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Effect of physical and chemical mutagens on *Staphylococcus aureus* cell wall

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By

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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صدق الله العظيم

سورة الرحمن

Dedication

Prise to Allah the lord of the universe, peace is upon Mohammed the messenger of Allah and upon his relatives.

This work is humbly dedicated to my parents for being who they are, the most companionable, forgiving, loving, caring, supporting, encouraging, sacrificing and tolerating parents ever. Thank you for your unconditional love.

To Ahmed whom always knew how to bring the best of me, thank you for your love that brought comfort to me during moments of weakness. You are the best brother that I can ever get.

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To Mariam whom always managed to draw a smile on my face when I exhaust all of my luck.

To all of you I owe my life, my happiness, whom I am and whom I will be.

Farrah

Supervisors Certification

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Summary

This study aimed to modify *Staphylococcus aureus* by using physical and chemical mutagens and subjections the cell wall to the hydrolytic activity of lysozyme. Chemical mutagenesis by N-methyle-N-nitro-N-nitroguanidine, was achieved by incubating the cell suspension of *Staphylococcus aureus* with 100µg/ml MNNG for different periods of time (5, 10, 15, 20, 25 and 30min). Aliquot of 100µl of the bacterial suspension subjected to the lethal and mutagenic effect of MNNG, then spread on BHI agar and incubated at 37˚C over night in dark. After incubation, 180 colonies were randomly selected and screened for their ability to grow on the same medium supplemented with 3.4 and 12.5µg/ml lysozyme to detect the lysozyme sensitive mutants. Results showed that all of the selected colonies were able to grow on 12.5µg/ml lysozyme containing medium except one colony (S1).

Physical mutagenesis by UV radiation was achieved by subjecting cell suspension of *S. aureus* to different doses of UV radiation (1, 2, 3, 4 and 5J/m^2). Aliquot of 100µl of the bacterial suspension was exposed to the lethal and mutagenic effect of UV radiation then spread on BHI agar and incubated at 37˚C over night in dark. After incubation, 160 colonies were randomly selected and screened for their ability to grow on the same medium but containing 3.4 and 12.5µg/ml lysozyme. Results showed that all of the selected colonies were able to grow on these media except four colonies (S2, S3, S4, and S5) which were unable to grow on 12.5µg/ml lysozyme containing medium, these four colonies were considered to be lysozyme sensitive mutants.

Plasmid profile of *S. aureus* was studied; the wild type and the lysozyme sensitive mutants (S1, S2, S3, S4, and S5) using alkaline lysis method, the total genomic DNA was extracted by lysozyme instead of lysostaphin which is known for its ability to lyse *S. aureus*, results showed the appearance of chromosomal band in addition to plasmid band from all of the lysozyme sensitive mutants (S1, S2, S3, S4, and S5), however the method was unable to extract DNA from the wild type because *S. aureus* cell wall is resistant to the hydrolytic effect of lysozyme, this result confirmed that chemical and physical mutagens can cause alternation in the structural genes responsible for the synthesis of the cell wall.

The synergism effect of penicillin and lysozyme on *S. aureus* cell wall was studied. Results showed that incubation of *S. aureus* with the minimal inhibition concentration MIC of penicillin (640u/ml) for different time periods (2, 3, 4 and 5 hours) then incubating the cells with 250µg/ml lysozyme for two hours causes the gradual lysis of the cell wall and finally the complete lysis as the incubation time progress by the formation of protoplast. Genomic DNA was extracted from these cells by alkaline lysis method using lysozyme instead of lysostaphin; results showed the appearance of chromosomal band and plasmid band on 0.7% agarose gel.

Lysozyme sensitive mutants isolated by UV and MNNG mutagenesis were characterized and the results were compared with the wild type by studying antibiotic sensitivity pattern for the wild type and for the five mutants resulted after mutagenesis with chemical and physical mutagens. Results showed variations in the antibiotic sensitivity pattern against the twelve antibiotics that were used in comparison with the wild type; these variations could be a result of the mutation in the genes responsible for the antibiotic resistance caused by the

physical and chemical mutagenesis. The possibility of lysing the cell wall of *S. aureus* mutant cells by lysozyme (50µg/ml) alone to the cell suspension results of observing the microscopic changes showed that *S. aureus* was converted into protoplasts after incubation for 2hours. This result confirms that the hydrolytic activity of lysozyme against mutant's cells of *S. aureus* can be used to acquire protoplast unlike the wild type in which the lysis of its cell wall requires a long period of incubation with penicillin and higher concentration of lysozyme. To identify the changes in the growth conditions of *S. aureus* after the chemical and physical mutagenesis in comparison with *S. aureus* wild type, mutant cells were incubated at different temperature (30, 37, 40, 43, and 45˚C). Results showed that unlike the wild type some of the mutants were unable to grow at high temperature (over 40°C), suggesting defects in cell surface structure and the membrane integrity caused by the mutagens effect.

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List of Abbreviations

Abbreviations	Meaning
TSST	Toxic Shock Syndrome Toxin
EFT	Exfoliation Toxin
Fc	Fragment crystalizable
NAM	N-acetylmuramic acid
NAG	N-acetyleglucosamine
PG	Peptidoglycan
PBP	Penicillin Binding Protein
oat A	O-acetyletransferase
MRSA	Methicillin Resistance *S. aureus*
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
UV	Ultraviolet
BHI	Brain-heart infusion
J/m^2	Joule/ square meter
rpm	Rotation per minute

Chapter One

1. Introduction and literatures review

1.1 Introduction

Staphylococcus aureus is one of the most frequently isolated bacterial pathogens, causing several morbidity, and often fatal infections. It has the ability to adhere to catheter and others indwelling devices (Cramton *et al*., 1999).

Staphylococcus aureus is a Gram-positive bacterium, which means that the cell wall of this bacterium consists of a very thick peptidoglycan; in addition cell wall is composed of teichoic acid and wall-associated surface proteins. The peptidoglycan arrangement of *Staphylococcus aureus* shows that peptide bridges are critical for the highly cross-linked murein architecture of this microorganism. Staphylococcal murein is composed of glycan and oligopeptide chains, both running in a plane that is perpendicular to the plasma membrane, with oligopeptide chains adopting a zigzag conformation this model confer a high degree of cross-linking that is the hallmark of the staphylococcal cell wall (Biswas, 2006).

Pathogenic bacteria, such as *Staphylococcus aureus*, express a wide variety of virulence factors that enable the organism to cause acute and chronic infections. The human defense system uses a variety of factors to destroy it that include bacteriolytic enzymes; one important widespread defense enzyme is lysozyme which is a PG hydrolyses that found in animals, plants, insects, viruses and bacteria (Piris, 2005). However *Staphylococcus*, in contrast to most of many other bacterial species is relatively resistance to lysozyme. Consequently, a great deal of attention has recently given to another bacterolytic enzyme; lysostaphin

that can cleave pentaglycine-cross bridges in the cell wall of *S. aureus* (Cramton *et al.,* 1999).

Mutagens are usually physical (e.g. UV radiation), and chemical compounds (e.g. MNNG). They can react with DNA and cause indirect structural changes affecting the resistance of *S. aureus* against lysozyme activity (Campbell *et al.,* 2005).

According to the high pathogenicity of *Staphylococcus aureus* and the complexity of its cell wall; this study was aimed to make this microorganism more susceptible to lysozyme by causing genetic modifications which can be achieved by subjecting *S. aureus* to different chemical and physical mutagens and selecting the lysozyme sensitive mutants, then trying to isolate the total genomic DNA using alkaline lysis method based on lysozyme instead of lysostaphin.

1.2 Literatures Review

1.2.1 *Staphylococcus*

Staphylococci are Gram positive spherical bacteria that occur in microscopic clusters resembling grapes, it is a normal inhabitant of the skin and mucous membrane in the nose of healthy human. 1n 1884, Rosenbach described the two pigmented colony types of *Staphylococcus* and proposed the appropriate nomenclature *Staphylococcus aureus* (yellow) and *staphylococcus albus* (white) which is now named *Staphylococcus epidermidis* (Skerman *et al.,*1980).

Although *Staphylococcus* genus include thirty-one species , they are mostly harmless found worldwide as a small component of soil microbial flora with the exception of *Staphylococcus aureus* and *Staphylococcus epidermidis* which are significant in their reaction with human (Holt ,1994).

Staphylococcus can cause a wide variety of diseases in humans and other animals through either toxin production or invasion. Staphylococcal toxins are a common cause of food poisoning, as it can grow in improperly stored food. Although the cooking process kills them, the enterotoxins are heat-resistant and can survive boiling for several minutes. Staphylococci can grow in foods with relatively low water activity (Ryan and Ray, 2004).

1.2.2 *Staphylococcus aureus*

Staphylococcus aureus, literally "Golden Cluster Seed" and also known as golden staph, is the most common cause of staph infections. It is a spherical

bacterium, frequently living on the skin or in the nose of a person. Approximately 20–30% of the general populations are "staph carriers" (Heyman, 2004).

S. aureus is a Gram-positive coccus, which appears as grape like clusters when viewed through a microscope and has large, round, golden yellow colonies, often with β-hemolysis, when grown on blood agar plates , *S. aureus* is a facultative anaerobe and opportunistic pathogen (Chambers, 2001).

1.2.3 Growth conditions of *Staphylococcus aureus*

The nutritional requirements of *S. aureus* are complex and vary from strain to strain (Kloos, 1980). In general, *S. aureus* grows between 7 and 47ºC, with an optimum of 30-37ºC. Enterotoxins are produced between 10 and 46ºC, with an optimum of 35-45ºC. Enterotoxin production is substantially reduced at 20-25ºC. It is generally accepted that enterotoxin production is unlikely to occur at temperatures below 10ºC (Bennett *et al*., 1986). The conditions under which this bacterium grows are shown in table (1-1).

The range of environmental conditions over which *S. aureus* will produce enterotoxins can be narrower than the range over which it will grow. It is possible for *S. aureus* to grow without producing enterotoxin. Optimum enterotoxin production occurs at pH 6-7 and is influenced by atmospheric conditions, carbon and nitrogen source and salt level. Reduced levels of water activity (a_w), may also inhibit toxin synthesis more than growth. Optimum growth and toxin production occur at a_w levels >0.99. Toxin production has been reported at as low as $a_w = 0.86$. *S. aureus* can grow in the presence or absence of oxygen, but grows best under aerobic conditions (Miller *et al.*, 1997).

Table 1-1: Physical parameters for *S. aureus* growth (Ash, 1997)

Physical parameters						
a_w minimum	pH		Salt%		Temperature ºC	
	minimum	maximum	minimum	maximum	minimum	maximum
0.86	4.0	9.8	7-10	20	6-7	45-47

a_w= water activity

1.2.4 Pathology

Staphylococcus aureus may occur commensally on human skin (particularly the scalp, armpits, and vagina); it also occurs in the nose and throat (in about 25% of the population) and less commonly in the colon and in urine. The occurrence of *S. aureus* does not always indicate infection and therefore does not always require treatment (Menichetti, 2005). *Staphylococcus aureus* however can cause many forms of infection as follows (Bowersox, 2007):

- Superficial skin lesions (boils, styes) and localized abscesses in other sites.

- Deep-seated infections, such as osteomyelitis and endocarditis and more serious skin infections.
- A major cause of hospital acquired infection of surgical wounds, and causes infections associated with indwelling medical devices.
- Food poisoning by releasing enterotoxins into food.
- Toxic shock syndrome by release of antigens into the blood stream.

1.2.5 *S. aureus* virulence factors

S. aureus expresses a variety of extracellular proteins and polysaccharides, some of which are correlated with virulence. Virulence results from the combined effect of many factors expressed during infection. Antibodies will neutralize staphylococcal toxins and enzymes, however vaccines are not available (Tenover *et al*., 1994).

1.2.5.1 *Staphylococcus aureus* Adherence to Host Proteins

Staphylococcus aureus cells express on their surface proteins that promote attachment to host proteins such as laminin and fibronectin that form part of the extracellular matrix. Figure (1-1) shows the virulence determinants of *Staphylococcus aureus*. Fibronectin is present on epithelial and endothelial surfaces as well as being a component of blood clots. In addition, most strains of *S. aureus* express a fibrinogen/fibrin binding protein (the clumping factor) which promotes attachment to blood clots and traumatized tissue (Foster and McDevitt, 1994).

S. aureus can adhere to the surface of cultured human endothelial cells and become internalized by a phagocytosis-like process and cause infections

associated with indwelling medical devices ranging from simple intravenous catheters to prosthetic joints and replacement heart valves (Vaudaux *et al.*, 1994).

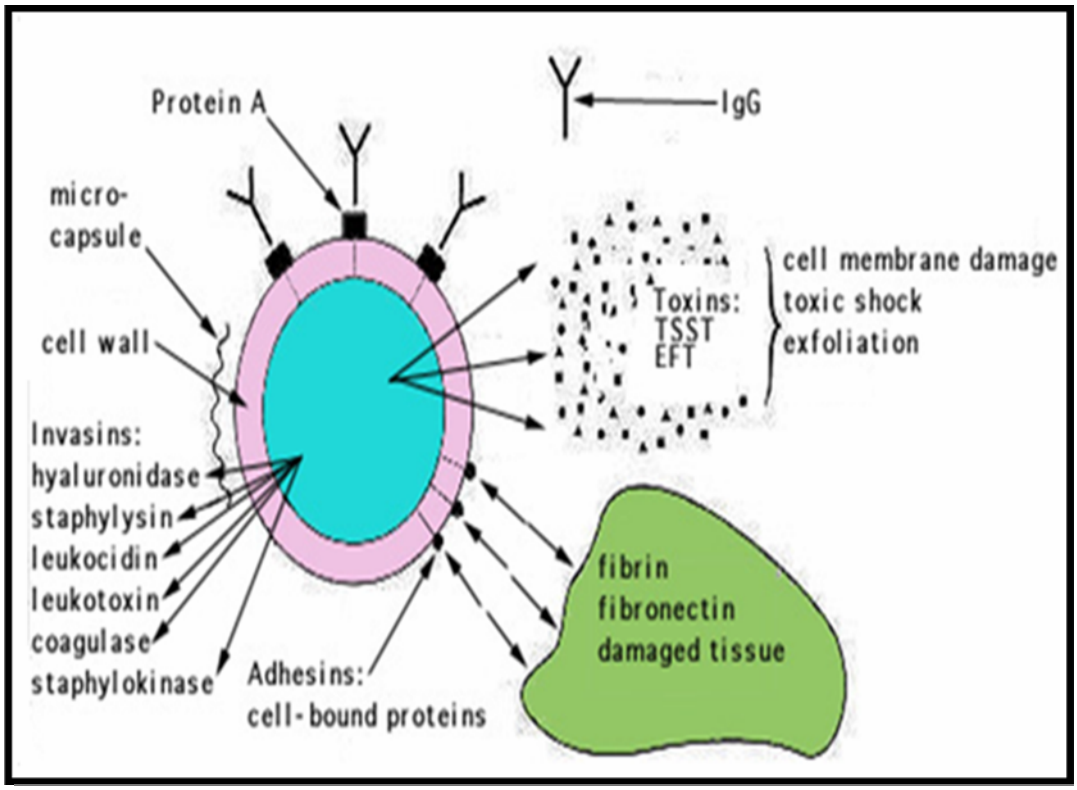


Figure (1-1) Virulence determinants of *Staphylococcus aureus* (Foster and McDevitt, 1994).

