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RESEARCH ARTICLE

# Effect of $H_2O_2$ and Ascorbic Acid in β- Hemolysin Production from Clinical Isolates of *Staphylococcus Aureus*

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## Abstract

Oxidizing agents and antioxidants are recently used in wound treatment and health care. The effect of such chemicals as $H_2O_2$ and ascorbic acid on beta hemolysin production by clinical isolates of Staphylococcus *aureus* (which were obtained from wound swabs) was investigated. Hemolytic activity was assayed semi- quantitatively (by measuring the diameter of the hemolytic zone around the wells in blood agar that contain the bacterial crude toxin) and quantitatively (hemolysis %) using the standard curve of osmotic hemolysis by Na Cl. The experiment was optimized by selecting the optimum culture medium, type of blood, pH of the medium, incubation temperature, number of rpm in shaker incubator and incubation time. The higher production of the hemolysin was obtained when *Staphylococcus aureus* grown in the medium containing (0.05 mM) of H2O2 and ( 5 MM) of ascorbic acid, whereas the toxin production decreased in the media containing these substances in lesser concentrations.

**Keywords:** *S. aureus, Hemolysin, $H_2O_2$, Ascorbic acid.*

## Introduction

*Staphylococcus aureus* is the most common pathogen that affects humans as colonizing to about 20% of the population in different parts of the body causing invasive infections [1, 2]. There are many virulence factors that are produced by *Staphylococcus aureus*, which assisted to cause many infections [3], represented adherence factors including surface cellular proteins and pilli as well as this bacterium secrets a group of extracellular proteins (Exoproteins) including: (Enzymes) such as nucleases, proteases, lipase and other enzymes; (Exotoxins) which characterized by their cytolytic activity such as: alpha hemolysin, beta hemolysin, gamma hemolysin, delta hemolysin and epsilon hemolysin. Staphylococcal hemolysins are pore forming toxins (PFT) so they considered as the most diverse and important group among the toxins produced by *Staphylococcus aureus* [4].

These toxins damage the membranes of red blood corpuscles releasing hemoglobin [5, 6]. Ratner and his coworkers [7] pointed that pores or holes formation through membranes of host cell is facilitated under ion gradient, so leading to loss of membrane integrity and consequently cell death, thus plays an important role in the pathogenesis of *Staphylococcus aureus*. Among multiple exotoxins that produced by *Staphylococcus aureus*, alpha hemolysin (α-hemolysin) is the first pore forming toxin known [7], it is also secreted by other bacteria such as *E.coli* [8]. Kernodle *et al* [9].

Found that α-hemolysin acts on red blood cells of rabbits, causes a partial or incomplete degradation that appears as dark green zone due to oxidation of hemoglobin [10]. Elek [11] pointed that it is not possible to diagnose the hemolytic zone of Gamma hemolysin on blood agar, whereas Delta hemloysin is the thermostable one [12]. Coagulase negative staphylococci also produce a hemolysin named Epsilon hemolysin (hemolysin) [13].

Beta hemolysin (ß- hemolysin) which is also called Sphingomyelinase an enzyme its action depend on the presence of magnesium ions ($Mg^{+2}$). [14, 15], has a molecular weight of approximately (35 KDa) [16]. This enzyme, which is an important virulence factor,

hydrolyzes sphingomyeline of host cell plasma membrane generating phosphocholine and Ceramide [15]. Ceramide has several effects on eukaryotic cells such as the stimulation of mitogen activated protein kinases (MAPK), modification of cell structure and induction of apoptosis [17].


The first who named ß-hemolyin were Glenny & Stevens. They identified this toxin through a distinctive colorless region that was observed around the bacterial colonies that grown on blood agar containing cattle blood [18]. The detection of ß- hemolysin was done by inoculating the bacteria on top of certain media to stimulate the production of beta-toxin, tryptic soy agar that contain 3% of cattle blood then incubating at 37°C for 24 hrs. And then 4°C for 16 hrs [12]. Whereas Dinges *et al.* [19] referred to the beta hemolytic activity at 10°C or less thus it is hot-cold hemolysin.

There was some relationship between the sphingomyelin content of the cell membrane and the susceptibility of the cells to the hemolytic action of this toxin, thus high sphingomyelin cells such cattle blood cells were more susceptible to beta hemolysin [20]. Beside antibiotics several routs were known in the treatment of wounds, such as using Antiseptics, Super oxidized water, Hydrogen peroxide and others Hydrogen peroxide has potential uses in health care for its bactericidal, virucidal, sporicidal, fungicidal properties at concentrations about (6-25%).

The antiseptic effect occur by producing hydroxyl free radicals, which effects on several cellular structures of many microorganisms such as, membrane lipids, DNA and others. *Staphylococcus aureus* which is highly catalase producing bacteria, requires 6% $H_2O_2$ for about (30-60 min) to redact about $10^8$ in cell counts [21], whereas 3% was used in post-operative wound care by mean of cleaning the laid open wound [22].

Loo and Halliwell [23] in 2011, found that $H_2O_2$ induces cell proliferation and migration in their keratinocyte scratch wound model, so, in 2012 they found that this chemical increases the re-epithelization of their wound model, leading to healing [24]. Vitamin C (hexuronic acid), the term that describes ascorbic acid a natural, water soluble, non-enzymatic, strong protective antioxidant by quickly reacting with high reactive hydroxyl radicals.

Vitamin C is an ideal antioxidant due to its low potential reduction of ascorbate and the oxidation products ascorbile radicals and its stability (low reactivity) [25]. Because Vitamin C is a strong regulatory co-factor in the collagen synthesis, it plays an important role in skin health [26, 27]. Kamer *et al.* [28] and Srivastava *et al* [29].

Suggested that ascorbic acid and antioxidants combo-treatment could confer benefits to tissue and accelerate wound healing by enhancing tissue hydroxyproline levels, epithelization, fibrogenesis (maturation of fibroblast), neovascularization, and deposition of collagen leading to regenerated wound tissue in rats.

Although expand usage of these substances in wound care and treatment in certain concentrations we designed this experiment to assessment that the exposure of

*staphylococcus aureus* to variable concentrations of these substances may cause oxidative stress a physical stress that may lead to alteration of some bacterial virulence factors especially hemolysin.

