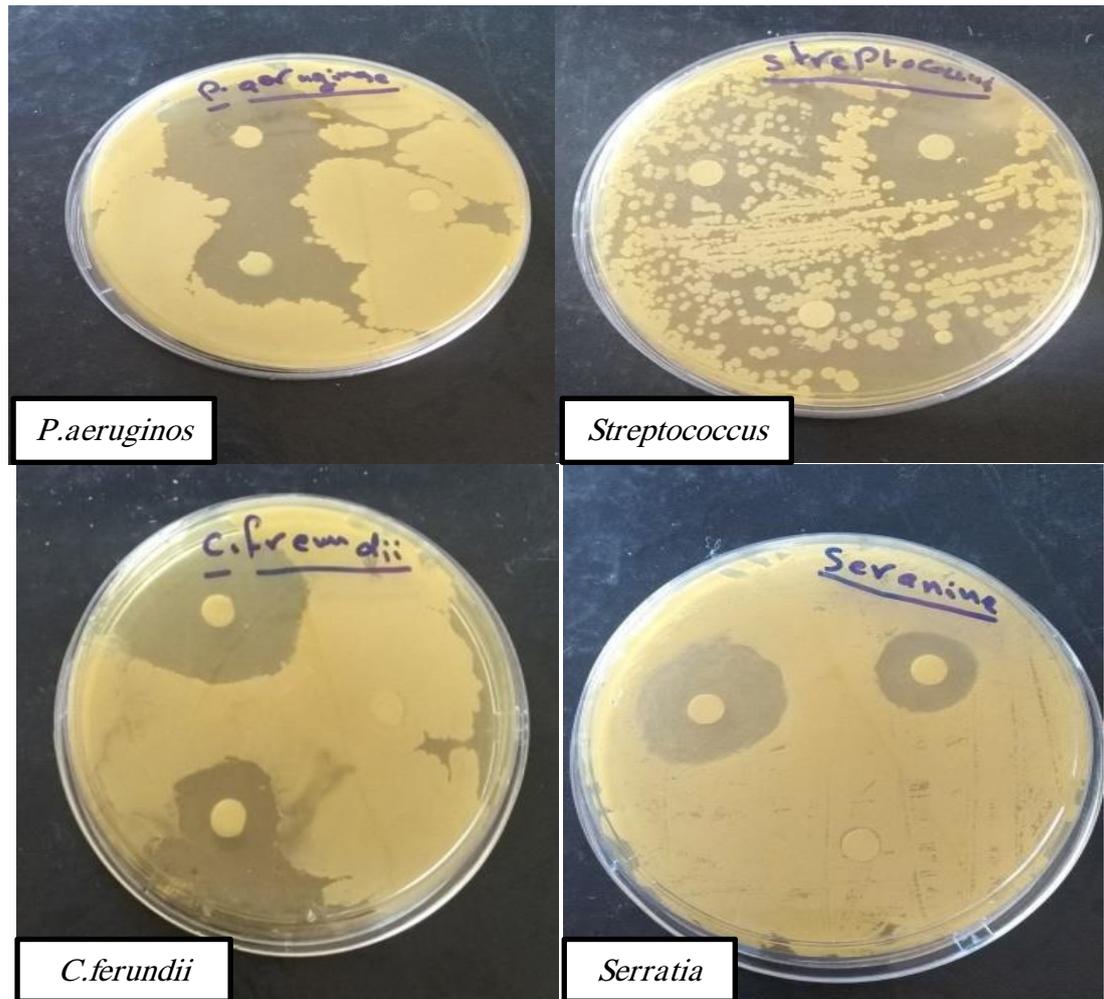
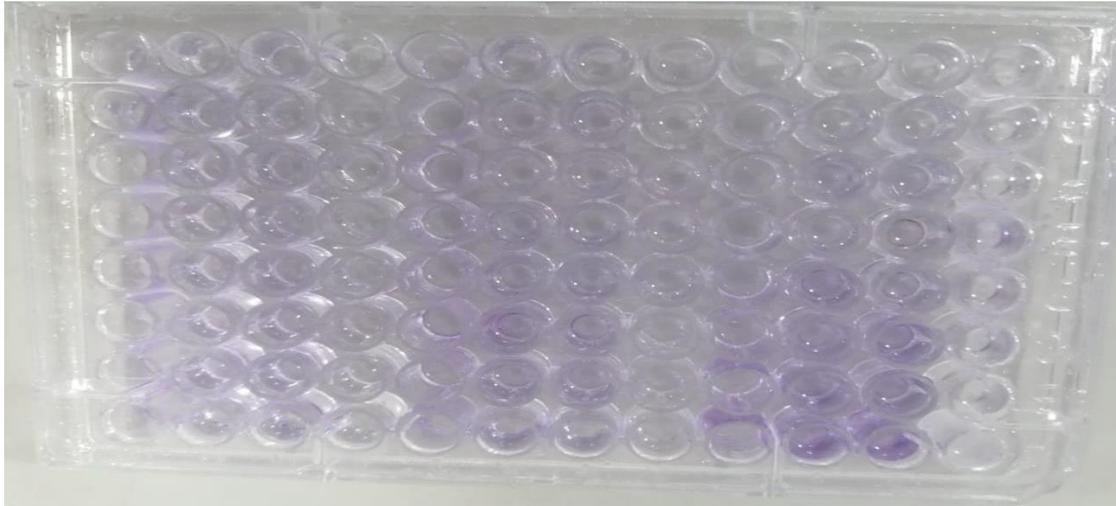