1.2.5.2 Avoidance of Host Defenses

S. aureus expresses a number of factors that have the potential to interfere with host defense mechanisms.

a- Capsular Polysaccharide

The majority of clinical isolates of *S. aureus* express a surface polysaccharide. This has been called a microcapsule because it can be visualized only by electron microscope, unlike the copious capsules of other bacteria which are visualized by light microscope. *S. aureus* isolated from infections expresses high levels of polysaccharide but rapidly loses it upon laboratory subculture. The function of the capsule is to impede phagocytosis; conversely, comparing wild-type and a capsule defective mutant strain in an endocarditis model suggested that polysaccharide expression actually impeded colonization of damaged heart valves, by masking adhesions (Easmon and Adlam, 1983).

b- Protein A

Protein A is a surface protein of *S. aureus* which binds immunoglobulin G molecules by the Fc region. In serum, bacteria will bind IgG. In principle this will disrupt opsonization and phagocytosis. Indeed mutants of *S. aureus* lacking protein A are more efficiently phagocytozed *in vitro*, and studies with mutants in infection models suggest that protein A enhances virulence (Prevost *et al*., 1995).

c- Exotoxins

Staphylococcus aureus can express several different types of protein toxins which are probably responsible for symptoms during infections. The exfoliatin toxin causes the scalded skin syndrome, which results in widespread blistering and loss of the epidermis, This is probably why healing occurs with little scarring although the risks of fluid loss and secondary infections are increased. There are two antigenically distinct forms of the toxin, exfoliatin toxin A (ETA) and exfoliatin toxin (ETB) (Todar, 2005).

1.2.5.3 Toxins

- α-toxin

The best characterized and most potent membrane damaging toxin of *S aureus* is α-toxin. It is expressed as a monomer that binds to the membrane of susceptible cells. In humans, platelets and monocytes are particularly sensitive to α -toxin. They carry high affinity sites which allow toxin to bind at concentrations that are physiologically relevant. The toxin causes the symptoms of septic shock that occur during severe infections caused by *S. aureus* (Bhakdi and Tranum, 1991).

- ß-toxin

The majority of human isolates of *S. aureus* do not express ß-toxin. ß-toxin causes deficiency of enzyme sphingomyelinase which result in the accumulation

of sphingomyeline lipid in the liver, spleen and other nerve tissues. The classical test for ß-toxin is lysis of sheep erythrocytes. (Bhakdi and Tranum 1991).

- δ-toxin

  Delta-toxin is a very small peptide toxin produced by most strains of *S. aureus*. It is also produced by *S. epidermidis*. The role of δ-toxin in disease is unknown (Bhakdi and Tranum, 1991).

- γ-toxin and leukocidin

The γ -toxin and the leukocidins are two-component protein toxins that damage membranes of susceptible cells. The proteins are expressed separately but act together to damage membranes. The γ -toxin locus expresses three proteins. The B and C components form a leukotoxin with poor hemolytic activity, whereas the A component is hemolytic and weakly leukotoxic.

The classical Panton and Valentine (PV) leukocidin is distinct from the leukotoxin expressed by the γ -toxin. It has potent leukotoxicity and, in contrast to γ -toxin, is non-hemolytic. Only a small fraction of *S. aureus* isolates (2% in one survey) express the PV leukocidin, whereas 90% of those isolated from severe lesions express this toxin. This suggests that PV leukocidin is an important factor in necrotizing skin infections (Todar, 2005).

- Superantigens: enterotoxins and toxic shock syndrome toxin

S. aureus can express two different types of toxin with superantigen activity, enterotoxins, of which there are six serotypes (A, B, C, D, E and G) and toxic shock syndrome toxin (TSST-1). Enterotoxins cause diarrhea and vomiting

when ingested and are responsible for staphylococcal food poisoning (Schlievert, 1993).

1.2.5.4 Role of pigment in virulence

The bright yellow pigmentation of *S. aureus* may be a factor in its virulence. When comparing a normal strain of *S. aureus* with a strain modified to lack the yellow coloration, the pigmented strain was more likely to survive than the mutant strain. Colonies of the two strains were also exposed to human neutrophils, the mutant colonies quickly were killed while many of the pigmented colonies survived. The pigmented strains created abscesses, wounds with the mutant strains healed quickly (Krinsky, 1993).

Some tests suggest that the yellow pigment might be the key to the ability of *S. aureus* to survive immune system attacks. Drugs that inhibit the bacterium's production of the carotenoids responsible for the yellow coloration may weaken it and renew its susceptibility to antibiotics (Liu *et al.,* 2005).

1.3 *Staphylococcus aureus* plasmids

S .aureus isolates and particularly those from hospitals, often carry one or more free or integrated plasmids. All types of *S. aureus* plasmids frequently carry genes that encode resistance to antibiotics, heavy metals, or antiseptics. Some virulence genes are also reported to be carried on plasmid, such as exfoliative toxin B and some superantigens, plasmids are broadly classified into three categories (Novich, 1990).

Class I plasmids: these are small plasmids (5 Kb or smaller) encode a single resistance determinant ,rarely carries two markers include tetracycline (TE) ,

erythromycin (E), chloramphenicol(Cm), streptomycin (S), kanamycin (km), bleomycin (BI), quaternary compound (Qa), and cadmium (Cd). These plasmids utilize a rolling circle, they are divided into four groups pT181, pC194, pSN2, and pE194 (Gruss and Erlich, 1989).

Class II plasmids: larger plasmids (15-30Kb) encode a multiple antimicrobial-resistance determinant such as β-lactam antibiotics, macrolides, and a variety of heavy metal ions, these plasmids are grouped into pI524, pII124, pI258, and pII071(Lacey,1980)

Class III plasmids: the largest plasmids (30-60Kb) are also multiresistance plasmids but are differentiated from those in class II by their ability to promote their own intercellular transmission via conjugation, these plasmids include Psk41, pG01, and pJE1, these plasmids carry resistance marker for gentamycin (Gm), penicillin (P), quaternary compound (Qa) (Thomas and Archer, 1989).

1.4 *Staphylococcus aureus* cell wall

Staphylococcus aureus is an important human pathogen that causes life threatening diseases including septicemia, endocarditis, toxic shock syndrome, and abscesses in organ tissues. The cell wall of the microorganism plays an important role in infectivity and pathogenicity (van Heijenoort and Gutmann, 2000).

Staphylococci are Gram-positive bacteria, and their cell walls are composed of teichoic acids, and wall-associated surface proteins, as shows in table (1-2). Stress-bearing murein represents continuous macromolecular units covering the whole cell. Murein consists of glycan strands, which are cross-

linked by peptide bridges furnishing the structural integrity of the sacculus (Höök and Foster, 2000).

The ability to evade host immune surveillance is a critical virulence determinant for any pathogen along with the capacity to defend against the immune defenses, and both of these aspects can be provided by the peptidoglycan ,a large polymer that provide much of the strength and rigidity to the bacterial cell wall. It consists a long glycan chains of alternating N-acetyleglucosamine (NAG) ,and N-actylmuramic acid (NAM) subunits, which are cross-linked via flexible peptide bridge, and it can confer resistant to the host bacteriolytic products; such as lysozyme (Boneca *et al.*, 2007).

Table (1-2): Function of each cellular structure of *Staphylococcus aureus* cell wall (Höök and Foster, 2000).

Structure	Function
Capsule	• Inhibit opsionazation and phagocytosis • Protects from leukocyte destruction
peptidoglycan	• Osmotic stability induce the production of endogenous pyrogen • Inhibit phagocytosis and chemotaxis
Protein A	• Binding IgG1,IgG2 and IgG4 fc receptors • Inhibit opsionazation and phagocytosis • Anticomplementary
Teichoic acid	• Regulate cationic concentration at cell membrane • Attachment site for mucosal receptors
Cytoplasmic membrane	• Osmotic barrier • Regulate transport in and out the cell • Site for biosynthetic and respiratory enzymes

The *Staphylococcus aureus* peptidoglycan comprises about 50% of the entire cell wall and forms a multilayered, highly cross-linked structure, which protects the bacterium from osmotic pressure and determines its shape, the peptidoglycan structure is shown in figure (1-2) (Bera *et al.*, 2005).

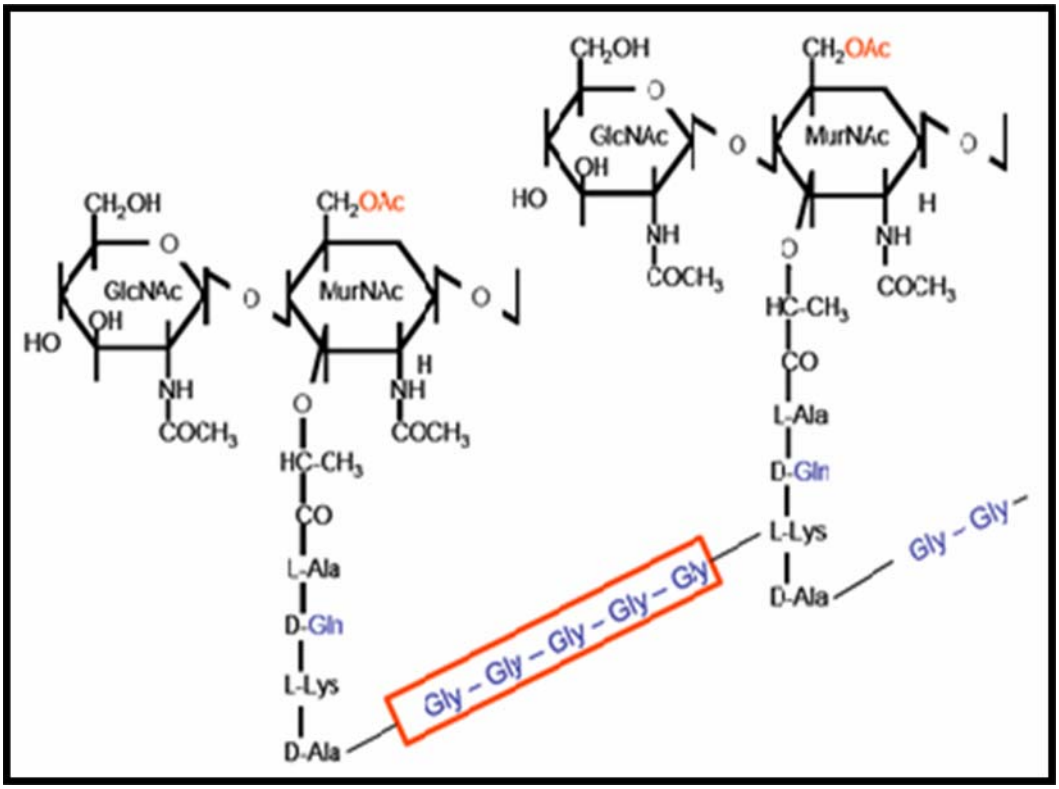


Figure (1-2): Structure of *S. aureus* peptidoglycan (PG). Staphylococcal peptidoglycan is composed of glycan strands, peptide subunits, and penta glycines cross bridges. The repeating disaccharide, N-acetylmuramic acid (NAM) and N-acetylglucosamine(NAG) are β, 1-4 linked with each other (Bera *et al.*, 2005).

The flexible interpeptide allows a high; three dimensional cross-linking of the peptidoglycan and characterize the staphylococcal cell wall, the biosynthesis of the interpeptide bridge is shown in figure (1-3). It also acts as attachment site

for various cell wall-sorted proteins, which are important in infection and virulence of this pathogen (Perry *et al*., 2002; Rohrer and Berger, 2003).

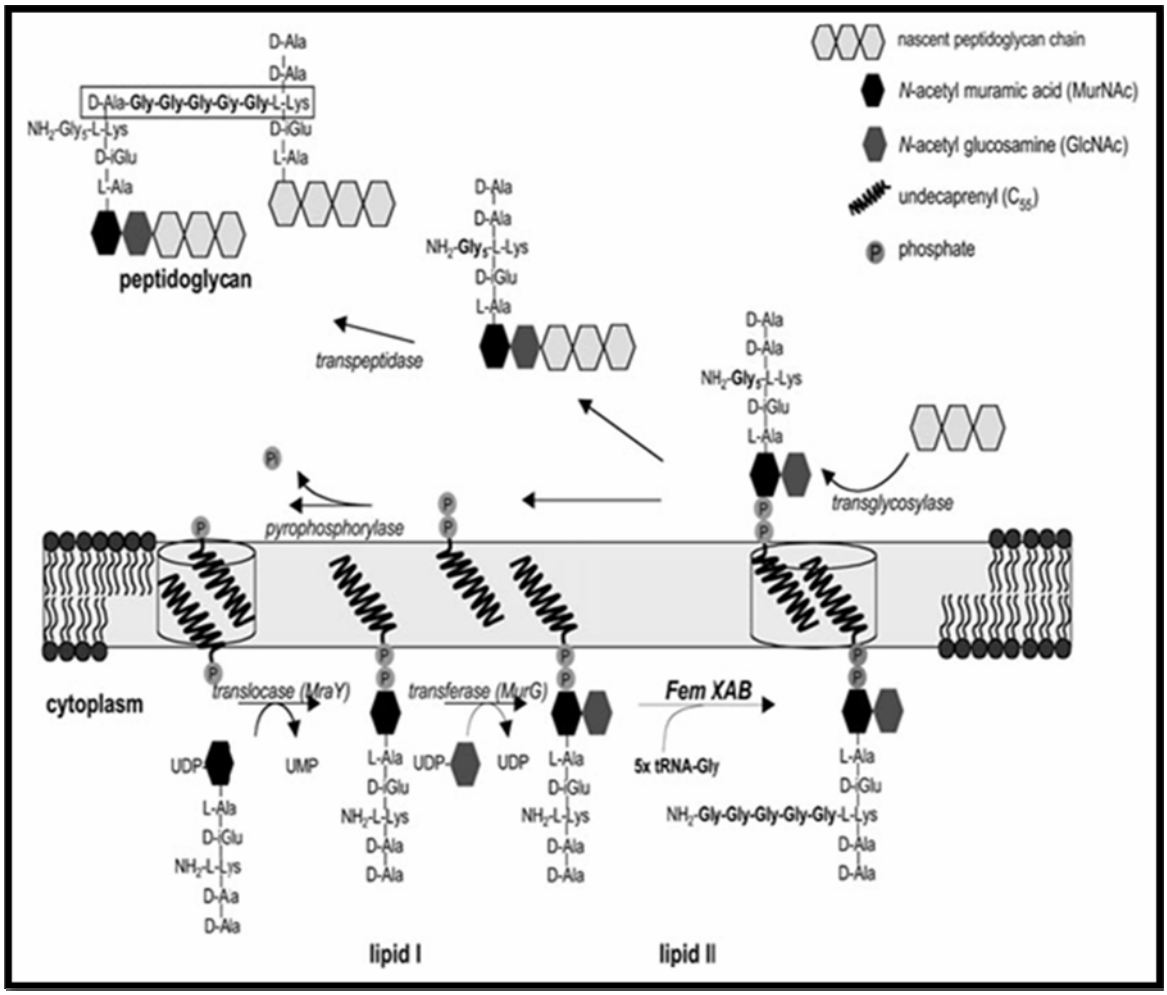


Figure (1-3): Cell wall biosynthesis of *S. aureus* and the formation of the pentaglycine interpeptide bridge (Rohrer and Berger, 2003).