## Materials and Methods

### Ethical Issues

This work was approved by the Board director of Al-Hussein hospital at holey Karbala province, informed consent was obtained from all patients.

### Bacterial Isolates

Clinical *Staphylococus aureus* (15 isolates) were isolated from wound swabs that obtained from patients in Al-Hussein hospital at holey Karbala province. Bacterial isolates were identified by biochemical and phenotypic tests according to Bergey's manual [30] and methods used by Macfaddin [31] and Collee et al [32].

### Assay of Hemolytic Activity

### Qualitative Assay of Hemolysin

Bacterial isolates were inoculated in the middle of the blood agar (as spots), plates were incubated at 37 ° C (24 ° C) for 24 hours, then at (4 ° C) for 16 hours, after that zones of blood hemolysis around the colonies were measured.

### Quantitative Assay of Hemolysin

The clinical isolates were grown in 5 ml of the chemically defined medium and then incubated at 37°C for 24 hrs. Using shaking incubator. Cultures were centrifuged by cooling centrifuge at 3000 rpm for 30 min., supernatants were filtered through 0.22 µm diameter filter paper, the filtrates are the crude extracts of the toxin.

### Semi-quantitative Assay of Hemolysin

Five microliters of filtrated supernatant of each bacterial isolate were put in a 5 mm diameter well in the center of blood agar, incubated, hemolysis zone around wells were measured.

### Quantitative Assay of Hemolysin

With regard to standerd curve of erythrocytes hemolysis by serial concentration of NaCl [33] Fig.1, Hemolytic activity of the crude hemolysins that produced by our isolates of *Staphylococcus aureus* were calculated by mean of hemolysis %. The hemolytic activities of bacterial supernatants were assayed quantitatively using serial twofold dilutions for each. The method was described by Santos *et al* [34]. Bizani and Brandelli [35] with a little modulation in the wave length used.

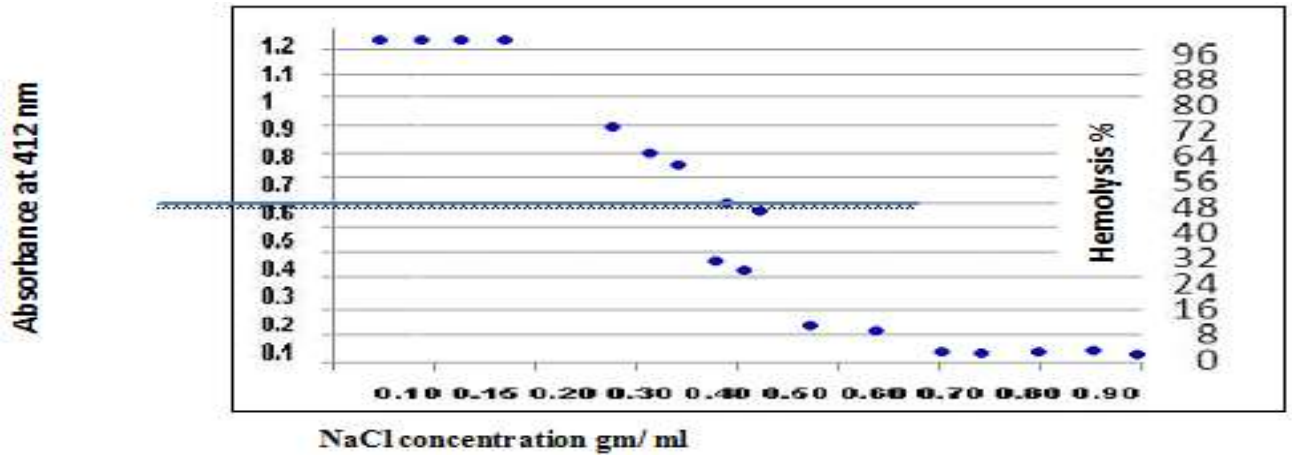



**Fig.1: Blood hemolysis % of serial concentrations of NaCl**

### Optimization of Hemolysin Production

The production of the toxin was optimized using different experimental conditions such as (components of culture media, temperature, and pH, type of erythrocytes, blood group, and incubation time in hours, stable or shaker incubator). For each experimental condition, hemolysin production was assayed as mentioned above.

### Optimal Medium for Hemolysin Production

The chemically defined medium (CDM) prepared as described by Snyder and Koch [36] and Trypton soy broth were used for optimal production of hemolysin

### Determination of the Optimum RBCs for Hemolysin Production

Bacterial isolates were inoculated by mean of spot in the middle of the blood agar containing various types of blood: Human (groups A, B, O, O, AB with both Rhs for each group), Rabbit, Sheep, Cows and Chicken.

### Determination of the Optimum Temperature for Hemolysin Production

Bacterial isolates were inoculated in the chemically defined medium (CDM) and incubated at different temperatures (25, 30, 37, 40˚C) using Shaking incubator.

### Determination of Optimal PH for Hemolysin Production

Buffers were prepared according to Colowick and Kaplan [37], buffers pH of about (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9) were used.

### Optimization of Shaking Incubation

By using Constant, Shaker incubator (100 and 200) rpm, effect of shaking on hemolysin production was determined.

### Determination of the Optimum Incubation Time for Hemolysin Production

Bacterial isolaes were inoculated in the chemically defined medium and incubated at optimum temperature, pH, shaking condition but in different incubation period (6, 18, 24, 48) hours.

### Effect of hydrogen peroxide (H2O2) in beta Hemolysin

Various concentrations of hydrogen peroxide (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5mM) in the chemically defined medium (10 ml for each), flasks were inoculated by bacterial suspension (1 ml for each) then incubated in optimum conditions.

### Effect of Ascorbic Acid (vitamin C) in beta Hemolysin

From stock solution various concentrations of ascorbic acid (0, 5, 10, 20, 40, 80, 160, 320, 640 mM) in the chemically defined medium (10 ml for each), flasks were inoculated by bacterial suspension (1 ml for each) then incubated in optimum conditions.