Figure (5): Antibacterial activity of Crud and purification bacteriocin against different spp bacteria on MHA by FDA for 72 hr. at 37°C in PH=7.

Appendixes

Appendixes(XI)



Figure(6):Anti-Biofilm formation that showed on application of *S. haemolyticus* to formation Biofilm after treatment bacteriocin.by microtiter plate method using crystal violet stain.

الخلاصة:

تعتبر مقاومة مضادات الميكروبات من أهم المخاطر على الصحة العامة العالمية في القرن الحادي والعشرين. وتشكل مقاومة مضادات الميكروبات (AMR) أحد الأخطار الدولية على التنمية والصحة. لذلك ، من الضروري التركيز على أكثر المواد المضادة للبكتيريا مقارنة بالمضادات الحيوية التقليدية الفعالة ضد مسببات الأمراض المقاومة للأدوية المتعددة. هدفت هذه الدراسة إلى استخلاص وتنقية وتوصيف البكتريوسين المعزول من المكورات العنقودية الحالة للدم المعزولة عن البول والسائل المنوي.

في هذه الدراسة ، تم جمع 150 عينة من (الأدرار 75 والسائل المنوي 75) من المرضى الذكور والمراجعين إلى من مرضى مستشفى اليرموك ومستشفى بغداد التعليمي ومن فئات عمرية مختلفة، للفترة من تشرين الاول وكانون الاول للعام 2022 . تم تشخيص 60 (40%) عزلة من بكتيريا *S.haemolyticus* من بين جميع العينات باستخدام طرق التشخيص التقليدية مثل فحص الخصائص الزرعية والمظهرية والاختبارات الكيميوحيوية وتم تأكيد التشخيص باستخدام نظام ال Vitek-2 والتشخيص على المستوى الجزيئي من خلال التحري عن وجود جين *Nuc*.

أظهرت نتائج الحساسية للمضادات الحيوية باستخدام نظام Viteck-2 أن *S.haemolyticus* لها مقاومة متعددة للمضادات الحيوية قيد الدراسة (بنزيل بنسيلين ، أوكساسيلين ، إريثروميسين ، فانيكو حمض ، نتراتريكالين ، جنتاميسين ، كلينداميسين ، موكسيفلوكساسين ، تريميثوبريم / سلفاميثوكسازول ، تيكوبلانيك ، ليفوفلوكساسين ، ريفامبيسين ، فانيكوفلانيك) عند (100%) 60 ، (100%) 60 ، (90%) 54 ، (86.6%) 52 ، (85 %) 51 ، (71.6 %) 43 ، (71.6%) 43 ، (85.3 %) 35 ، (43.3%) 26 ، (28.2 %) 17 ، (26.6%) 16 ، (26.6 %) 16 ، (20%) 12 على التوالي ،

بينما أعلى حساسية لـ Linezolid ، Tigecycline كانت (100%) 60 و (96.6%) 58 حساسة للنيتروفوراننتوين.

كان معدل انتشار العزلات البكتيرية المنتجة للغشاء الحيوي (95%) 57/60 بواقع (15.7%) 9/57 لها القابلية على إنتاج غشاء حيوي قوي ، (52.6 %) 30/57 أنتجت غشاء حيوي معتدل ، (31.5 %) 18/57 (%) عزلة أنتجت غشاء حيوي ضعيف و (5%) 3/60 كانت غير منتجة للغشاء الحيوي.

أوضحت الدراسة أن 34 / 57 (59.6%) من عزلات السائل المنوي و 23 / 57 (40.3%) عزلة من عزلات الادرار كانت تحمل جينات المشفرة لقابلية الالتصاق داخل الخلايا *icaADB*.