Peptidoglycan strands were approximated as long cylinders or stretched ropes. The approximated strands were arranged parallel to the plasma

membrane and to each other to simulate a peptidoglycan layer. To achieve a maximal degree of cross-linking, the cell walls of Gram positive bacteria exhibit a wide diversity from simple to very complex structures with variably high degrees of cross-linking. Staphylococcal cell walls have a rather extraordinary type of architecture, belonging to the most highly cross-linked type; this scaffold model, in contrast, to other Gram positive bacteria such the walls of bacilli in which exhibit about 50 to 55% cross-linking, readily guarantees 80 to 90% cross-linking (van Heijenoort, 2001).

1.5 Lysozyme

The antibacterial nature of lysozyme was first witnessed by Sir Alexander Fleming, British bacteriologist and Nobel laureate, best known for his discovery of penicillin; in 1922 he discovered lysozyme (Fleming, 1922), lysozyme is a PG hydrolyses that is found in animals, plants, insects, viruses and bacteria. Because lysozyme is cationic, it closely adheres to bacteria through electrostatic interactions with negatively charged tiechoic and lipotiechoic acids and phospholipids on the bacterial surface this interaction can result in bacterial lysis by hydrolyzing the bond between NAG and NAM, It is a highly specific enzyme in hydrolyzing the β1-4 bond where the linking oxygen atom resides between the N-acetylmuramic acid (NAM) and the N-acetylglucosamine (NAG) (figure 1-4) (Varki *et al.,* 1999).

Although, lysozyme is known to be bactericidal to certain bacteria but its antimicrobial function is limited to certain Gram positive bacteria and is less effective against Gram negative ones, owing to the differences found in their membrane structure (Cheetham *et al.,* 1992).

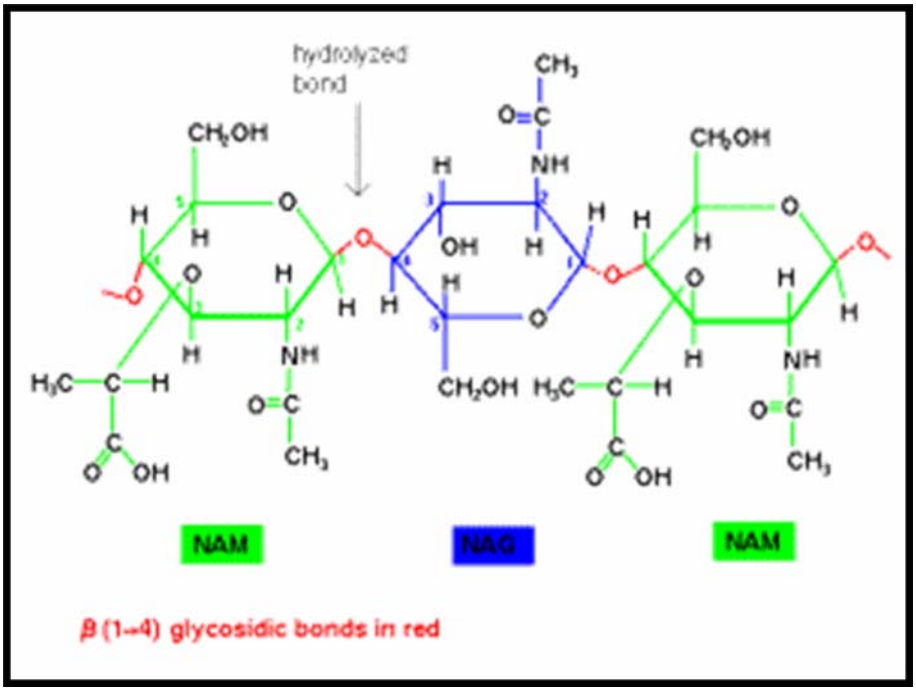


Figure (1-4):Hhydrolytic site between the NAM-NAG in the polysaccharide (Koonin *et al.*, 2003).

Modifications of peptidoglycans can affect their sensitivity to peptidoglycan hydrolyses. *Bacillus cereus* peptidoglycan is sensitive to lysozyme because of the unacetylated amino groups on the majority of its glucosamine residues. Conversely, lysozyme resistance of the peptidoglycans of other organisms is due to O-acetylation of amino sugars, and these peptidoglycans can be made lysozyme sensitive by de-O-acetylation. Accessory cell wall polymers, such as teichoic acids or lipoteichoic acids, can also affect the susceptibility of bacteria to a number of peptidoglycan hydrolyse (Heckels *et al*., 1988).

Staphylococcus aureus peptidoglycan (PG) is completely resistant to the hydrolytic activity of lysozyme because of the modifications in PG by O acetyltransferase (oatA) responsible for O actylation and this leads to resistance to the muramidase activity of the lysozyme, a mutation in (oatA) will make the cell wall little susceptible to lysozyme as it is shown in figure (1-5), however there are some suggestions claim that other factors such as wall teichoic acid, and a high degree of cross-linking also contribute in the cell wall resistance (Collins *et al.*, 2002).

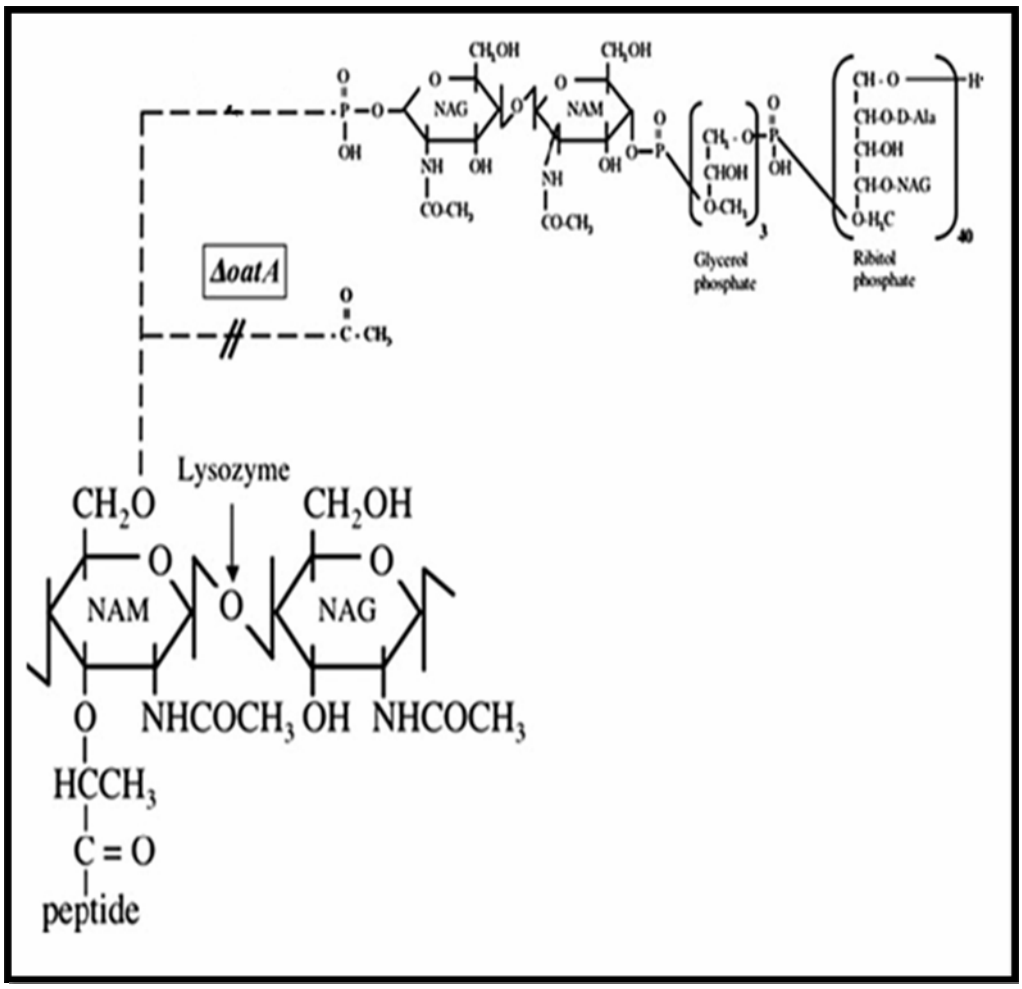


Figure (1-5): Possible modifications at the C-6 position of the NAM in *S.aureus* PG. lysozyme cleaves the beta-1, 4-glycosidic bond between NAM and (NAG) (Collins *et al*., 2002).

1.6 Lysostaphin

Staphylococcus simulans produces an extracellular glycylglycine endopeptidase (lysostaphin) that lyses other *staphylococci* by hydrolyzing the cross bridges in their cell wall peptidoglycan (Novick, 1991).

Lysostaphin, a bacteriocin with the intent of selectively killing microbes to compete for limited resources by cleaving pentaglycine cross bridges in the cell wall of *Staphylococcus aureus* as it is demonstrated in figure (1-6). The Gram positive bacterium *S. aureus* is a human pathogen that colonizes the human skin and nares. Over the past three decades, treatment of staphylococcal infections has become increasingly difficult because of colonization with strains that are resistant to virtually all antimicrobial agents, however recent data demonstrate the effectiveness of lysostaphin in the clearance of Staphylococcal associated infections (Akira and Sato, 2003).

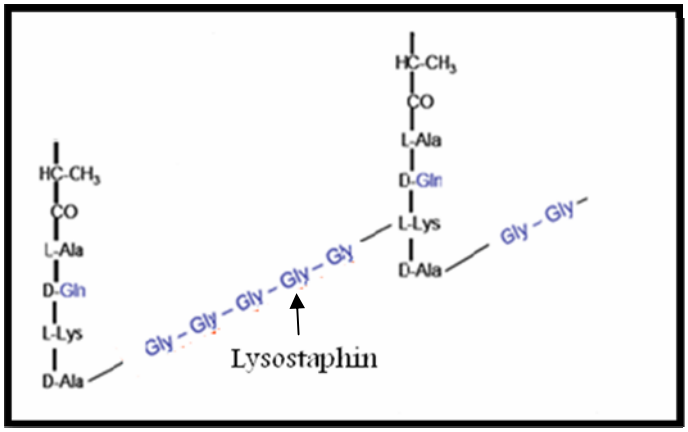


Figure (1-6): *Staphylococcus aureus* peptidoglycan, indicating the cleavage sites lysostaphin (Akira and Sato, 2003).

1.7 Resistance of Staphylococci to Antimicrobial Drugs

S. aureus showed a remarkable ability to survive antibiotics treatment by developing new resistance mechanisms against them within a short time of its introduction. Some strains are now resisting most conventional antibiotics; it is worrisome that it seems to be there are no new antibiotics on the horizon and any recent antibiotic developments are merely a modification to existing drugs; *S. aureus* is known to be notorious in their acquisition of resistance to new drugs. Many strains of *S. aureus* carry a wide variety of multi-drug resistant genes on plasmids (Uwaezuoke and Aririatu, 2004).

S. aureus has responded to the introduction of new drugs by rapidly acquiring resistance by a variety of genetic mechanisms including:

i. Acquisition of extrachromosomal plasmids or additional genetic information in the chromosome via transposons or other types of DNA insertion.

ii. By mutations in chromosomal genes.

Table (1-3) shows different mechanisms in which *staphylococcus aureus* use to resist antimicrobial agents, Staphylococcal resistance to penicillin is mediated by penicillinase (a form of β-lactamase) production; an enzyme which breaks down the β-lactam ring of the penicillin molecule (Lyon and Skurray, 1987).

Table (1-3): different mechanisms in which *staphylococcus aureus* use to resist antimicrobial agents (Lyon and Skurray, 1987).

Antimicrobial	Resistance mechanism	Genetic basis
Penicillin	B-lactamase enzymatic inactivation of penicillin	Plasmid
Methicillin	Expression of new penicillin resistant; penicillin binding protein	Chromosomal gene
Tetracycline	• Efflux from cell • Modification of ribosome	Plasmid Chromosomal gene
Chloramphenicol	Enzymatic inactivation	Plasmid
Erythromycin	Enzymatic modification of ribosomal RNA, preventing drugs from binding to ribosome	Plasmid Trasposon
Streptomycin	• Mutation in ribosomal protein prevents drug binding • Enzymatic inactivation	chromosomal gene Plasmid

Gentamycin	Enzymatic inactivation	Plasmid Transposon

All clinical isolates of methicillin-resistant *Staphylococcus aureus* contain an extra penicillin binding protein (PBP) in addition to four PBPs present in all staphylococcal strains. The mechanism of resistance to methicillin is by the acquisition of the mecA gene, which codes for an altered penicillin-binding protein (PBP) that has a lower affinity for binding β-lactams (penicillins, cephalosporins and carbapenems). This confers resistance to all β-lactam antibiotics and obviates their clinical use during MRSA infections (James *et al.,* 2001).

The antibiotic vancomycin is useful against Gram-positive pathogens. However, with its increased use, resistance has been reported in various species of bacteria, mainly enterococci. Vancomycin-resistant *Staphylococcus aureus* (VRSA) is strains with reduced susceptibilities to vancomycin have been isolated in several countries, staphylococcal strains showed variable levels of resistance to several antimicrobial agents, including oxacillin, and unstable resistance to vancomycin. The thickening of the cell wall in these staphylococcal strains may be an important contributor to vancomycin resistance (Cui *et al*., 2000).

1.8 Effect of mutagenesis

Mutagenesis plays a central role in our lives. A low level of mutagenesis is advantageous, and ensures the survival of species by promoting evolution. Programmed mutagenesis of immunoglobulin genes promotes diversity and

provides a dynamic defense against invading pathogens. However, many human diseases, including most cancers, arise as a consequence of mutations that occur either spontaneously, or are induced by copying errors in the genetic material during cell division, by exposure to ultraviolet or ionizing radiation, chemical mutagens (N-Methyl-N nitro-N'-nitrosoguanidine), or viruses (Ellis *et al.,* 2001).

Mutagens are usually physical (e.g. UV radiation), and chemical compounds (e.g. MNNG). Mutagens can be divided into different categories according to their effect on DNA replication, Some mutagens act as base analogs and get inserted into the DNA strand during replication in place of the substrates, or react with DNA and cause structural changes that lead to miscopying of the template strand when the DNA is replicated, and others work indirectly by causing the cells to synthesize chemicals that have the direct mutagenic effect (Campbell *et al.,* 2005).

1.8.1 Ultraviolet radiation

Ultraviolet (U.V.) is an effective mutagenic agent producing point mutations and chromosomal changes. It is readily absorbed by superficial layers of cells in tissues; therefore, special experimental procedures are necessary for induction of mutations in animals, plants. U.V. is, however, suitable for mutagenesis in microorganisms because their cells are small, permitting the radiation to reach the nuclei. Action spectrum studies reveal that U.V. mutagenesis results from absorption of the radiation by nucleic acid (Huang and Toledo, 1982).

The most prominent alteration in DNA following absorption of U.V. is dimerization of pyrimidines, chiefly thymine. Such a change not only retards

DNA replication but results in mutations, It has an in indirect effect as well through the formation of chemical intermediates such as oxygen and hydroxide radicals which interact with DNA to form strand breaks (Nolan, 2003). UV is normally classified in terms of its wavelength (Pledger *et al*., 1994):

- UV-C (180-290 nm) the most energetic and lethal, it is not found in sunlight because it is absorbed by the ozone layer.
- UV-B (290-320 nm), major lethal/mutagenic fraction of sunlight.
- UV-A (320 nm--visible) also has deleterious effects primarily because it creates oxygen radicals but it produces very few pyrimidine dimers.