### Statistical Analysis

Completely Randomized Design (C.R.D.) with five replicates were used, L.S.D. and means were calculated according to Al-Rawi [38]

## Results and Discussion

Fifteen clinical isolates of S. aureus were collected from wound swabs including (wounds, burn wounds, post-operative wounds) from patients of various ages and both sexes. In order to choose the effective isolate(s) for studying the effect of oxidizing and antioxidant agent on the hemolytic activity of these isolates, optimization of hemolysin production was done.

The hemolytic activiteis of Staphylococcus aureus isolates were assayed by measuring the diameter of the beta hemolytic zone shown on agar containing blood from different sources including rabbits, sheep, cows, chicken, human of various blood groups A, B, AB, O).

**Table 1: Diameters of hemolytic zones of *Staphylococcus aureus* using blood from different animals**

| Bacterial isolates | Diameter of hemolysis zones (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Human $A^+$ | Human $B^+$ | Human $AB^+$ | Human $O^+$ | Human $O^-$ | Chicken | Cattles | Sheep | Rabbits |
| $C_1$ | 9 | 12 | 13 | - | - | - | 5 | 16 | - |
| $C_2$ | 9.5 | 12.5 | 14 | 6.5 | 6 | 8.5 | 6.5 | 20.5 | - |
| $C_3$ | 9 | 12 | 14 | 6.5 | 6 | 8 | 5 | 18 | - |
| | | | L.S.D= 1.8 | | | | | L.S.D= 2.4 | |

No hemolysis

Red blood cells taken from the different animals differed in their susceptibility to the beta hemolysin of Staphylococcus aureus (Table 1) and the sheep RBCs were the most susceptible towards this enzyme, showed clear hemolytic zones ranging from16 to 20.5 mm in diameter. Whereas red blood cells of the AB + group showed clear hemolytic zones ranging from 13 to 14 mm in diameter. Red blood cells of rabbit did not show any susceptibility. The results confirmed that red blood cells of the sheep were the most susceptible to beta hemolysin whereas the less susceptible were RBCs of rabbits. In analyzing the above data statistically, the

hemolytic zones in sheep blood showed a significant difference at the probability level (0.05). Most studies of hemolycin are carried out with RBCs, due to the ease of metabolism furthermore these cells can be obtained free of other cells. These cells will also be the primary target of bacterial pathogens that secrete hemolysins. [39].

**Fig. 2: Semi-quantitative assay ( wells assay ) of beta hemolysis in blood agar containing sheep RBCs, the clear zone around the well indicated beta hemolysis activity of bacterial supernatant (crude toxin)**

Certainly, the failure of hemolysin to lyse RBCs of rabbits, chickens and human (group O) was probably due to mutations in membrane Glycosyl phosphatidyl inositol (GPI) recepeors. These mutations make the susceptibility of RBCs to beta toxin 1000 times lower [40]. The hemolytic activity % of the toxin reach (99,98 an d95%) for clinical isolates C1, C2 and C3 respectively, at 37° C. (L.S.D. 2.2) while decreased when cultures incubated at 40° C (Table 2). A reduction of toxin activity in the incubation at 25° C and 30° C were observed. Temperature has an important effect on the production of the enzymes by microorganisms. Rising of the temperature increases the kinetic energy of the molecules and the speed of enzymatic reactions in the cell which may lead to an increase in energy activation. Thus increasing the activity or the reactions of synthesis pathway of the enzymes. The higher the temperature, the greater the change in the normal structure of the enzyme, ie, the enzyme loses its activity at high temperatures, while in the low temperature (less than 30° C), growth and metabolism were ceased. When the semi-quantitative assay of hemolysin was performed, plates were incubated at 37° C for 24 hours and then at 4° C for 16 hours.

**Table 2: Diameters of hemolytic zones of *Staphylococcus aureus* at various temperatures**

| Temperature | Diameter of hemolytic zones (mm), at 37° C for 24 hrs. | | | Diameter of hemolytic zones (mm), at 37° C for 24 hrs and then at 4° C for 16 hrs. | | |
|---|---|---|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ | $C_1$ | $C_2$ | $C_3$ |
| 25° C | 5 | 5.5 | 5 | 5.5 | 6 | 7.5 |
| 30° C | 6.5 | 8.5 | 8 | 7.5 | 10.5 | 9.5 |
| 37° C L.S.D.= 2.2 | 10 | 17 | 15 | 13 | 19.5 | 19 |
| 40° C | 6 | 6.5 | 6 | 8 | 8 | 8.5 |

It was noted from the results shown in (table 2) that the highest hemolytic zones of the enzyme was at 37° C (10, 17 and 15 mm) for isolates C1, C2 and C3 respectively, while the diameters of hemolysis decreased to 6.5, 6, when plates where incubated at 40° C. It was also observed that there were a decrease in the diameters of the hemolysis at incubation temperatures 25° C and 30° C.

The incubation of the plates at 4° C for 16 hours increases the hemolysis diameters of beta-toxin (hot-cold toxin). In other words, at 37° C, the toxin act on the membranes of RBCs but there is no degradation or it may be incomplete but it is clearer after incubation at 4° C (Fig. 3). Our results indicated a clear increase in the diameters of the hemolytic zones after the completion of incubation at 4° C for 16 hours. Actually, 37° C was the most appropriate for clinical isolates for beta-toxin production.

It represents the optimal temperature for the production of this enzyme but the process of RBCs membranes hemolysis seem clear only after incubation at 4° C for 16 hrs. The results of the present study are consistent with the results of Javed et al. [41], which showed that the temperature 37° C is the

optimal temperature for the production of hemolysin while the product is less effective at the lower temperatures. Wang *et al.* [42] reported that the highest level of toxin production by their isolates were at 37° C. The other researcher, Tsai *et al.* [43] found that the optimum temperature of hemolysin production by *Aeromonas hydrophila* was 28° C.

