WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 7, Issue 01, 111-122. Research Article ISSN 2277–7105

EFFECT OF PROTEASES SECRETED BY *STAPHYLOCOCCUS AUREUS* ON BIOFILM INTEGRITY

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Article Received on
09 Nov. 2017,
Revised on 30 Nov. 2017,
Accepted on 20 Dec. 2017
DOI: 10.20959/wjpr20181-10498

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ABSTRACT

This study aimed to use protease enzymes produced by *Staphylococcus aureus* in medical applications through the investigation of their antimicrobial activity on biofilm integrity. For such purpose, a total of 100 samples were obtained from patients of both genders and various ages who were referred to two teaching hospitals in Baghdad suffering from general infections of different severity and location of injuries in their body. Only 98 (98%) of the samples were positive for giving bacterial growth. The samples were cultured on blood agar and mannitol salt agar as a primary step for cultivation of bacteria. The results showed that more than 40 bacterial isolates were obtained. After the identification of these isolates by cultural, microscopic and biochemical examination, the results showed that 34 isolates were identified as *Staphylococcus aureus*. Moreover, Vitek 2 system was used which ensured that all of the 34 isolates belonged to this species. When the ability of these isolates to produce proteases was examined, the results showed that all the 34 isolates of *S. aureus* were able to do so but with variable degrees of production. Among them, an isolate symbolled *S. aureus* S28 was the most superior in protease production with a value of specific activity reaching 41.93 U/mg proteins. When the 34 *S. aureus* isolates were subjected to the antibiotics susceptibility test toward eleven different antibiotics, the results declared that all the isolates were totally resistance to penicillin, vancomycin, cephalothin, gentamicin and tetracycline, but completely susceptible to amikacin, ciprofloxacin, chloramphenicol, imipenem, novobiocin and

rifampicin. To improve its production of proteases, the selected isolate S28 of *S. aureus*, was subjected to investigate its optimization conditions. The results declared that after supplementation of pH 8.5 with 0.5% lactose (as a carbon source), 1.5% peptone (as a nitrogen source), and 0.1% NaH_2PO_4 (as a phosphate source) when incubated at 37ºC in a shaker incubator (150 rpm) for 24 h, the maximum protease production was reached. To order to investigate their ability to form biofilm, fifteen 15 of the *S. aureus* isolates that gave the highest specific activity of proteases were used. The results showed that 14 of the isolates were able to produce a moderate production of biofilms at a rate of 93.3%, while the remaining one was a weak biofilm producer with a rate of only 6.7%. Upon studying the activity of proteases secreted by the selected *Staphylococcus* isolate on the biofilm integrity, the results declared that proteases treatment with different concentrations decreased the biofilm formation as the concentration increases. The lowest percentage of residual biofilm (29.4%) was achieved by using 1000 µg/ml of protease.

KEYWORD: Staphylococcus aureus, Protease, Biofilm.

1. INTRODUCTION

Staphylococcus aureus, a Gram-positive bacterium, is frequently colonizes humans and other warm-blooded animals. It is persistently colonized in approximately 25% of the human population, and the other 75% are intermittently or not colonized.[1, 2] Colonization occurs primarily in the anterior nares, while the throat, skin, axilla, perirectal area, and groin are potential secondary sites.[3] Though a human commensal, *S. aureus* acts as an opportunistic pathogen and carriage is associated with an increased risk of a subsequent infection.[4]

Biofilm formed by the opportunistic pathogen *S. aureus* is one of the important factors contributing to the establishment of chronic infection.[5] *S. aureus* is able to readily form biofilms on host surfaces such as bones,[6] cartilages, and heart valves,[7] foreign body implants, including catheters and orthopedic devices.[8] The biofilm, when matured, is composed of a community of cells encased in an extracellular matrix. This structure provides inherent resistance to the innate immune system and other antimicrobials, so it promotes bacterial persistence.[9, 10]

Several *Staphylococcus* species produce a variety of extracellular proteases, which are critical to the maintenance of cellular function.[11] Proteases, for example, are hydrolytic enzymes that attach the peptide bonds in the primary structure of proteins and peptides. These enzymes

are present in a wide variety of living organisms. They also show different physiological, chemical and biological functions in their environments on the earth.

2. MATERIAL AND METHODS

2.1 Isolation of *S. aureus*

A total of 100 swab samples were obtained from various clinical samples (Throat, ear, nose and wound) from two hospitals in Baghdad and were screened by biochemical test and Vitek2 system to isolate *S. aureus*.