1.8.2 N-methyl-N-nitro-N-nitrosoguanidine (MNNG)

In 1974 scientists found that this compound is as much as lethal as mutagenic that has an effect on DNA *in vitro* and plant chromosome (Ehrenberg and Wachtmeister, 1977).

MNNG is toxic, carcinogenic, and mutagenic in experimental test systems. Its primary use is for tumor induction and related research in experimental animals and as research mutagen; it is responsible for most of the mutations induced, MNNG belongs to a class of agents that form methylated bases in DNA both *in vitro* and *in vivo*, this activity alters base direction causes the incorporation of either thymine or cytosine, without blocking DNA replication, resulting in GC-to-AT transition mutations. The chemical structure of MNNG is shown in figure (1-7) (Mendle and Greenberg, 1960).

MNNG induce gene mutations in cells, this mechanism accounts for the known propriety of MNNG to cause multiple closely linked mutations in the

replication fork (Lábaj *et al.*, 2003). MNNG action can cause depurination (loss of purine base), the action of error prone repair system known as SOS repair allows the replication to proceed past the gap, with the incorporation of the wrong base into the new DNA strand (Kat *et al.*, 1993).

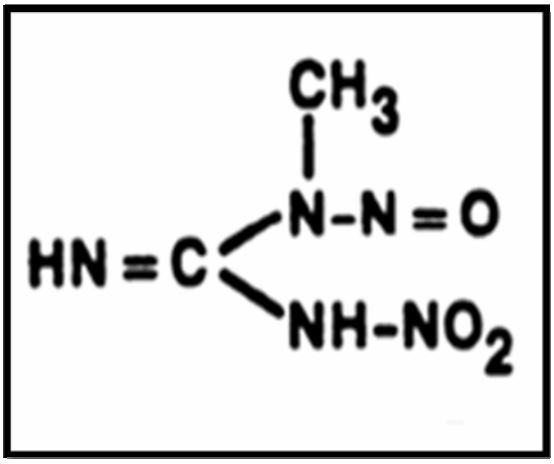
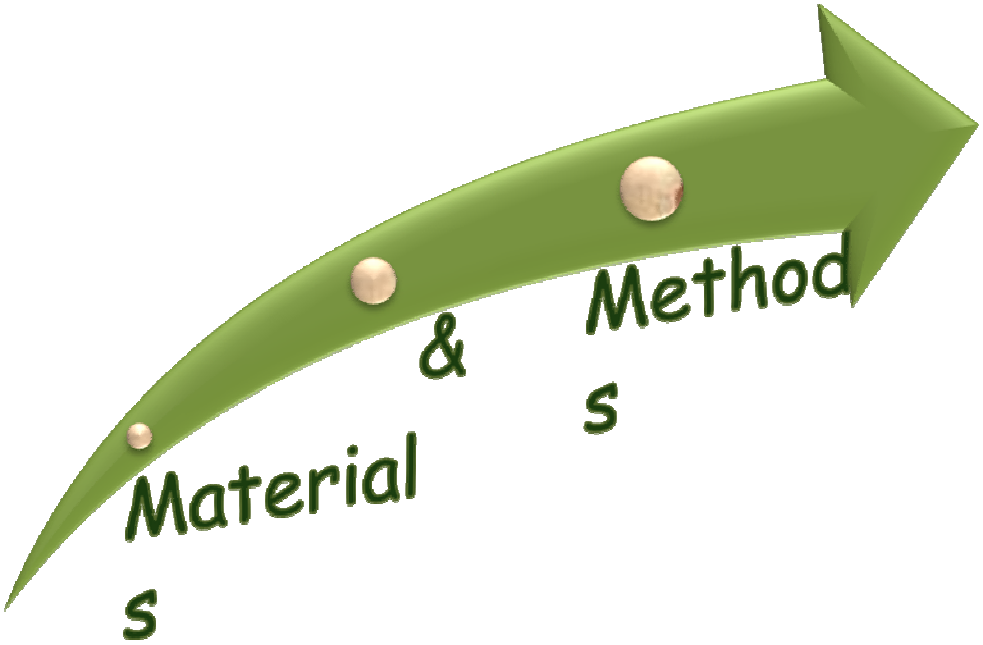


Figure (1-7): Chemical structure of MNNG, MW =147.09 (Ehrenberg and Wachtmeister, 1977)

Chapter Two



2. Materials and methods

2.1 Materials

2.1.1 Equipment

The following equipment was used in this study:

Equipment	Company/Origin
Autoclave	Karl Kolb/ Germany
Sensitive balance	Mettler /USA
Centrifuge	MSE/England
Cooling Centrifuge	Harrier/UK
Distillator	Gallenkamp/England
Incubator	Memmert/ Germany
Shaker incubator	GFL/England
Visible spectrophotometer	Milten roy /USA
Oven	Gallenkamp
Vortex mixer	Griffen/Germany
pH meter	Mettler /USA
Power supply	LKB/Sweden
Gel electrophoresis unit	LKB
Compound microscope	Olympus/Japan
Micropipette	Brand/Germany
Laminar air flow	Memmert
UV transilluminator	Ultraviolet product /USA
Millipore filter unit (0.22μm)	Millipore Filter Corp/USA

2.1.2 Chemicals

The following chemicals were used in this study:

Chemicals	Supplier/Origin
Isoamyl alcohol,Chloroform Ethanol, Ethidum bromide Isopropanol, Agarose, Crystal violet, Iodine, Safranin,KH_2PO_4,K_2HPO_4 ,Peptone, Acetone	BDH/England
Sodium dodecyle sulfate	LKB/Sweden
Hydrogen peroxide, Agar, N, N, N, N-tetra methyl p-phenylene- diamine dihydrochloride,	Difico/USA
Antibiotic discs	Bioanalyse /Turkey
NaCl, Tris-HCl, NaOH EDTA, HCl	Merk/Germany
Yeast extract	Biolife Italy
N-methyl-N-nitro- N-nitrosoguanidine MNNG	Fluka/Switzerland

2.1.3 Bacterial Isolate

Staphylococcus aureus isolate used in this study was obtained from the Department of Biotechnology/College of Science/Baghdad University; it was isolated locally in 2007.

2.1.4 Media

2.1.4.1 Ready to use media

The following media were prepared according to the instruction of the manufacturing company; pH was adjusted to 7.0 and sterilized by autoclaving at 121ºC.

Medium	Supplier/Origin
Blood agar base	BDH/England
Brain-heart infusion agar	
Brain-heart infusion broth	
DNase agar	Difico/USA
Mannitol salt agar	
Nutrient agar	FLUKA/Germany
Nutrient broth	

2.1.4.2 Laboratory prepared media (Atlas *et al.,* 1995).

- **Blood agar**

It was prepared by dissolving 37g of blood base agar in 950 ml of D.W. and autoclaved after pH was adjusted to 7.6. After cooling to 50ºC, 5% of the blood was added, mixed well, and distributed into petri-dishes under aseptic conditions.

- **Luria –Bertani broth**

This broth medium contained the following components:

Component	Quantity(g)
Peptone	10
NaCl	10
Yeast extract	5

All components were dissolved in 950ml D.W, pH was adjusted to 7.5, and the volume was completed to one liter before sterilization by autoclaving.

2.1.5 Antibiotic discs

The following antibiotic discs were used for antibiotic sensitivity test, Results of inhibition zone of *S. aureus* were then compared with those indicated in NCCLS (2002) for *S. aureus* as shown in table (2-1).

Table (2-1): Antibiotic discs used for antibiotic sensitivity test of *S. aureus* local isolate, and diameter of inhibition zone according to NCCLS (2002).

Antibiotic	Symbol	Concentration (µg/disk)	Diameter of Inhibition zone (mm)		
			R	I	S
Gentamycin	Gm	10	12	13-14	15
Cephalothin	CL	30	14	15-17	18
Cefotaxime	CTX	30	14	15-22	23
Erythromycin	E	15	13	14-17	18
Penicillin	P	10	20	21-28	29
Amoxicillin	Amx	25	11	12-19	20
Fucidic acid	FA	10	14	15-22	23
Imipenem	IPM	10	13	14-15	16
Tetracycline	TE	30	14	15-18	19
Pipracillin	PPI	100	17	18-20	21
Streptomycin	S	10	11	12-14	15
Vancomycin	VA	30	9	10-11	12

R:Resistant I:Intermediate S: Sensitive

2.1.6 Buffers and solutions

Buffers and solutions used in this study were prepared as follows:

2.1.6.1 Solutions used for *S. aureus* identification

All solutions used for *S. aureus* identification were prepared according to Atlas *et al.,* (1995) and as follows:

- **Catalase reagent**

This reagent was prepared to be consisting of 3% hydrogen peroxide and used for catalase production test.

- **Oxidase reagent**

This reagent was prepared by dissolving 1 % N, N, N, N-tetra methyl p-phenylen-diamine dihydrochloride in 100 ml distilled water and kept in a dark bottle at 4ºC until use .

2.1.6.2 Mutagenesis solutions

All solutions used to induce *S. aureus* mutation were prepared according to Atlas *et al.,* (1995) and as follows:

- **Normal saline solution 0.85%**

This solution was prepared by dissolving 0.85g NaCl in 100ml distilled water, and then sterilized by autoclaving.

- **Phosphate buffer solution (0.2M)**

  This solution was prepared by dissolving 9.52g of K_2HPO_4 and 6g of KH_2PO_4 in 950 ml, pH was adjusted to 7, and then the volume was completed to 1000 ml with, and sterilized by autoclaving.

- **N-methyl-N-nitrosoguanidine (MNNG) stock solution(100µg/ml)**

  Stock solution of MNNG was prepared by dissolving 10mg in 10 ml of phosphate buffer solution.

- **Lysozyme Stock solution (500µg/ml)**

  Stock solution of lysozyme was prepared by dissolving 10mg of lysozyme in 20 ml sterilized distilled water, and then sterilized by filtration.

2.1.6.3 DNA extraction solutions

All solutions used for DNA extraction from *S. aureus* were prepared according to Sambrook and Russell (2001) and as follows:

- **Tris-EDTA buffer solution(TE)**

  This solution was prepared to be consisting of 10mM Tris HCl (pH 7.4) and 1mM EDTA (pH 8).

- **Sodium chloride Tris-EDTA buffer solution (SET)**

  This solution was prepared to be consisting of 100mM NaCl, 1mM EDTA and 10mM Tris-HCl (pH 8).

- **Sodium dodecyle sulphate solution (SDS) 10%**

  This solution was prepared by dissolving 10g of SDS in 100 ml of distilled water.

- **Tris-borate Buffer solution TBE(1X)**

  This solution was prepared Sambrook and Russell (2001) to be consist of 0.098 M Tris- HCl, 0.089M boric acid and 0.002 M EDTA.

- **Agarose gel (0.7%)**

  Agarose gel was prepared by dissolving 0.35 g of agarose in 50 ml of Tris borate (1X). Crystals of agarose were completely dissolving under heating.

- **Ethidium bromide**

  Ethidium bromide solution was prepared by dissolving 0.05g of ethidium bromide in 10 ml of distilled water.

- **Loading buffer**

  This solution was prepared to be consist of 30 % sucrose, TBE (1X), 20% distilled water and 0.25% bromophenol blue.

2.2 Methods

2.2.1 Sterilization methods

- **Autoclaving**

Media and solutions were sterilized by autoclaving at 121ºC for 15 minute (15 psi pressure).

- **Oven sterilization**

Glasswares were sterilized in an oven at 180ºC for 3 hours.

- **Filter sterilizing**

Lysozyme solution was sterilized by filtration using 0.22µm Millipore filter unit.

2.2.2 Re-identification of *S. aureus* local isolate

Staphylococcus aureus isolate that was obtained in a previous study was re-identified by conducting a few morphological and biochemical tests.

2.2.2.1 Cultural characteristics (Koneman *et al.*, 1992)

Different Cultural characteristics (color, shape, edge, and size) of *S. aureus,* was studied on brain heart infusion agar. After 16 hours of incubation at 37ºC.

- **Gram's stain (Harley and Prescott, 1996)**

Single colony of the bacterial isolate was smeared on a clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with 70% alcohol, and counterstained with safranine, then examined under light microscope.

2.2.2.2 Biochemical tests

- **Catalase test (Atlas *et al*., 1995)**

A drop of hydrogen peroxide solution (3%) was placed onto single colony of *S. aureus* on a clean and sterilized microscopic slide. Production of gaseous bubbles indicates a positive result.

- **Oxidase test (Atlas *et al*., 1995)**

This test was performed by moistening filter paper with few drops of a freshly prepared N,N,N,N-tetra methyl p-phenylen-diamine dihydrochloride solution, then a single colony of *S. aureus* was smeared on the moisten filter paper. The development of violet or purple color within 10 minutes indicates a positive result.

- **Coagulase test (Benson , 2002)**

In this test, loopful of fresh culture of *S. aureus* was used to inoculate human plasma in sterile test tubes, and incubated at 37°C. Solidification of plasma within 6 hours indicates a positive result.

- **DNase test (Harley and Prescott, 1996)**

This test was achieved by streaking DNase agar plates with fresh culture of *S. aureus* for 6 hours, then drops of 0.1N HCl were added to each plate. Appearance of clear zone around the colonies indicated a positive result.

- **Hemolysin production test (Cruickshank *et al*., 1975)**

This test was achieved by streaking freshly prepared blood agar plate with the fresh culture of *S. aureus*. Appearance of clear zone around the colonies after 16 hours of incubation at 37ºC indicates a positive result.

- **Mannitol fermentation test (Benson, 2002)**

This test was achieved by streaking *S. aureus* isolates on Mannitol salt agar plate and incubated at 37ºC for 16 hour. Changing in color from red to yellow indicates a positive result.

- **Growth at 45 ºC (Atlas *et al*., 1995)**

Test tube containing BHI broth was inoculated with overnight culture of bacterial isolate, incubated at 45 ºC for 24 hours. Bacterial growth (turbidity) was regarded as a positive result.

- **Growth at 15 ºC (Atlas *et al*., 1995)**

Test tube containing BHI broth was inoculated with overnight culture of bacterial isolate, incubated at 15 ºC for 24 hours. Bacterial growth (turbidity) was regarded as a positive result.

2.2.3 Maintenance of bacterial strains

The isolate of *S. aureus* used in this study was maintained according to (Maniatis *et al.,* 1982) as follow:

- **Short term storage**: bacterial isolate was maintained for few weeks on nutrient agar plates. The plates were tightly wrapped in parafilm and stored in refrigerator at 4ºC.

- **Medium term storage**: bacterial isolate was maintained in stab culture for few months .Such culture was prepared in screw-capped bottles containing 5-8 ml of nutrient agar and stored in refrigerator at 4ºC.

- **Long term storage**: bacterial isolate was maintained for few years in media containing 15% glycerol at low temperature without significant loss of viability, this was done by adding 1.5 ml of glycerol to 10 ml of an exponential growth culture of the bacterial isolate in sterile screw capped bottle with final volume 10ml and stored at -20 ºC.