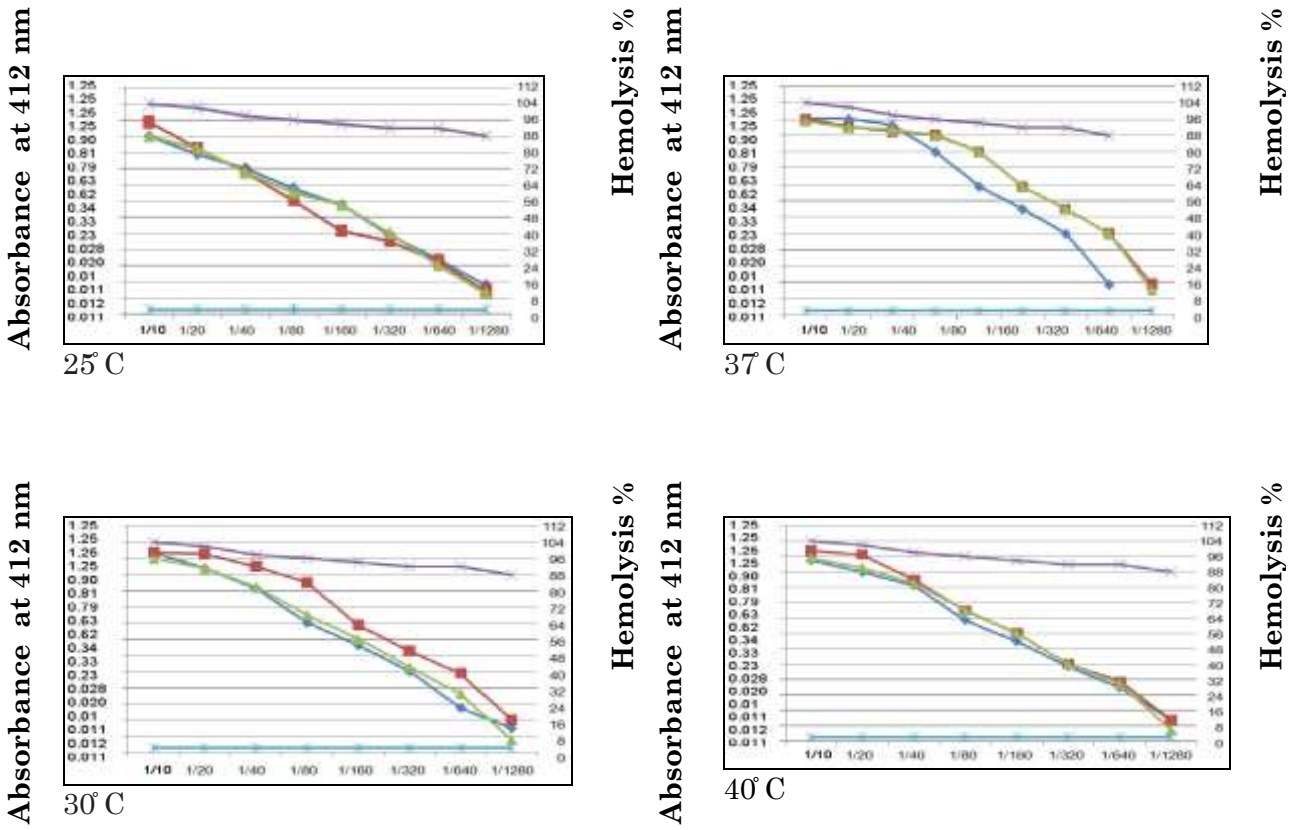



**Fig. 3: Hemolytic activity of *S.aureus* after incubation at various temperatures.**
**◇C1 □C2 △C3 × +ve control ✳ -ve control**

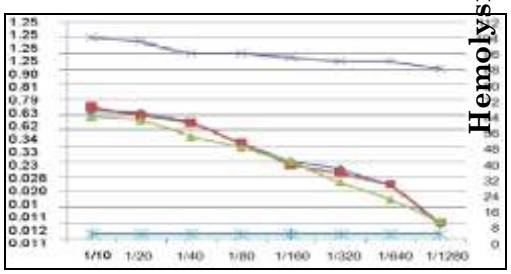
By semi-quantitative assay, we obtained results similar to those of quantitative assay. The highest diameters of hemolytic zone were recorded at pH (7.5), these are (17.5, 18 and 19 mm) for the three isolates C1, C2 and C3 respectively, the diameters decreased at lower pH (Table 3). Once again the incubation of blood agar plates at 4° C for 16 hrs was an important step to detect beta hemolysis. Statistical analysis showed that pH (7.5) had a significant effect, indicating that the growth of the microorganisms and their enzymes production occurs at natural to basic pH values. At these values the medium has a significant effect on the free and ionizable groups of the enzymes affecting the enzyme's activity or biological reactions. On the other hand the decrease of enzyme's activity at pH values below or above the optimum is due to change in the natural ionic structure of the enzyme or the substrate or both [44].

**Table 3: Diameters of hemolytic zones of *Staphylococcus aureus* after incubation in wide rang pH (4-9)**

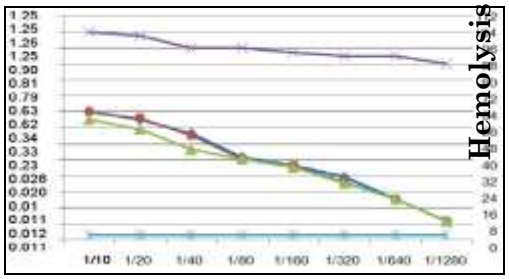
| pH values | Diameter of hemolytic zones (mm), at 37° C for 24 hrs. | | | Diameter of hemolytic zones (mm), at 37° C for 24 hrs and then at 4° C for 16 hrs. | | |
|---|---|---|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ | $C_1$ | $C_2$ | $C_3$ |
| 4 | 5 | 6.5 | 5.5 | 5.5 | 7 | 6.5 |
| 4.5 | 6 | 7 | 6 | 7 | 7.5 | 7 |
| 5 | 7 | 8 | 7.5 | 7.5 | 8.5 | 8 |
| 5.5 | 8 | 8.5 | 8 | 9 | 10 | 9 |
| 6 | 6 | 7 | 6.5 | 7 | 8 | 7 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6.5 | 11 | 12.5 | 12 | 12 | 13.5 | 13.5 |
| 7 | 17 | 17.5 | 15 | 18 | 19.5 | 17.5 |
| 7.5<br>L.S.D= 0.9 | 17.5 | 19 | 18 | 18.5 | 20.5 | 20 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8.5 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 |