أظهرت جميع عزلات *S.haemolyticus* تحلل خلايا الدم الحمراء البشرية بواسطة Beta-Hemolysin على وسط الدم ، وباستخدام تقنية PCR للجين المشفر للهيموليسين في *S.haemolyticus* باستخدام بادئات محددة يمكن ملاحظة أن جينات *hla_haem* كانت موجودة في جميع العزلات .

تم الكشف عن إنتاج البكتيريا للبكتريوسين باستخدام وطريقة الانتشار بالحفر (AWD) وطريقة اقراص ورق الترشيح (FPD). باستخدام طريقة ال FPD أظهرت النتائج أن حوالي 95% من *S.haemolyticus* كانت منتجة للبكتريوسين بينما 5% فقط من *S.haemolyticus* لوحظ انتاجها للبكتريوسين بواسطة AWD.

بينت النتائج بان الظروف المثلى لتخليق البكتريوسين في وسط نقيع القلب والدماغ (BHIB) المضاف له 1% كلوكوز برقم هيدروجيني 7 عند درجة حرارة 37 درجة مئوية لمدة 72 ساعة. وكذلك أظهرت النتائج أن قدرة عملية النمو على إنتاج البكتريوسين تأثرت بشكل كبير بإضافة مصادر النيتروجين.

أظهرت نتيجة تأثير درجة حرارة الحضان أن الحد الأقصى للإنتاج قد تم الحصول عليه بين 30 إلى 37 درجة مئوية مع مناطق تثبيط 32 و 39 مم لكل سلالة من البكتريا المفحوصة على التوالي.

كذلك بينت النتائج تأثير فترات الحضانة أن أكبر مناطق تثبيط كانت 26 و 39 مم في العزلات المعزولة من السائل المنوي بعد 48 و 72 ساعة على التوالي ، في حين كانت مقدار التثبيط هي 37 ، 45 مم في العزلات البكتيرية المعزولة من الادرار.

كانت أقصى مستويات إنتاج للبكتريوسين والنمو البكتيري لـ *S. haemlyticus* عند قيم الأس الهيدروجيني بين 6 و 8.

كانت نتيجة دراسة العلاقة بين إنتاج البكتريوسين وحجم اللقاح البكتيري الذي يحتوي على 1.5×10^8 CFU/ml حيث اعطت مناطق التثبيط ما بين 25 إلى 38 مم.

تم في هذه الدراسة تنقية البكتريوسين عن طريق الترسيب باستخدام كبريتات الامونيوم وباعلى فعالية بنسبة اشباع بلغت 70% بينما كانت اقل فعالية بنسبة اشباع عند 40-60% .

تم تحديد الوزن الجزيئي لـ *S. haemolyticus* bacteriocin بحوالي (29 كيلو دالتون) بواسطة الترحيل الكهربائي للهلام (SDS-PAGE) Sodium Dodecyl Sulfate-polyacrylamide. كما تم فحص الخصائص الفيزيائية للبكتيريوسين ، وأظهرت النتائج أنها كانت مستقرة بمدى درجة حموضة واسعة (4-9) ومدى درجة حرارة (80 و 60 و 40 درجة مئوية). أما بالنسبة لدراسة تأثير التخزين على فعالية *S.haemolyticus* bacteriocin فقد كان ذو فعالية وثبات بعد التجميد لمدة ثلاثة اشهر تخزين مع أقصى تثبيت للمنطقة (30 مم) من عزلات السائل المنوي و (25 مم) من عزلات البول عند 20- درجة مئوية.

تم تقييم التأثير السام للبكتيريوسين المنقى على الخلايا الحية في المختبر باستخدام اختبار MTT ، والذي تم استخدامه لقياس النشاط السام للخلايا للبكتيريا على خطوط الخلايا ، وكانت نتيجة قيم IC لخلايا PC3 HdFn, و 122.5- 61.82 (3.763-168.2) ميكروغرام ميكرومتر.



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم للبنات
قسم علوم الحياة

الدراسة الجزيئية والفعالية التضادية للبكتريوسين
المستخلص والمنقى من
Shaemolyticus haemlyticus
المعزولة من العينات السريرية

رسالة مقدمة الى
مجلس كلية العلوم للبنات / جامعة بابل كجزء من
متطلبات نيل درجة الماجستير في العلوم / علوم الحياة

من قبل
رغده عباس رزاق
بكالوريوس علوم حياة / كلية العلوم للبنات / جامعة بابل / 2018

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