2.2.4 Mutagenesis of *Staphylococcus aureus*

Staphylococcus aureus was subjected to chemical mutagenesis using (N-methyl-N-nitro-N-nitrosoguanidine) and physical mutagenesis using (ultraviolet radiation) to induce mutation as follows:

2.2.4.1 Chemical mutagenesis

Mutagenesis by MNNG was achieved according to Matsuhisa e*t al.,* (1980) .To prepare for mutagenesis , L-broth media was inoculated with 0.1 ml of fresh culture of *Staphylococcus aureus,* and incubated over night at 37ºC, from this culture 0.1 ml was used to inoculate 5ml of L-broth and incubated for 3 hours with shaking (150 rpm),then cells were centrifuged for 10 minutes at 3000 rpm ,washed twice with normal saline and resuspended in 5 ml of phosphate buffer (pH7.0) containing 100µg/ml of N-methyl-N-nitro-N-nitrosoguanidine and incubated at 37ºC for 30 min, during this period 0.1 ml aliquots of the cell suspension was taken every 5 min, diluted to appropriate dilution and spread on brain-heart infusion agar plates. Plates were then incubated at 37ºC over night to determine viable count and survivals of *Staphylococcus aureus*. Lysozyme sensitive mutants were screened by replica plating on brain heart infusion agar plates containing 3.4µg/ml and 12.5µg/ml of lysozyme.

2.2.4.2 Physical mutagenesis

Mutagenesis by UV irradiation was done according to Chapple *et al*., (1991) by subjecting fresh culture of *Staphylococcus aureus* suspended in phosphate buffer solution (pH7.0) to UV radiation in a dark place using the UV-transilluminator. The tray of the irradiation approximately 15X25 cm exposes sample in glass Petri dish and the distance between the UV source and irradiated suspension was 11 cm.

The suspension of *S. aureus* was prepared by inoculating 5 ml of L-broth with single colony of *S. aureus* over night at 37ºC , cells were then precipitated at 3000 rpm for 15 min, and washed twice with normal saline and resuspended in 5 ml of phosphate buffer (pH 7), the cell suspension were poured in sterilized

Petri dishes and subjected to 1, 2, 3, 4, and 5 J/m² UV of irradiation, then 0.1ml of cell suspension was taken after each treatment diluted to appropriate dilution and spread on brain-heart infusion agar plates, plates were then incubated over night at 37ºC to determine the viable count and survivals of *Staphylococcus aureus*. Lysozyme sensitive mutants were screened by replica plating on brain heart infusion agar plates containing 3.4µg/ml and 12.5µg/ml of lysozyme.

2.2.5 Combined effect of penicillin and lysozyme on *S. aureus* cell wall

To reduce the resistant of *S. aureus* to the hydrolytic activity of lysozyme, the isolate cell wall was weakened by incubating the bacterial cells with the minimum inhibitory concentration of penicillin and then treated with lysozyme.

2.2.5.1 Minimum inhibitory concentration (MIC) test (Atlas *et al.*, 1995)

Inocula of *S. aureus* isolate was grown on 5ml nutrient broth, then 0.1ml of the culture was inoculated in series of 5ml fresh nutrient broth containing various concentration of penicillin (10, 20, 40, 80, 160, 320, 640, and 1280u/ml), then all tubes were incubated at 37ºC for 24 hr. then of 100µl aliquot from each tube were taken and spread on brain-heart infusion agar plates were spread on brain heart infusion agar and incubated at 37ºC for 24 hr. the lowest concentration of the antibiotic solution that inhibited the growth of bacterial isolate considered the Minimum inhibitory concentration (MIC).

2.2.5.2 Hydrolysis *S. aureus* cell wall

Synergistic effect of penicillin and lysozyme on *S. aureus* cell wall was achieved according to Aldrich and Sword (1963), lysis of *S. aureus* cell wall was accomplished by adding 10 ml of 8-12 hour culture to five flasks containing 30

ml of nutrient broth contain the minimum inhibitory concentration of penicillin prepared as described in 2.2.5.1 and incubated with shaking at 37˚C. Optical density (OD_{600}) for growth cultures was measured before and after the addition of penicillin for 1, 2, 3, 4, 5 hours.

Each culture treated with penicillin in addition to the control of five hours incubation, were incubated with lysozyme at a final concentration of 250µg/ml for two hours and the optical density (600 nm) were measured. Conversion of *S. aureus* cells to protoplasts was observed by microscope after each treatment.

2.2.6 Plasmid profile

Total DNA of *S. aureus* was extracted using Birnboim and Doly, (1982) alkaline lysis method and as follows:

- Hundred ml of fresh culture of *S. aureus* in BHI broth was centrifuged in centrifuge at room temperature for 5min.
- Cell pellets were resuspended in the same volume of SET buffer solution (pH 8), the cells were recenterifuged, suspended in 0.5ml ice-cold acetone, and kept on ice for 5 minutes.
- The cells were centrifuged again and the acetone was decanted and residual acetone was removed with gentle stream of air.
- Bacterial pellets were resuspended in lysis buffer. Lysozyme was then added at final concentration 200µg/ml and the mixture was incubated at 37°C for 30 minutes.

- Lysis was achieved by adding 0.4 ml of SDS (10%). Cellular debris was removed by centrifugation 12000rpm for 15min.
- The supernatant was then extracted twice with an equal volume of a mixture of chlorophorm- isoamyl alcohol (24:1 v/v).
- The DNA was precipitated with (0.6 v/v) isopropanol alcohol; the mixture was incubated at -20°C overnight and recentirfuged 12000rpm for 15 minutes.
- The supernatant was decanted and precipitate was washed with 70% ethyl alcohol, centrifuged again and the precipitate was dried with gentle stream air.
- TE buffer (0.025 ml) was added for gel electrophoresis analysis.

2.2.7 Agarose gel electrophoresis

Agarose gels (0.7%) were run horizontally in Tris Borate- EDTA buffer (TBE 1 X), samples of extracted DNA were mixed with loading buffer in 1:10 ratio and added to the wells on the gel. Generally, gel was run for 2-3 h at 5 $v.cm^{-1}$ and the agarose gel was stained with ethidium bromide by immersing them in ethidium bromide solution (0.5 µg/ml) for 30-45 min, DNA bands were visualized by U.V in transilluminator cabinet, and photographed.

2.2.8 Characterization of lysozyme sensitive mutants

2.2.8.1 Antibiotic sensitivity test (Atlas *et al*., 1995)

The disc diffusion method was used to test the antibiotic sensitivity of the bacterial isolate .A sterile cotton swap was applied in to the inocula (fresh culture for 18 hour) and the entire surface of the brain heart infusion agar plates was swabbed three times by rotating the plate approximately 60º after each streaking to ensure even distribution. Then the disc of antibiotics were applied on cultured media and incubated at 37ºC. The zone of inhibition was measured after incubation for 16 hour.

2.2.8.2 Protoplasts formation of *S. aureus* mutants by lysozyme

Protoplast formation of *S. aureus* involves the conversion of selected mutant cells into protoplast using lysozyme alone was achieved according to Yabu and Huempfner, (1974).

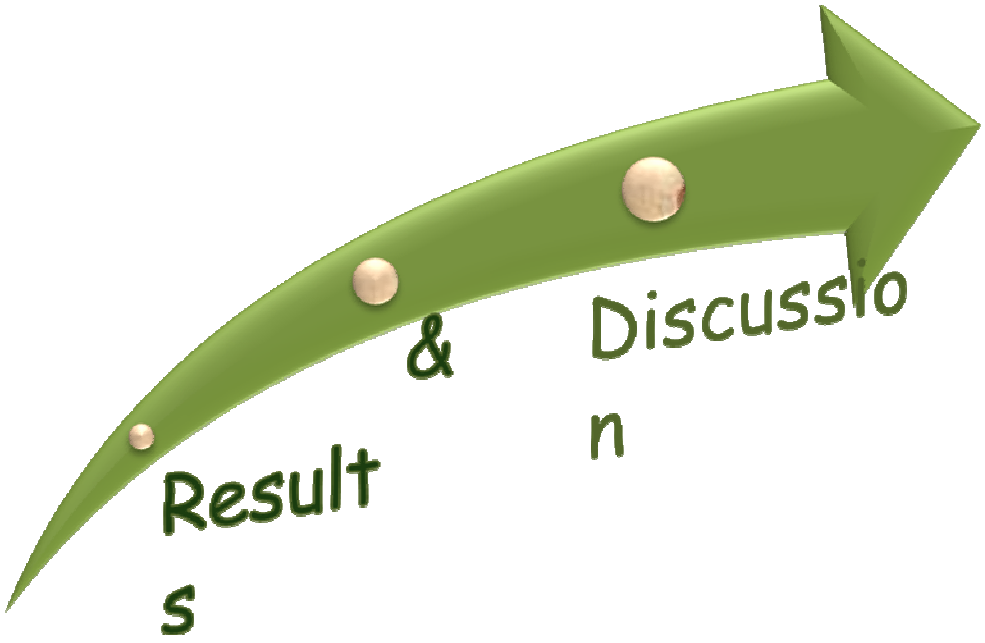
Two lysozyme sensitive mutants of *S. aureus* obtained after mutagenesis by MNNG (S1) and UV radiation (S2) were selected by replica plating on brain heart infusion agar as described previously in 2.2.5.1 and 2.2.5.2 and were inoculated into 50ml of brain heart infusion broth and incubated over night at 37˚C with shaking until optical density of the growth medium at OD_{600} measured 0.50, then five ml of each growth culture were centrifuged at 3000rpm for 10min and resuspended in phosphate buffer (pH 7). Then lysozyme was added to the cell suspension at final concentration of 50µg/ml and incubated at 37˚C with shaking 150rpm for 2 hours, the formation of protoplasts was observed by light microscope.

2.2.8.3 Temperature sensitive growth

Temperature sensitive growth was tested in lysozyme sensitive mutants of *S. aureus* and compared with the wild type the procedure was described by Dyer and Iandolo, (1983).

Single colony of *S. aureus* (wild type) and the mutants cells arose after MNNG mutagenesis (S1) and UV radiation mutagenesis (S2, S3, S4, S5) which were selected as described previously in 2.2.5.1 and 2.2.5.2 by replica plating on brain heart infusion agar, were inoculated into 50ml brain heart infusion broth over night at 37˚C, then 0.1ml of each culture spread on brain heart infusion agar and incubated over night at 30, 37, 40, 43 and 45˚C, then the growth was observed and survivals were counted after each incubation.

Chapter Three



3. Results & Discussion

3.1 Re-identification of *S. aureus*

3.1.1 Morphological characteristics

Staphylococcus aureus was isolated and identified in a previous study (Zeidan, 2007). It was re-identified and examined for some cultural and biochemical characteristics. Results of re-identification showed that this isolate was Gram positive cocci, appears as grape like clusters when viewed under light microscope, has yellow, round and large colonies, when cultured on brain heart infusion agar plates.

The colonies on solid media are smooth, raised, and circular. Colonies size of 6-8 mm diameter was found on non-selective media (BHI).

3.1.2 Biochemical tests

In order to confirm that this isolate was *S. aureus,* some biochemical tests were performed. Result indicated in table (3-1) showed that this isolate gave a positive results for catalase (because it was able to degrade hydrogen peroxide to oxygen and water), Coagulase (because it was able to coagulate the blood), and Dnase (because it was able to degrade deoxyribonucleic acids), while it was negative for oxidase.

Results also showed that this isolate was able to produce hemolysin that caused blood hemolysis (type β- hemolysis) when it was cultured on blood agar medium. Other biochemical tests showed that this isolate was able to utilize

mannitol salt, and grow at both 15ºC and 45ºC. These results were in agreement with those mentioned by Morse, (1981) and Seifert *et al.*, (2003).

Table (3-1): Biochemical characterization identification of *S. aureus*

Test	Result
Oxidase	-
Catalase	+
Coagulase	+
DNase	+
Hemolysin production	+
Mannitol fermentation	+
Growth at 45 ºC	+
Growth at 15 ºC	+

Positive test + Negative test -

3.2 Mutagenesis of *Staphylococcus aureus*

Staphylococcus aureus was subjected to mutagenesis by using two types of mutagens, the chemical mutagen (N-methyl-N-nitro-N-nitrosoguanidine) and the physical (Ultraviolet radiation). These lethal and mutagenic treatments were

employed to make *S. aureus* cell wall more susceptible to lysozyme hydrolyzing action.

3.2.1 Chemical mutagenesis

Results indicated in figure (3-1) shows the killing effect of MNNG on bacterial cells. Total viable count of *S. aureus* was decreased from $104x10^6$ CFU/ml zero time of subjection to mutagen to $30x10^6$ after incubation with MNNG in a concentration of 100µg/ml for 5 min. Survival percentages were decreased to 28.8, 20, 19.2 ,15.4 , 13.5 and 2.4%after subjection to MNNG for 5, 10, 15, 20, 25 and 30 minutes respectively.

According to Manson *et al.*, (1998) survivals obtained after subjection to the killing effect of mutagen (caused 90% killing and above) where screened for mutation.

In this study 90% killing was achieved after subjection to MNNG for 30 minute the colonies were spread on brain heart infusion agar and incubated at 37°C for 24 hours in order to screen the lysozyme sensitive mutants. After incubation, 180 colonies were selected and replica plated on brain heart infusion agar containing 3.4 and 12.5µg/ml lysozyme, results showed that all of these colonies were able to grow on brain heart infusion agar containing 3.4µg/ml lysozyme. It has been reported that the acetyl groups linked to the peptidoglycan in certain strains of *Micrococcus*, prevented the action of lysozyme. The same O-acetyl groups have been found in the cell wall peptidoglycan of *S. aureus* and may be responsible for lysozyme resistance. Other structural properties of the *S. aureus* peptidoglycan may also be responsible for the lysozyme resistance (Peschel *et al*, 1999).

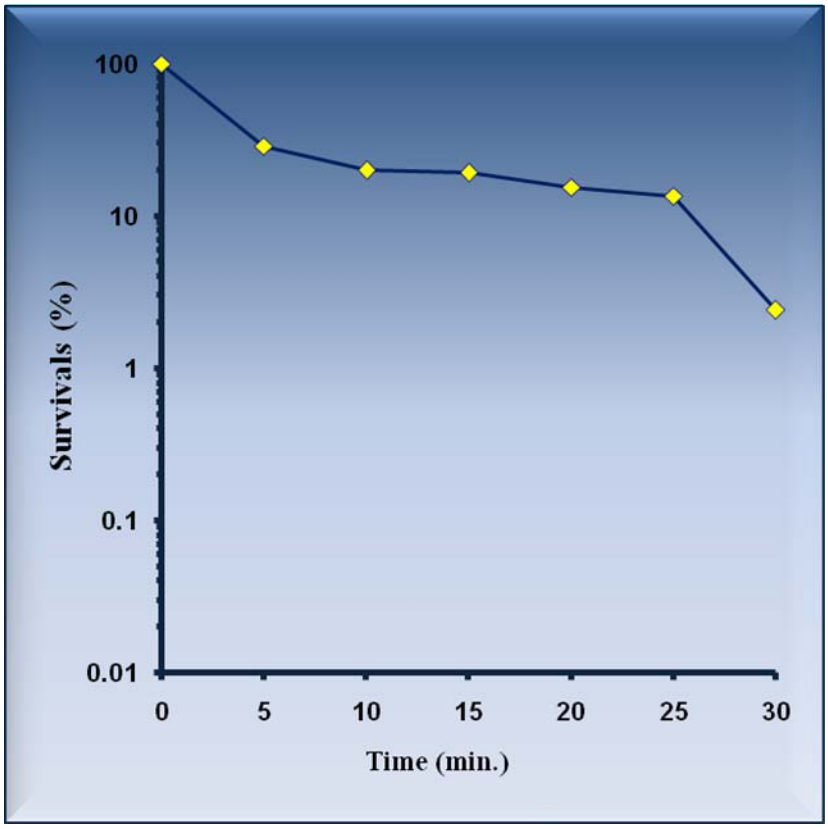


Figure (3-1): Survival curve of *S. aureus* after subjection to mutagenesis with MNNG (100 µg/ml).