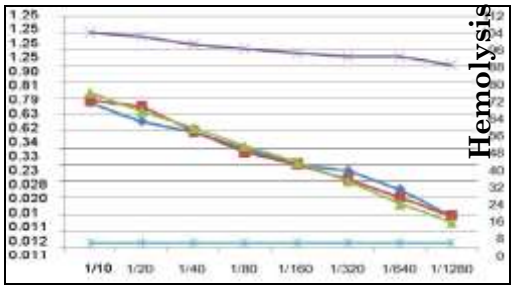
Absorbance at 412 nm Absorbance at 412 nm Absorbance at 412 nm Absorbance at 412 nmAbsorbance at 412 nm Absorbance at 412 nm



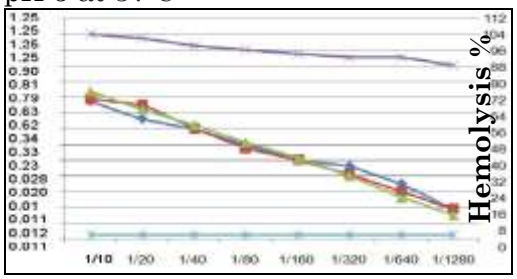

pH 4 at 37° C



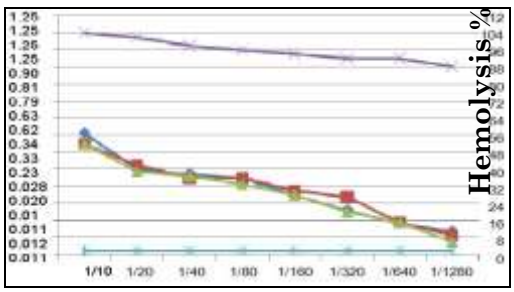

pH 4.5 at 37° C




pH 5 at 37° C

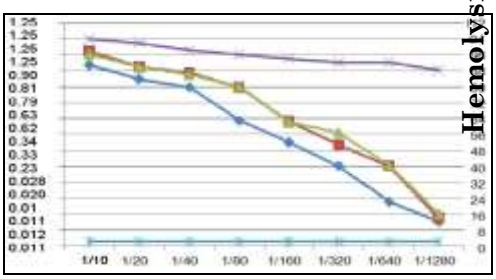



pH 5.5 at 37° C

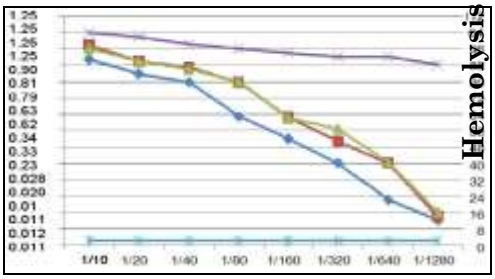



pH 6 at 37° C

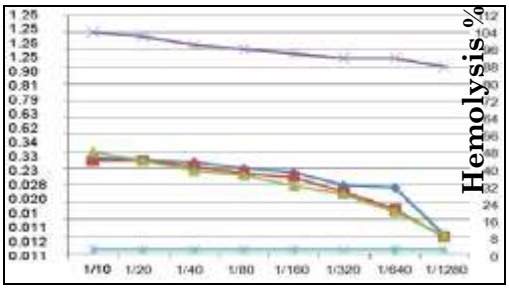
Absorbance at 412 nm Absorbance at 412 nm Absorbance at 412 nm Absorbance at 412 nmAbsorbance at 412 nm Absorbance at 412 nm



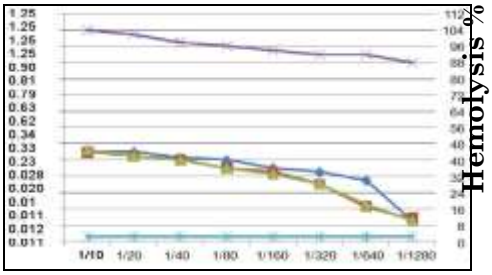

pH 7 at 37° C



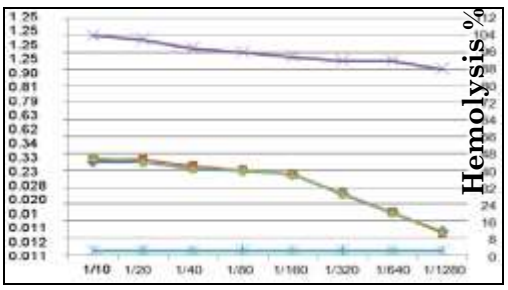

pH 7.5 at 37° C



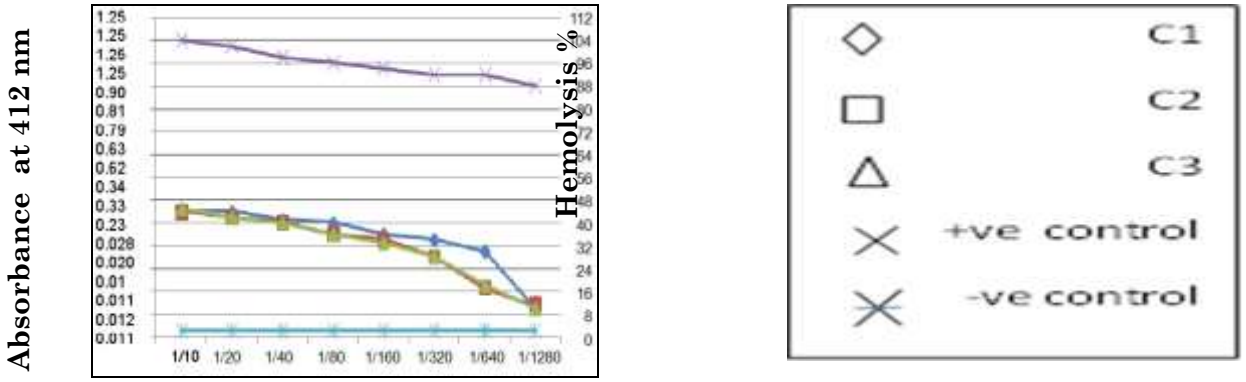

pH 8 at 37° C




pH 8.5 at 37° C




pH 9 at 37° C




pH 6.5 at 37° C

**Fig 4: Hemolytic activity after incubation in media of various pH**

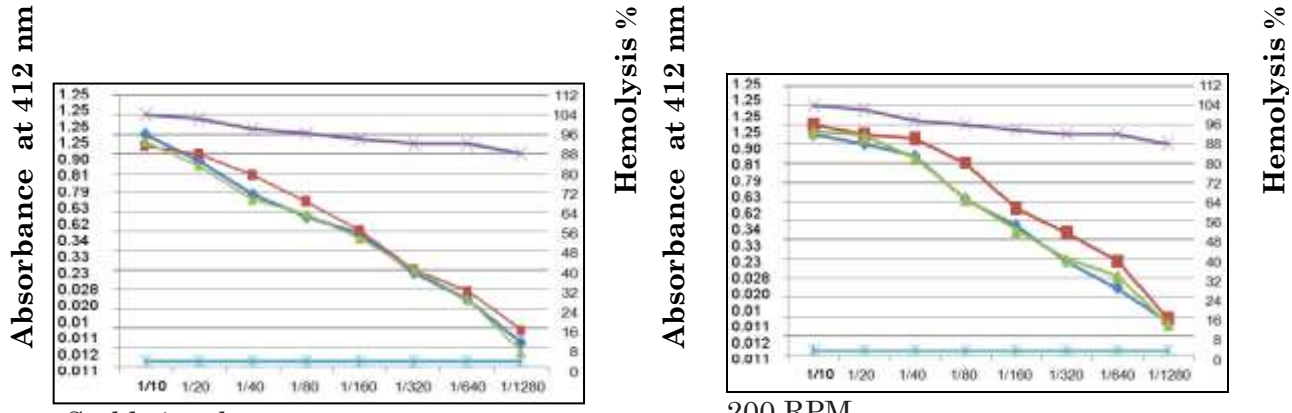
Our results confirmed that the production of hemolysin using the stable incubator is less than that of the shaking incubator (100 RPM) which had a significant effect.

The reduced hemolytic activity at (200 RPM) probably due to the change in enzyme by denaturation.

**Table 4: Diameters of hemolytic zones of *Staphylococcus aureus* after incubation in stable and shaker incubators**

| RPM | Diameter of hemolytic zones (mm), at 37° C for 24 hrs. | | | Diameter of hemolytic zones (mm), at 37° C for 24 hrs and then at 4° C for 16 hrs. | | |
|---|---|---|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ | $C_1$ | $C_2$ | $C_3$ |
| Stable | 6 | 8.5 | 8 | 7.5 | 9.5 | 8.5 |
| 100 RPM L.S.D= 1.7 | 10 | 12.5 | 11 | 11.5 | 14.5 | 12 |
| 200 RPM | 6.5 | 9 | 8.5 | 8 | 10.5 | 10.5 |

The statistical analysis of the data listed in Table (4), indicated a significant difference in the diameters of the hemolytic zones when culture media incubated in (100 RPM) after the completion of incubation at 4° C, (L.S.D. =1.7). These findings indicated that the use of the shaker incubator stimulates the production of the enzyme in large quantities as well as providing good conditions for aeration and thus yield good growth. These results were somewhat consistent with the researcher's Muslim [45], who emphasized that the conditions of the shaking incubator were much better than those of the fixed incubator in the production of hemolysin. Kothary *et al.* [46] indicated that the best production of hemolysin occurs at (100 RPM) using shaker incubator.




Stable incubator

200 RPM

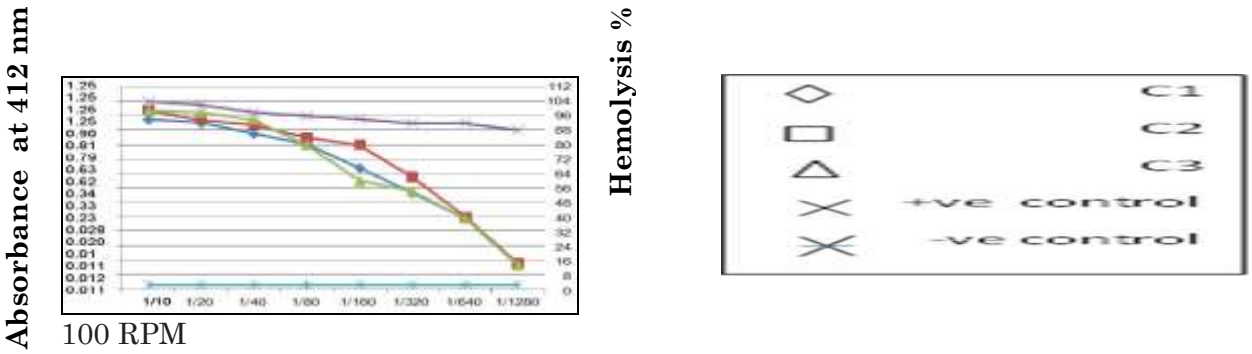



**Fig 5: Hemolytic activity after incubation in stable and shaker incubators**

The highest hemolytic activiteis % were recorded at the incubation period (24 hr), this was indicated by the statistical analysis of the data listed in (Table 5; Fig 6). Others found that the optimum incubation time for the production of this toxin is 18hrs but the maximum production reached at 24 hrs [45], While Kothary *et al.* [46] noted that the production of hemolysin by his isolates were obtained after incubation for 17 hrs.