Moreover result in figure (3-2) showed that one of the colonies was unable to grow on the medium containing lysozyme 12.5µg/ml. such results indicates that this colony may be lysozyme sensitive mutant, due to the structural changes in the cell wall caused by MNNG, which is known to be a methylating agent that can alkylate DNA at many sites producing a wide variety of lesions; it can cause misspairs during DNA replication and are therefore mutagenic. While its lethal

effect results throughout its ability to interfere with unwinding of the DNA during replication and transcription (Hix *et al*., 1995).

Many studies aimed to use MNNG in order to isolate lysozyme sensitive mutants from different bacterial species (*Mycobacterium smegmatis*, and *Corynebacterium glutamicum*) with complex cell wall nature that makes them relatively resistant to the hydrolytic action of lysozyme (Yabu and Huempfner, 1974; Hirasawa *et al*., 2000).

Figure (3-2): Screening lysozyme sensitive mutants of *S. aureus* after subjection to chemical mutagenesis by MNNG.
A: colonies plated on BHI agar (master plate).
B: colonies plated on BHI agar containing 12.5µg/ml lysozyme.

In order to confirm that this mutant symboled S1 was lysozyme sensitive mutant because of the structural changes in its cell wall, total genomic DNA was extracted from S1 by the alkaline lysis method using lysozyme instead of lysostaphin to complete lysis of cell wall.

3.2.2 Physical mutagenesis

Another type of mutagenesis was used to generate lysozyme sensitive mutant using physical mutagen by UV radiation which is the most potent physical mutagen. DNA is one of the key targets of UV-induced damage; therefore, organisms have developed a number of repair mechanisms to counteract the DNA damage caused by UV. These repair mechanisms will however be unable to cope if the UV dose applied is higher than the repair capacity (Danon *et al*., 2004).

Physical mutagenesis was achieved using UV radiation by subjection fresh cultures of *S. aureus* to different doses of UV ray. Results indicated in figure (3-3) showed that this mutagen has a lethal effect on bacterial cell; this can be noticed from the reduction of the viable count of bacterial cells from $46x10^7$ CFU/ml at the zero time to $13x10^7$ CFU/ml after exposure to the first dose of UV ray ($1J/m^2$). Percentage of survivals after subjection to the first dose of UV was decreased to 28.2%, then exposure to the next doses of UV ray (2, 3 and $4J/m^2$) causes high reduction in survival percentages to 6.9, 2.6, and 1.3%, respectively, while the survivals was decreased to zero% after subjection to the final dose ($5J/m^2$) of UV ray.

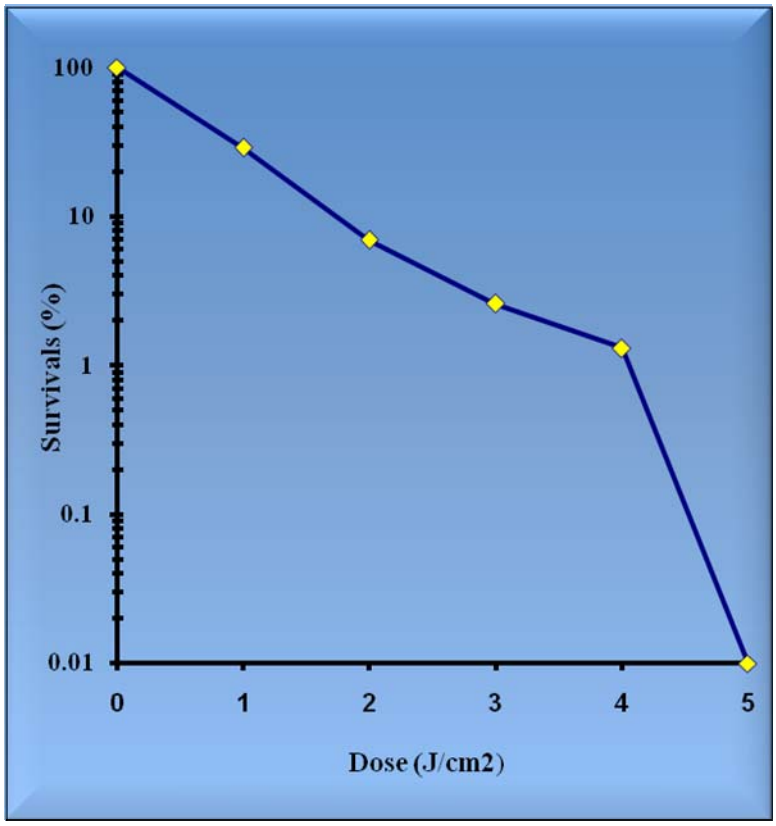


Figure (3-3): Survival curve for *S. aureus* after subjection to different doses of UV radiation (J/m^2).

After subjection to different doses of UV ray, 100ml aliquots of the cell suspension subjected to the killing effect of mutagen 90% killing and above was taken and spread on brain heart infusion agar plates then incubated at 37°C for 24 hours in order to screen the lysozyme sensitive mutants. After incubations,

160 colonies were selected and replica plated on brain heart infusion agar containing 3.4 and 12.5µg/ml lysozyme, Results showed that all of the selected colonies were able to grow on brain heart infusion agar containing 3.4µg/ml lysozyme. While results in figure (3-4) showed that four of the total colonies were unable to grow on the medium containing 12.5µg/ml lysozyme. These colonies were probably affected by the mutagenic activity of UV radiation that may caused alterations in deoxyribonucleic acid (DNA) (Matsumura and Ananthaswamy, 2004).

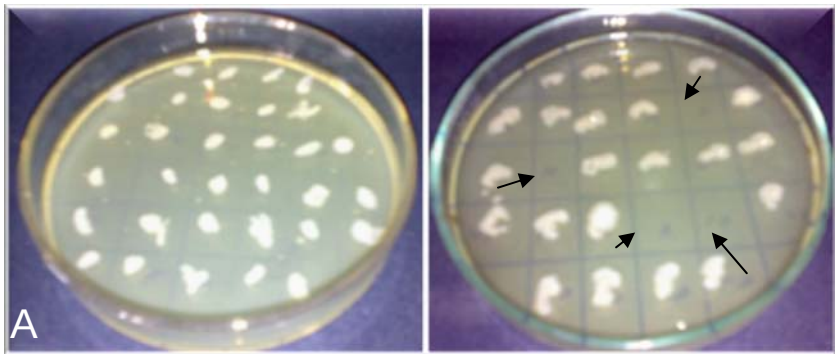


Figure (3-4): Screening lysozyme sensitive mutants of *S. aureus* after subjection to physical mutagenesis by UV radiation.
A: colonies plated on BHI agar (master plate).
B: colonies plated on BHI on agar containing 12.5µg/ml lysozyme.

The biochemical and genetic properties of mutant *S. aureus* caused by UV radiation were described, and their usefulness for studies of staphylococcal plasmids was discussed by Inoue *et al*., (2001). Mutant cells produced as a result

of defect in deoxyribonucleic acid repair. The differences among *S. aureus* isolates in regard to UV radiation susceptibility may relate to their pigmentation, since pigmented isolates were found to be more resistant to the effect of UV radiation (Sheagren, 1984). UV radiation causes DNA damage and mutagenesis of the bacterial chromosome via affecting the genes included in the SOS regulatory system (Dunny *et al*., 1995). It was applied as mutagen to isolate osmotically fragile mutant from *Bacillus subtilis* (Slotboom *et al.,* 1970) and to count the survivals of *E. coli* (Battista *et al*., 1990).

In a previous study Neuhaus and Baddiley, (2003) stated that with the proper mutation, *S. aureus* cells were rendered to lysozyme hydrolysis. Recently, Biswas *et al*., (2006) reported that *S. aureus* cell wall with a deletion mutation in oat A and/or tag O is more susceptible to lysozyme than the wild type. These two genes and their corresponding enzymes were identified for their contribution to the resistance of *S. aureus* against hydrolytic activity of lysozyme. oat A catalyzes O-acetylation modification in the cell wall , while tag O catalyze the synthesis of wall teichoic acid (Goerke, 2005).

In order to confirm that many lysozyme-sensitive mutants could be isolated after the subjection to mutagenesis by chemical (MNNG) and physical (UV radiation) mutagens, genomic DNA was extracted from the wild type; lysozyme sensitive mutants arose after subjection to mutagenesis by MNNG (mutant S1) and UV (mutants S2, S3, S4, and S5).

As shown in figure (3-5), lysozyme sensitive mutants (S1, S2, S3, S4, and S5) showed clear chromosomal and plasmid DNA bands on agarose gel which indicates lysis of these mutants; the wild type, on the other hand, was resistant to

lysis by lysozyme. These results confirmed that lysozyme sensitive mutants isolated by chemical and physical mutagenesis have altered bacterial cell wall structure caused by the genetic mutations that may occur in the structural genes responsible for the construction of bacterial cell wall. It appears that lysozyme sensitive mutants provide obvious economical advantages over the wild type strain for the preparation and study of macromolecules.

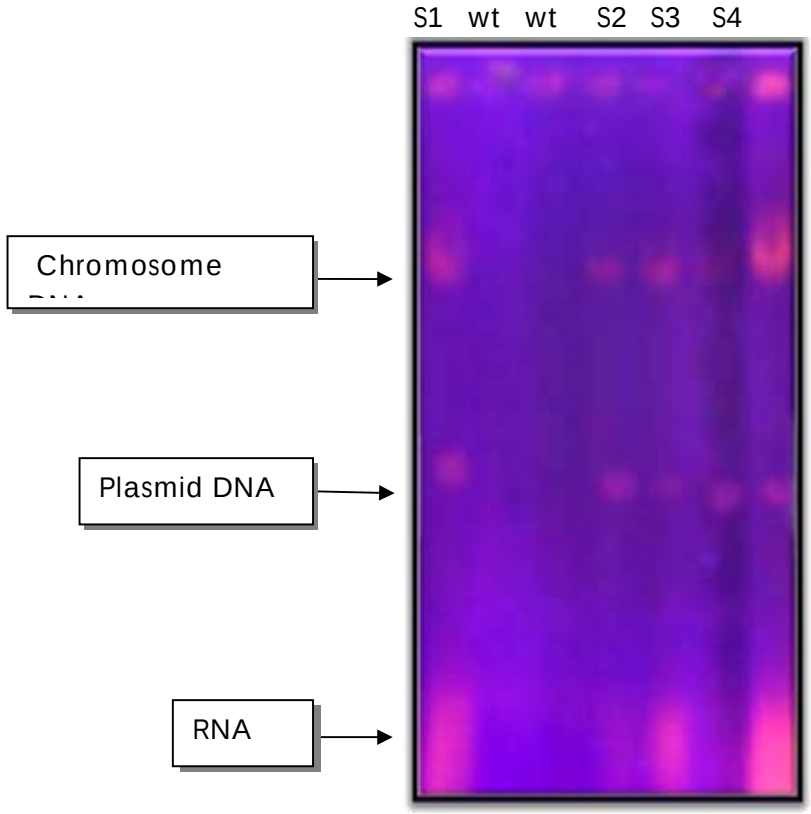


Figure (3-5) Genomic DNA extracted from *S. aureus* after subjection to mutagenesis by UV and MNNG. on agarose gel (0.7%) electrophoresed for 2hours at 5v/cm.
Lane 1: DNA extracted from lysozyme sensitive mutants S1.
Lane 2, 3: DNA extracted from wild type.
Lane 4: DNA extracted from lysozyme sensitive mutants S2.
Lane 5: DNA extracted from lysozyme sensitive mutants S3.

Lane 6: DNA extracted from lysozyme sensitive mutants S4.
Lane 7: DNA extracted from lysozyme sensitive mutants S5.

3.3 Combined effect of penicillin and lysozyme on *S. aureus* cell wall

Staphylococcus aureus are not easily lysed by mechanical or chemical treatments. Penicillin can inhibit *S. aureus* cell wall and can cause formation of fragile cell wall; hence addition of lysozyme in combination with penicillin was studied to facilitate cell fractionation and extraction of DNA (Fahlgren *et al*., 2003).

3.3.1 Minimum inhibitory concentration of penicillin against *S. aureus*

Penicillin decreases cross-linkages of bacterial cell wall by inhibiting transpeptidase that catalyzes the final step in cell wall biosynthesis. Incubating *S. aureus* with lysozyme and the MIC concentration of penicillin may result in increasing the bacterial cell wall sensitivity against lysozyme (Keshav *et al*., 1991)

Results of minimum inhibitory concentration of penicillin showed that *S. aureus* isolate was still resistant to wide range of penicillin concentrations between (10-640u/ml), but it was sensitive to the next concentrations (1280u/ml). As shown in table (3-2), although the degree of *S. aureus* sensitivity against penicillin isolated from different sources varies. It was reported that 97% of *S. aureus* isolates were resistant to the penicillin (Uwaezuoke and Aririatu, 2004; Farzana *et al*., 2004).

Table (3-2): Minimum inhibitory concentration (MIC) of penicillin against the parent isolate of *S. aureus*

Penicillin concentration (u/ml)	Growth
10	+++
20	+++
40	+++
80	++
160	++
320	+
640	+
1280	-

- No growth +++ Very good growth ++ Good growth + Low good

Johnson, (2001) stated that *Staphylococcus aureus* has become resistant to many commonly used antibiotics, only 2% of all *S. aureus* isolates are sensitive to penicillin. The β-lactam antibiotics (oxacillin, cloxacillin and flucloxacillin) were developed to treat penicillin-resistant *S. aureus* and are still used as first-line treatment. Methicillin was the first antibiotic in this class to be used which

was introduced in 1959, but only two years later, the first case of methicillin-resistant *S. aureus* (MRSA) was reported in England.

3.3.2 Hydrolyzing *S. aureus* cell wall

Synergistic hydrolyzing of *S. aureus* upon exposure to penicillin and lysozyme was accomplished by partially removing cell wall of the exposed cells to minimum inhibitory concentration of penicillin (640u/ml). *S. aureus* culture with optical density OD_{600}= 0.2 was incubated with the MIC of penicillin for 1, 2, 3, 4, and 5 hour, and the optical density was measured hourly. Results were recorded as follow: 0.32, 0.37, 0.36, 0.3 and 0.22. The higher optical density value was achieved after incubating the culture for two hours, and the lowest OD was when incubated with the MIC of penicillin for 3, 4 and 5 hour.

Since the maximal sphere formation occurred about two hours after incubation with penicillin, this time was selected to start exposing *S. aureus* cells to lysozyme treatment for two hours. Results indicated in figure (3-6) showed the effect of lysozyme on cell suspension of *S. aureus* treated with MIC of penicillin. Addition of lysozyme (250µg/ml) to cell suspension of *S. aureus* treated with penicillin and incubated for 2, 3, 4, and 5 caused rapid lysis of the cell wall of penicillin-treated bacterial cells. Optical densities were decreased to 0.35, 0.3, 0.24, and 0.2, respectively; meanwhile bacterial cells incubated under the same conditions without penicillin treatment (positive control) were not affected by lysozyme.

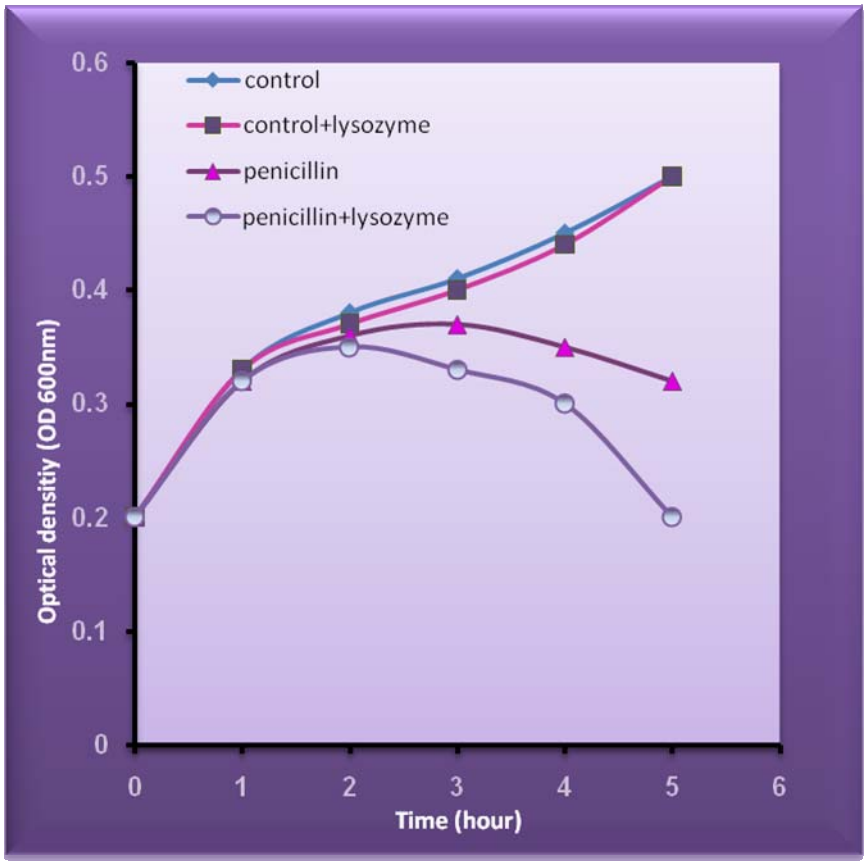


Figure (3-6): Effect of lysozyme and penicillin on the lysozyme resistance of *S. aureus* for different time of incubation at 37˚C.

Hunter *et al*., (2005) mentioned that cell wall of *S. aureus* can be altered after penicillin treatment because bacterial cells loss their ability to resist lysozyme. This study showed that cells incubated with penicillin and lysozyme showed increase lysis in comparison with untreated cells.

Lysis of *S. aureus* after incubation with lysozyme and penicillin was also observed by changes in the turbidity from turbid growth to clear as it was shown in figure (3-7, c and d); the decrease in number of spheres indicates cell lysis as time progresses.

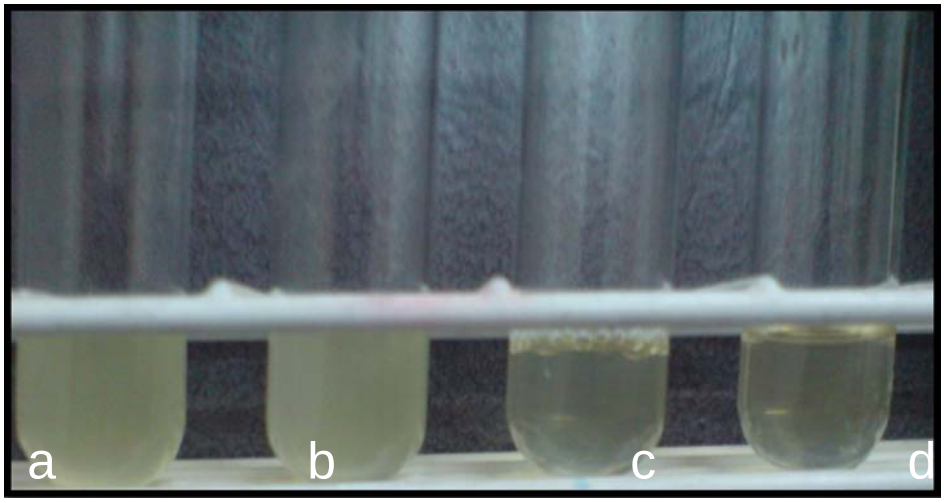


Figure (3-7): Changes in turbidity of *S. aureus* suspension incubated at 37˚C in BHI broth media.

a: After incubation of penicillin treated *S. aureus* for 2hour with (250µg/ml)lysozyme.

b: After incubation of penicillin treated *S. aureus* for 3hour with (250µg/ml)lysozyme.

c: After incubation of penicillin treated *S. aureus* for 4hour with (250µg/ml)lysozyme.

d: After incubation of penicillin treated *S. aureus* for 5hour with (250µg/ml)lysozyme.

Staphylococcus aureus cells exposed to penicillin (for 2, 3, 4, and 5 hour) and incubated with lysozyme (250µg/ml) for two hours, respectively, causes morphological changes. When these cells were examined under light microscope, they appeared in tetrad, and grape like, after the first treatment, then converted to pairs, and slightly enlarged. Morphological changes were more apparent after the third addition when the cells became more transparent, swollen and less compact. However, protoplasts formation was not observed until the addition of lysozyme to the cell suspension incubated with penicillin (for 5hours). Intracellular membrane emerged from the ruptured cell wall and became visible with the tendency to form irregular shapeless clusters. The formation of protoplasts is demonstrated in figure (3-8).

In order to confirm that combined effect of penicillin and lysozyme lysis of *S. aureus* cell wall, genomic DNA was extracted from the wild type and fresh cell suspension incubated with MIC of penicillin (640µg/ml), then treated with lysozyme in a concentration of 250µg/ml for two hours by alkaline lysis method described by Brinboim and Doly (1982).

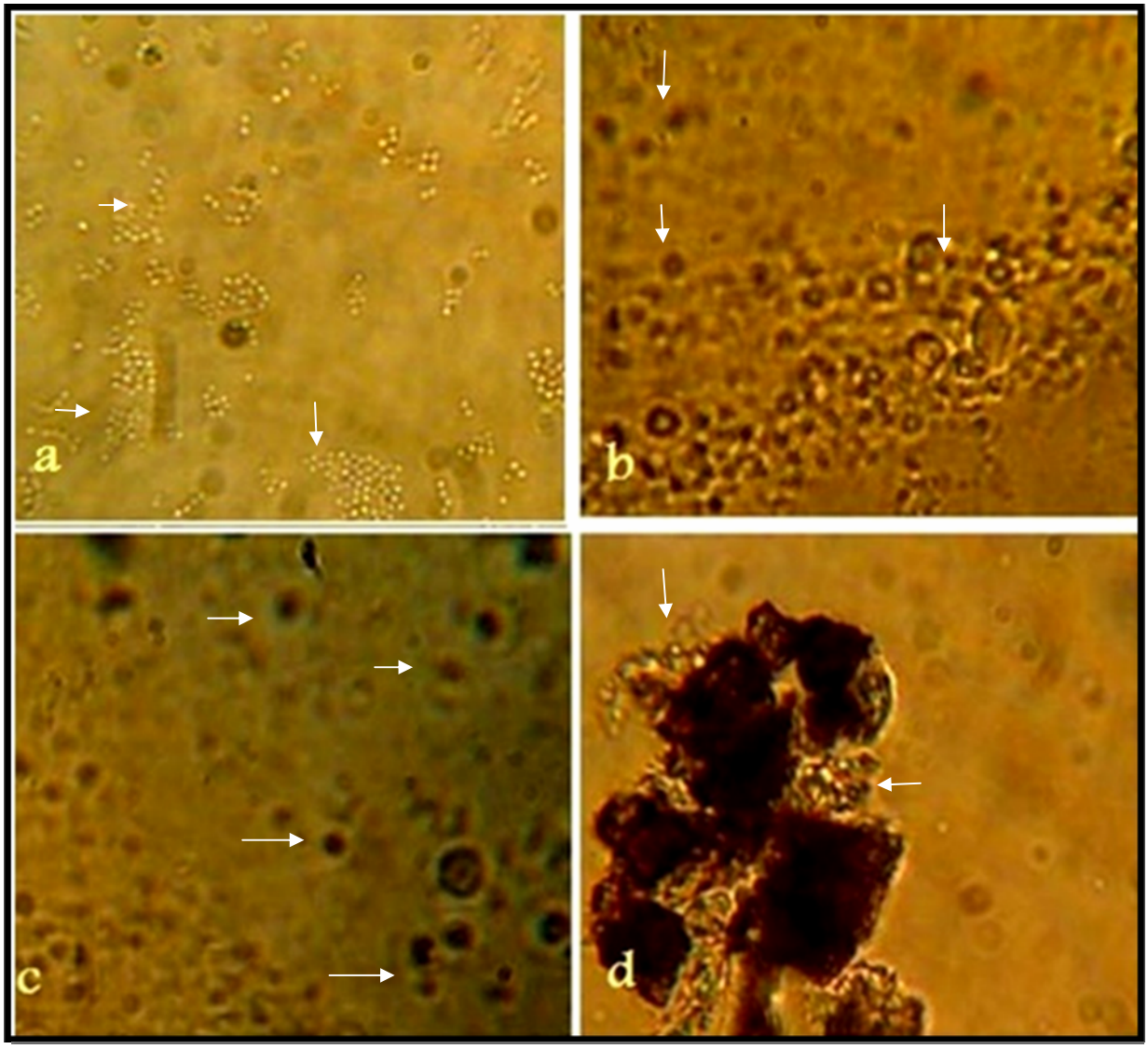


Figure (3-8) Protoplast formation of *S. aureus* after incubation with penicillin and treatment with lysozyme under light microscope

a. **Protoplast incubated with penicillin (640u/ml) for two hours and treated with 250µg/ml lysozyme.**
b. **Protoplast incubated with penicillin (640u/ml) for three hours and treated with 250µg/ml lysozyme.**
c. **Protoplast incubated with penicillin (640u/ml) for four hours and treated with 250µg/ml lysozyme.**
d. **Protoplast incubated with penicillin (640u/ml) for five hours and treated with 250µg/ml lysozyme.**

Results indicated in figure (3-9) showed clear bands of the chromosomal and plasmid DNA on agarose gel extracted from *S. aureus* pre-treated with penicillin and lysozyme, while the wild type was still resistant to the hydrolytic effect of lysozyme.

Staphylococcus aureus is difficult to lyse by the procedures commonly used for other bacterial species, and mechanical means of cell disruption may degrade deoxyribonucleic acid. The combined penicillin-lysozyme treatment facilitates staphylococcal lysis, while avoiding the dangerous associated with mechanical disruption (Archer, 1998).

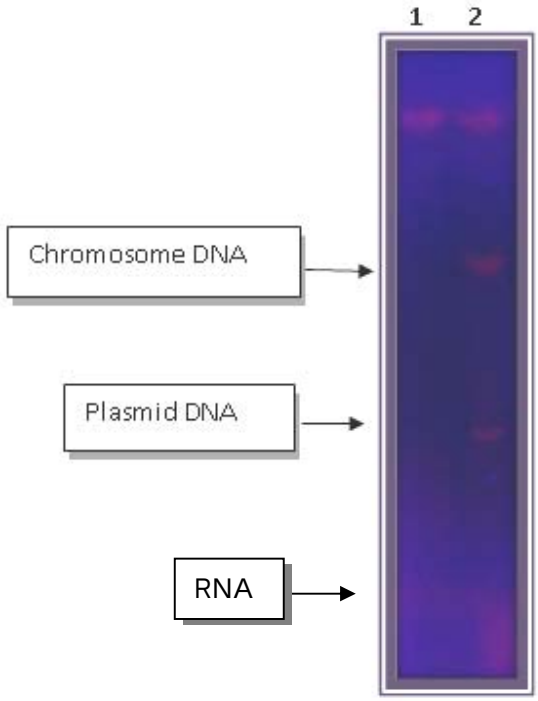


Figure (3-9): Total DNA extracted from *S. aureus* incubated with penicillin (640u/ml) for two hours at 37˚C. On agarose gel (0.7%) electrophoresed for 2hours at 5v/cm.
Lane 1: Total DNA extracted from wild type.
Lane2: Total DNA extracted from cells treated with penicillin and lysozyme.

The combined effect of penicillin and lysozyme was studied in *E. coli* and *Clostridium botulinum* to facilitate the lysis of their cell wall, penicillin blocked the cross linking of the peptidoglycan and gives arise protoplast like spheres, while lysozyme hydrolysis β 1-4 bond. The presence of lysozyme could contribute to produce better protoplasts by making possible an earlier weakening of the defective cell wall being synthesized (Dancer, 1980).

3.4 Characterization of lysozyme sensitive mutants

Staphylococcus aureus mutants (S1, S2, S3, S4 and S5) obtained after chemical and physical mutagenesis showed differences in some of their characteristics, from the wild type along with increasing sensitivity to the lysozyme effect.

3.4.1 Antibiotic sensitivity test

Antibiotics sensitivity test was performed on wild type and mutants of *S. aureus* in order to reveal that the changes resulted from the effect of MNNG and UV treatment on the antibiotics sensitivity pattern of the wild type, for this purpose twelve antibiotic discs were used in this test.

Results in table (3-3) showed that the wild type of *S. aureus* was resistant to gentamycin, cephalothin, cefotaxime, erythromycin, penicillin, streptomysin, vancomycin, pipracillin and fucidic acid, while sensitive to impenem, amoxicillin and tetracycline. These results were constitute with the findings of Maree, (2007) who stated that *S. aureus* has become resistant to many commonly used antibiotics, only 2% of all *S. aureus* isolates were sensitive to penicillin, due to the penicillinase (a form of β-lactamase).

Table (3-4) Antibiotic sensitivity of the wild type and mutants of *S. aureus* after subjection to UV mutagenesis and MNNG mutagenesis

Antibiotic		Wild type	MNNG induced mutant	UV induced mutants			
Type	Conc. µg/ml		S1	S2	S3	S4	S5
Gentamycin	10	R	R	S	R	R	R
Cephalothin	30	R	R	S	R	S	S
Cefotaxime	30	R	S	S	S	S	S
Erythromycin	15	R	S	S	S	S	S
Penicillin	10	R	R	R	R	R	R
Amoxicillin	25	S	S	S	S	S	S
Fucidic acid	10	R	R	R	R	R	R
Imipenem	10	S	S	S	S	S	S
Tetracycline	30	S	S	S	S	S	S
Pipracillin	100	R	R	R	R	R	R
Streptomycin	10	R	R	R	R	R	R
Vancomycin	30	R	S	R	S	R	R

R: Resistance S: Sensitive

However, lysozyme sensitive mutants obtained after mutagenesis with MNNG and UV were variable in their resistance to these antibiotic; for example, *S. aureus* S1 mutant obtained after mutagenesis with MNNG lost its ability to resist three different antibiotics, the other mutants (S2, S3, S4, and S5) obtained after UV radiation lost their ability to resist 4, 3, 3 and 3 different antibiotics respectively.

These results may be due to the genetic alternations in different genes that confer antibiotic resistance to *S. aureus*, occurred after mutagenesis with UV radiation and MNNG (de Jonge *et al.,* 1992).

In other study, lysozyme-sensitive mutant isolated from *Staphylococcus epidermidis* induced by (50µg/ml) of MNNG for 30minute showed same level of sensitivity in comparison with the wild type against rifampin, novbiocin, chloromophenicol, tetracycline, streptomycin, and SDS (Strominger and Ghuysen, 1967).