**Table 5: Diameters of hemolytic zones of *Staphylococcus aureus* after incubation for different times**

| Incubation time | Diameter of hemolytic zones (mm), at 37° C for 24 hrs. | | | Diameter of hemolytic zones (mm), at 37° C for 24 hrs and then at 4° C for 16 hrs. | | |
|---|---|---|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ | $C_1$ | $C_2$ | $C_3$ |
| 6 hr | 5.5 | 6 | 6 | 6.5 | 8 | 8 |
| 18 hr | 9 | 9.5 | 8 | 9.5 | 11.5 | 11.5 |
| 24 hr<br>L.S.D= 1.1 | 13.5 | 20.5 | 15 | 14.5 | 21.5 | 21.5 |
| 48 hr | 7.5 | 7 | 6.5 | 8.5 | 10 | 10 |

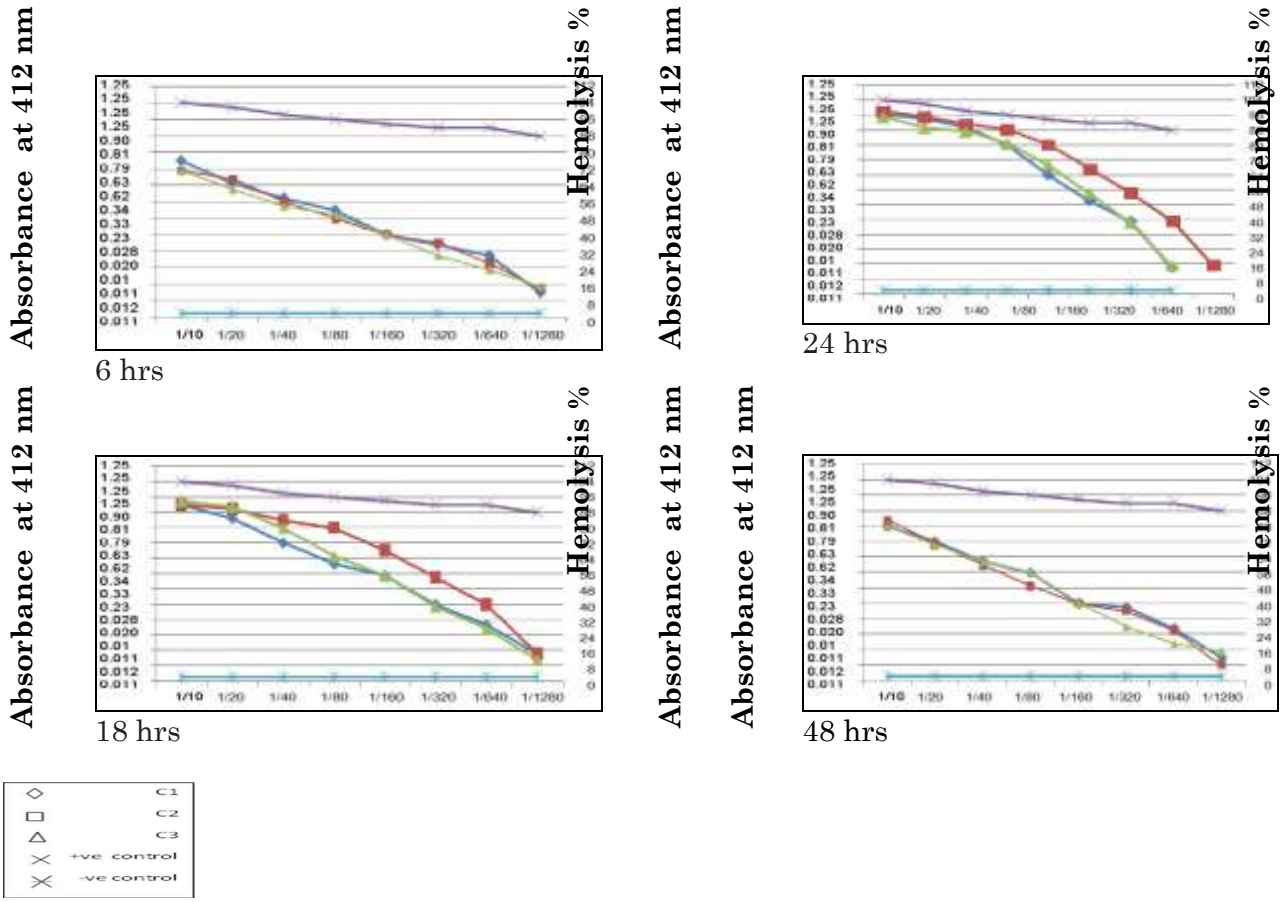



**Fig. 6: Hemolytic activity after incubation for different times**

## Effect of Various Concentrations of Hydrogen Peroxide in the Production of Beta-Hemolysin

Hydrogen peroxide ($H_2O_2$) is an oxidizing agent that causes high concentrations of free radicals that cause the lipid peroxidation of cellular membranes, free lipids and proteins, leading to oxidative stress, which destroys cells toxicity and disrupts their functions [47]. Hydrogen peroxide has been used as a wound antiseptic, helping to form keratinocytes and wounds healing. The wounds treated with this substance have been shown to be free of infectious microbes [48]. *S. aureus* isolates have been treated with various concentrations of $H_2O_2$. Treatment with low concentrations or exposure to oxidative stress has a significant effect on the production of the enzyme by these bacteria, which causes a strong reduction in the quantity of hemolysin produced by the studied isolates.

**Table 6: Diameters of hemolytic zones of *Staphylococcus aureus* after incubation with various concentrations of $H_2O_2$**

| Bacterial isolates | $H_2O_2$ conc. (mM) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 | 0.45 | 0.5 |
| $C_1$ | 16 | 14.5 | 14 | 13.5 | 12 | - | - | - | 7 | 8 |
| $C_2$ | 22<br>L.S.D=2.2 | 22<br>L.S.D=2.6 | 16 | 14 | 13 | - | - | - | 6.5 | 8.5 |
| $C_3$ | 22 | 21.5 | 13.5 | 12 | 11 | - | - | - | 8 | 9 |

**No hemolysis**

**Table 7: Hemolytic activity of *Staphylococcus aureus* after incubation with various concentrations of $H_2O_2$**

| $H_2O_2$ conc. (mM) | Hemolysis% | | |
|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ |
| 0 | 97 | 96 | 92 |
| 0.05 | 98.4 | 95 | 94.8 |
| 0.10 | 98.8 | 98 | 95.6 |
| 0.15 | 95.2 | 88.8 | 66.4 |
| 0.20 | 61.6 | 55 | 56 |
| 0.25 | 60.8 | 62 | 59.2 |
| 0.30 | 48 | 45 | 46 |
| 0.35 | 46 | 46 | 42.4 |
| 0.40 | 47.2 | 46.4 | 45 |
| 0.45 | 52 | 60 | 50.4 |
| 0.50 | 58.4 | 64 | 65.6 |

The highest hemolytic activity % was recorded at 0.05 mM of $H_2O_2$ (Tables 6, 7) which were (98.4, 95 and 94.8%) for C1, C2 and C3 respectively as a comparison to control. The activity began to decrease gradually with the concentration of hydrogen peroxide solution increasing to 0.40 mM, in which no hemolytic activity was seen compared to positive control.

The results showed that concentrations of 0.10, 0.15.0.20, 0, 25, 0.30, 0.35 and 0.40 mM had an inhibitory effect on the production of hemolycin by *Staphylococcus aureus*, that is, this enzyme is sensitive to these concentrations. These concentrations of hydrogen peroxide may cause a repression of the gene responsible for the transcription or synthesis of hemolysin [49]. For the concentrations 0.45, 0.50 mM, it was noted that there was a slight increase in hemolysis. Statistically, these concentrations showed significant effects in hemolysin production by bacterial isolates. The highest concentration of protein was recorded at the lowest concentration of hydrogen peroxide (0.05 mM) (Table 8), which was 210, 160,160 µg/ml for isolates C1, C2 and C3, respectively.

The detection of catalase production from the studied isolates was done by slide test using these various concentrations of hydrogen peroxide, it was negative for all. This result was identical to the results of previous research, Ingraham *et al*. [50], who noted that lower concentrations of hydrogen peroxide were insufficient to stimulate catalase production, but higher concentrations of 0.50 molar could induce catalase production.

**Table 8: Protein concentration of *Staphylococcus aureus* Crude extracts after incubation with various concentrations of $H_2O_2$**

| $H_2O_2$ conc. (mM) | Protein conc.(µg/ml) | | |
|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ |
| 0 | 162 | 162.5 | 212 |
| 0.05 | 160 | 160 | 210 |
| 0.10 | 155 | 157.5 | 185 |
| 0.15 | 147.5 | 152.5 | 135 |
| 0.20 | 120 | 150 | 102.5 |
| 0.25 | 75 | 108 | 90 |
| 0.30 | 30 | 104 | 40 |
| 0.35 | 25 | 97.5 | 10 |
| 0.40 | 17.5 | 82.5 | 7.5 |
| 0.45 | 32.5 | 112.5 | 25 |
| 0.50 | 45 | 120 | 30 |
| L.S.D | 0.17 | 0.2 | 0.1 |

## Effect of Various Concentrations of Ascorbic Acid in the Production of Beta-Hemolysin

The addition of variable concentrations of ascorbic acid (vitamin C) has a significant effect on hemolysin produced by *Staphylococcus aureus*. We found that the percentage of hemolysis decreased to (96.2, 94.4 and 91.4%) for C1, C2, C3 respectively when treated with 5 mM of vitamin C while they were ( 91, 92 and 91%) when vitamin concentration increased to 10 mM. But when reached to 20 mM, the hemolytic activities were (64, 74 and 83%) for the three isolates respectively. At 640 mM, any hemolysis were detected (Table 9 and 10), indicating that the bacteria have lost their ability to damage red blood cells at this concentration of vitamin C. This antioxidant has the potentiality to induce the loss of many plasmids from bacteriai cells [51] and may include plasmid responsible for encoding this enzyme.

**Table 9: Diameters of hemolytic zones of *Staphylococcus aureus* after incubation with various concentrations of Vit.C**

| Bacterial isolates | Vit. C conc. (mM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 | 10 | 20 | 40 | 80 | 160 | 320 | 640 |
| $C_1$ | 19 | 16.5 | 15 | 13.5 | 12 | - | - | - |
| $C_2$ | 19.5 | 18 | 16 | 14.5 | 13 | - | - | - |
| $C_3$ | 18<br>L.S.D.= 1.6 | 17 | 15.5 | 13.5 | 11.5 | - | - | - |

No hemolysis

Vitamin C is the most powerful non-enzymatic natural antioxidant and acts as an effective oxygen filter and has a protective effect against the inhibition of antioxidant enzymes such as catalase and glutathione peroxidase [52]. This vitamin reduces the risk of destruction by free radicals [53]. In addition, this vitamin has biologic functions, which include alteration in the properties of DNA and the generation of oxidizing factor hydrogen peroxide and hydroxyl radicals by autoxidation of cellular membrane components [54].

**Table 10: Hemolytic activity of *Staphylococcus aureus* after incubation with various concentrations of Vit.C**

| Vit.C conc. (mM) | Hemolysis % | | |
|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ |
| 0 | 97 | 96 | 92 |
| 5 | 96.2 | 94.4 | 91.4 |
| 10 | 91 | 92 | 91 |
| 20 | 64 | 74 | 83 |
| 40 | 60.8 | 56 | 52 |
| 80 | 53 | 50.4 | 51.2 |
| 160 | 44 | 44 | 45 |
| 320 | 40 | 42 | 44 |
| 640 | 40.2 | 40 | 42 |

The concentration of protein was measured after incubation with variable concentrations of vitamin C, The highest concentration of protein was recorded at the concentration (5 mM) of vitamin C, while gradually decreased with the increase of the concentration of this

vitamin (Table 11) the highest concentration (640 mM) in which the lowest protein production was observed compared to control. As vitamin C, antioxidants break down the disulfide bonds in protein yielding thiol groups, leading to an exchange of the cross bridge sulfides bonds which may abort the biological activity of the protein [55].

**Table 11: Protein concentration of Staphylococcus aureus Crude extracts after incubation with various concentrations of Vit.C**

| Vit.C conc. (mM) | Protein conc. | | |
|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ |
| 0 | 158 | 170 | 192.5 |
| 5 | 157 | 167.5 | 187.5 |
| 10 | 145 | 152.5 | 167.5 |
| 20 | 127.5 | 105 | 137.5 |
| 40 | 140 | 125 | 150 |
| 80 | 122.5 | 105 | 135 |
| 160 | 132.5 | 125 | 142.5 |
| 320 | 47.5 | 60 | 55 |
| 640 | 15 | 25 | 32.5 |
| L.S.D | 0.20 | 0.25 | 0.22 |

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