3.4.2 Protoplast formation of *S. aureus* mutants by lysozyme treatment

Bacterial protoplasts and spheroplasts are osmotically fragile forms that lack rigid or partially rigid cell walls. A conversion of bacterial cells into protoplasts or spheroplasts may be achieved normally by the addition of lysozyme, to the growth medium. Removing the cell wall by lysozyme has been used with success on a variety of bacteria. However, protoplasts of *Mycobacterium smegmatis*, *S. aureus*, and *Clostridium pasteurianum* cells were not readily prepared by lysozyme alone (Yabu and Huempfner, 1974).

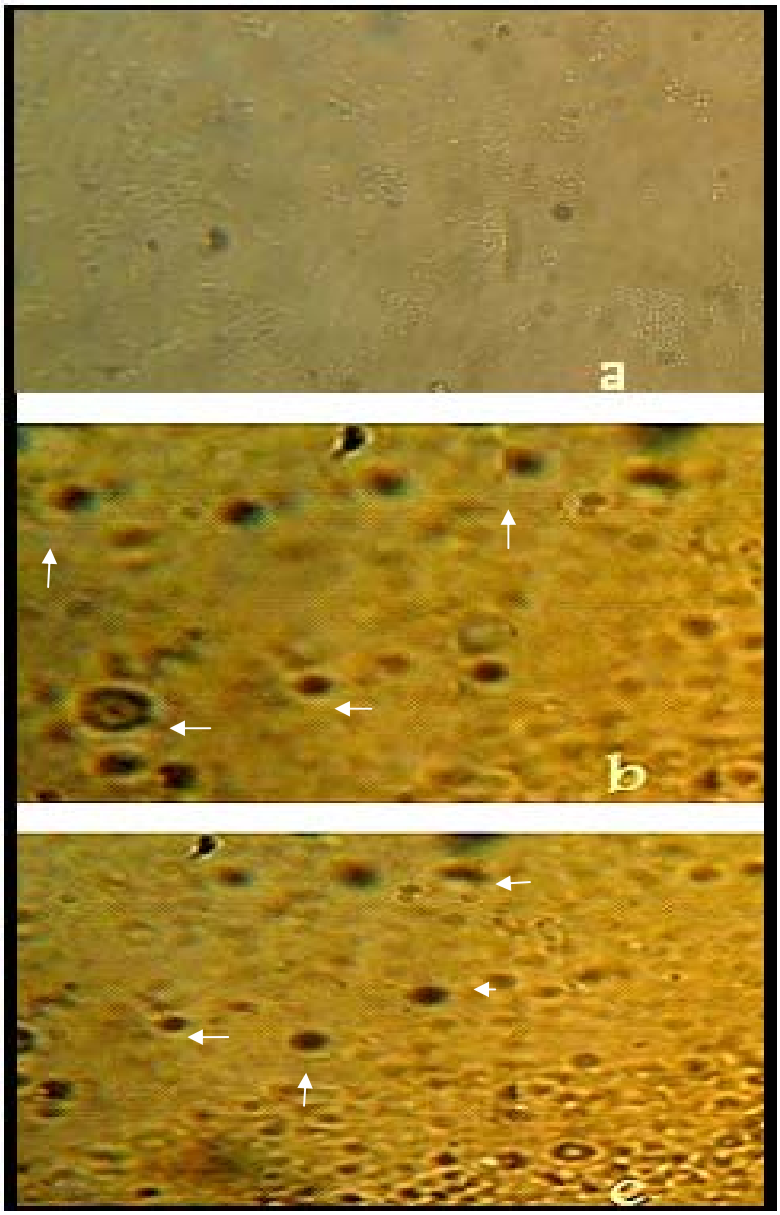


Figure (3-10): *Staphylococcus aureus* cells under light microscope.
a: wild type

b: lysozyme sensitive mutant S1 obtained after chemical mutagenesis by MNNG.

c: lysozyme sensitive mutant S2 obtained after physical mutagenesis by UV radiation.

Two lysozyme sensitive mutants of *S. aureus* isolated by MNNG (S1) and UV radiation (S2) mutagenesis were treated with lysozyme at concentration of 50µg/ml, and incubated for 2 hours alone without the addition of penicillin. The formation of protoplasts was observed by light microscope. Cells appeared as spherical bodies of various sizes and density. They were transparent and swollen in comparison with the wild type. Figure (3-10) showed the morphological changes of *S. aureus* cells.

However, prolonged incubation may be necessary to lead to cell division and loss of the cell wall, which result in protoplast formation. As a result of this treatment, the cell contents would be enclosed only by a cell membrane.

Many studies described the formation of protoplasts from mutants with defective membranes by lysozyme. Protoplast isolated from osmotically fragile *Bacillus subtilis* mutant has a very active enzyme system for the catabolism of phospholipids in comparison with the wild type. Recombinant DNA technology developed super strains of *B. thuringiensis* for more efficient production of δ-endotoxin as a result of protoplasts fusion of mutant cells (Slotboom *et al*., 1970; Samsonov and Padron, 1997).

3.4.2 Temperature-sensitive growth

Hirasawa *et al*., (2001) stated that lysozyme sensitive mutants isolated by MNNG and UV mutagenesis might show temperature sensitive growth when

incubated at elevated temperatures. *S. aureus* mutants were investigated for temperature sensitive growth and compared with the wild type.

Results indicated in table (3-4) shows the temperature sensitive growth of *S. aureus*. The wild type and mutants obtained after UV radiation (S4 and S5) showed growth at 30, 37, 40, 43 and 45˚C. While the mutant obtained after MNNG treatment and two of the mutants obtained after UV radiation (S1, S2, and S3) were unable to grow at high temperature (above 40˚C), suggesting defects in cell surface structure and the membrane integrity as results of mutagenesis (Hsieh *et al*., 1998).

MNNG and UV mutagenesis cause point mutation that produce defect in RNase. Temperature sensitive growth considered to be caused by simultaneous defect of RNase which is related to the synthesis and maintaining of the cell surface of this bacterium (Kunst *et al*., 1997).

Kaito *et* al., (2002) reported that the initiation step of DNA replication is halted in lysozyme sensitive mutants grown at non-permissive temperature. This statement was in agreement with previous findings in *B. subtilis* (Imai *et al.*, 2000).

Temperature-sensitive lethal mutants isolated from *Staphylococcus aureus*, *Bucillus subtilis* and other gram positive bacteria caused by the mutagenic effect of MNNG were found to be more susceptible to some of antibiotics classes (macrolides and lincosamides); although not all of the class were affected by this mutant (beta-lactam and glycopeptides) (Ling and Bachi, 1998).

Table (3-5): Lysozyme sensitive mutants of *S. aureus* incubated at different temperatures.

Bacterial Isolate	Growth Temperature (˚C)				
	30	37	40	43	45
WT	++	+++	++	+	+
S1	++	+++	++	-	-
S2	++	+++	++	-	-
S3	++	+++	++	-	-
S4	++	+++	++	+	+
S5	++	+++	++	+	+

+++ Very good growth ++ Good growth + Low growth - No growth

WT Wild type of *S. aureus*

S1, S2, S3, S4, S5 Lysozyme sensitive mutants

Conclusions & Recommendations

Conclusions

- Subjection of *S. aureus* to physical and chemical mutagenesis led to produce lysozyme-sensitive mutants.
- Combined effect of penicillin MIC (640u/ml) with lysozyme causes the lyses of *S. aureus* cell wall and conversion of the treated cells into protoplast.
- Total DNA can be easily extracted from lysozyme-sensitive mutants and from *S. aureus* protoplasts (produced by sublethal concentration of penicillin and lysozyme) by using lysozyme.
- Chemical and physical mutagens altered the antibiotic susceptibility of *S. aureus* to different antibiotics.
- Lysis of *S. aureus* mutant cell wall and the formation of protoplast were achieved by 50µg/ml lysozyme without the addition of penicillin.
- Broth of *S. aureus* mutants was temperature-sensitive in comparison with the wild type.

Recommendations

- Determine the type of changes in bacterial cell wall after mutagenesis with physical and chemical mutagens.
- Determine the site of mutations in chromosomal DNA for different mutants arose after physical and chemical mutagens.
- Curing plasmid DNA of *S. aureus* mutants to investigate the antibiotic resistance profile using lysozyme instead of lysostaphin by alkaline lysis method.

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الخلاصه

هدفت هذه الدراسه الى تحوير الجدار الخلوي لبكتريا *Staphylococcus aureus* باستخدام المطفرات الكيميائيه والفيزيائيه يجعل الجدار الخلوي قابل للتحلل بفعل انزيم اللايسوزايم. أجريت عملية التطفير الكيميائي بمادة النايتروسوكواندين (MNNG) وذلك بحضن عالق بكتريا *S. aureus* مع المطفر (MNNG) بتركيز ١٠٠ مايكروغرام/مل لفترات زمنيه مختلفه (٥، ١٠، ١٥، ٢٠، ٢٥ و٣٠ دقيقه)، نشر بعدها ١٠٠ مايكرولتر من عالق الخلايا المعرضه للتأثير القاتل والمطفر لهذه الماده على وسط نقيع الدماغ المتصلب بمادة الاكار وحضنت بدرجة حرارة ٣٧ م لمدة ٢٤ ساعة في الظلام ، تم بعدها الأنتقاء العشوائي لـ ١٨٠ مستعمره من المستعمرات الناميه على الوسط وغربلة قابليتها على النمو في نفس الوسط الحاوي على اللايسوزايم بتركيز ١٢,٥ مايكروغرام/مل للتحري عن الطافرات البكتيرية الحساسة للايسوزايم ، وقد تمكنت جميع المستعمرات البكتيريه من النمو في الوسط بأستثناء مستعمره واحدة رمز لها S1 عدت حساسه للايسوزايم.

أجريت عملية التطفير الفيزيائي بأستخدام الاشعه فوق البنفسجيه وذلك بتعريض عالق بكتريا *S. aureus* الى جرعات مختلفه من هذه الاشعه (١، ٢، ٣، ٤ و٥ جول/م^{٢})، نشر بعدها ١٠٠ مايكرولتر من عالق الخلايا المعرضه للتأثير القاتل والمطفر للأشعه على وسط نقيع القلب الدماغ المتصلب بمادة الاكار وحضنت بدرجة حرارة ٣٧ م لمدة ٢٤ ساعة في الظلام ، تم بعدها الأنتقاء العشوائي لـ ١٦٠ مستعمره من المستعمرات الناميه على الوسط وغربلة قابليتها على النمو في نفس الوسط الحاوي على اللايسوزايم بتركيز 3.4 و ١٢,٥ مايكروغرام/مل للتحري عن الطافرات البكتيرية الحساسة للايسوزايم ، وقد أشارت النتائج الى أن جميع المستعمرات البكتيريه الناتجه من النمو في الوسط بأستثناء اربعة مستعمرات رمز لها S2 ,S3, S4, S5 عدت حساسه للايسوزايم.

درس النسق البلازميدي لبكتريا *S. aureus* النوع البري والطافرات البكتريه S1,S2,S3, S4 , S5 الناتجة من التطفير الكيميائي والفيزيائي وذلك بأستخلاص الدنا البلازميدي بطريقة التحلل القاعدي بأستخدام اللايسوزايم بدلا عن اللايسوستافين المعروف بفعاليته الحاله لجدار بكتريا *S .aureus* وقد اشارت النتائج الى ظهور حزمه بلازميديه واحده فضلا عن الحزمه الكروموسوميه في

كل من هذه الطافرات البكتيريه S5, S4, S3, S2, S1 في حين لم تتمكن الطريقه من استخلاص الدنا البلازميدي من النوع البري وذلك لمقاومة جدارها الخلوي لأنزيم اللايسوزايم ، مما يعد دليلا على حدوث الطفرات في الجينات التركيبيه المسؤوله عن بناء الجدار الخلوي.

درس التأثير التآزري لكل من البنسلين واللايسوزيم على بكتريا *S. aureus* حيث أشارت النتائج الى ان حضن البكتريا مع التركيز المثبط الادنى للبنسلين (MIC) بتركيز ٦٤٠ وحده/مل لفترات زمنيه مختلفه (٢، ٣، ٤، ٥ ساعه) و لمدة ساعتين مع انزيم اللايسوزام بتركيز ٢٥0 مايكروغرام/مل أدى الى حدوث التحلل التدريجي للجدار الخلوي بعد اضافة اللايسوزايم وذلك من خلال نشوء الخلايا المنزوعة الجدار (البروتوبلاست) ،ثم التحلل التام بزيادة فترة الحضن. تم التحري عن النسق البلازميدي لهذه الخلايا بتحليل جدرانها الخلويه بطريقة التحلل القاعدي بأستخدام اللايسوزايم بدلا من اللايسوستافين ، وقد أشارت النتائج ايضا الى ظهور حزمة بلازميديه وكروموسوميه واحده على هلام الأكاروز بتركيز٠,٧%.

شخصت الطافرات الحساسة لانزيم اللايسوزايم الناتجه بعد تعريض *S. aureus* لتأثير المطفرات الكيميائيه و الفيزيائيه (النايتروسوكواندين والاشعه فوق البنفسجيه) وتم مقارنة النتائج مع النوع البري. بعدها ذلك درس نمط المقاومة لمضادات الحياة للطافرات البكتيريه الخمسه الناتجه عن التطفير الكيميائي والفيزيائي وقد اشارت النتائج الى ظهور تغاير في نمط مقاومة المضادات لـخمسة مضادات حيويه من المضادات المستخدمه في هذه الدراسه بالمقارنة مع النوع البري، وذلك لأمكانية حدوث الطفرات الوراثيه في الجينات المسؤوله عن صفات المقاومة لمضادات الحياة بعد تعرضها للتطفير الكيميائي والفيزيائي. كما وتمت دارسة امكانية تحلل الجدار الخلوي لطافرات بكتريا *S. aureus* بعد اضافة اللايسوزايم (٥٠ مايكرغرام/مل) الى العالق البكتيري و اظهرت النتائج بعد فحص الخلايا بواسطة المجهر الضوئي نشوء خلايا منزوعة الجدار (البروتوبلاست) بعد ساعتين فقط من الحضن مما يؤكد على امكانية الحصول على البروتوبلاست من الخلايا المطفره خلافا للنوع البري حيث يتطلب تحلل جداره الخلوي الى فترات طويله من الحضن مع البنسلين وتركيز اعلى من انزيم اللايسوزايم.

ولغرض التعرف على التغيرات الحاصله في ظروف النمو للطافرات الناتجه عن التطفير الكيمائي والفيزيائي بالمقارنه مع النوع البري ، تم حضن النوع البري و الطافرات في درجات حراريه

مختلفه (٣٠، ٣٧، ٤٠، ٤٣، ٤٥ م) وقد اظهرت النتائج ان بعض العزلات الطافره غير قادره على النمو في درجات الحراره العالية (٤٣،٤٥ م) خلافا للنوع البري الذي اظهر نموا ،مما يؤكد على حساسية هذه الطفرات للنمو في درجات الحراره العاليه.

جمهورية العراق
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كلية العلوم

تأثير المطفرات الكيميائية والفيزيائية على تركيب الجدار الخلوي لبكتريا *Staphylococcus aureus*

رسالة

مقدمة الى كلية العلوم / جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

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بكالوريوس تقانة أحيائيه / كلية العلوم / جامعة النهرين (٢٠٠٦)

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