2.2 Testing ability of S. aureus isolates for protease production

A) Semi-qualitative screening

Each isolate of *S. aureus* was streaked on nutrient agar medium and incubated at 37 °C for 24h. After incubation, a single colony was placed on a skim milk agar medium plate. The plate was incubated at 37°C for 24h. Ability of the isolate to produce protease by formation of clear halo zone around the colony was recorded.

B) Quantitative screening of protease

Production of protease was achieved by determining the enzyme activity and specific activity.[12]

2.3 Optimum conditions for protease production

1- Optimizing carbon source.
2- Optimizing concentration of carbon source.
3- Optimizing nitrogen source.
4- Optimizing concentration of nitrogen source.
5- Optimizing phosphate source.
6- Optimizing concentration of phosphate source.
7- Optimizing pH.
8- Optimum incubation temperature.

2.4 Antibiotics susceptibility test[13]

Antibiotics susceptibility test was performed by using Kirby Bauer's disc diffusion method[14] according to the Manual on Antimicrobial Susceptibility Testing (MAST) (2004). Results were compared with to those of the Clinical Laboratory Standards Institute (CLSI) (2012).

2.5 Detection of bacterial ability to biofilm formation

The ability of staphylococci to biofilm formation was tested by the tissue culture plate (TCP) method[15] on the fifteen *S. aureus* isolates that gave the highest specific activity of protease enzyme.

2.6 Effect of protease on biofilm integrity

The activity of protease secreted by the *Staphylococcus* isolate on biofilm integrity was investigated by inoculating its culture in the modified biofilm production medium (BM) and incubated for 48–72 hours under static conditions at 37 °C.[16] After incubation, 200 µl of the bacterial suspension was dispensed in the first well on the TC- plate (polysterol) which was considered as the control. In the second well, 200 µl of the suspension and 10 µg/ml of crude protease were added. In the third, 200 µl and 100 µg/ml were added; while in the last well 200 µl and 1000 µg/ml were added. All plates were incubated at 37ºC for 24 h. After incubation, the culture supernatant was discarded, and the wells were washed three times with phosphate-buffered saline (PBS) to remove the non-adherent cells. The plates were air dried for 20 min, and the surface attached cells were stained with 200 µl of 1% crystal violet solution for 20 min. Subsequently, the crystal violet was discarded (in the same way) before washing three times with tap water. After 30 min, they were air dried, then 200 µl of 96% ethanol was added to dissolve the cell-bound crystal violet, and the absorbance was measured by a micro ELISA auto reader (model 680, Bio rad) at a wavelength of 570 nm.

3. RESULTS

3.1 Isolation of *S. aureus*

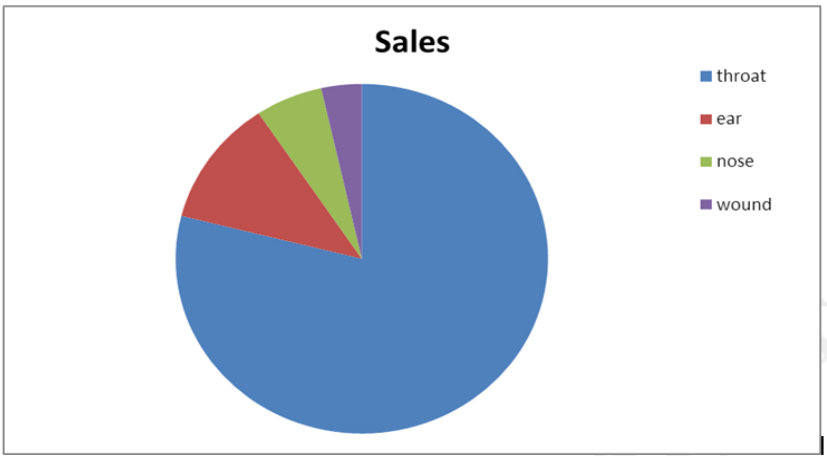

Figure (3-1): Pie shape of suspected Staphylococci isolates distributed according to the types of patient infections.

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

Test	Result
β-hemolysis blood agar	Positive
Catalase	Positive
Citrate	Positive
Coagulase	Positive
DNase production	Positive
Gram stain	Positive
Indole	Negative
Lactose fermentation	Positive
Mannitol salt agar	Positive
Methyl red	Positive
Motility	Negative
Oxidase	Negative
Spore forming	Negative
Urease	Positive
Voges-proskauer	Positive

3.2 The ability of *S. aureus* isolates to produce proteases

A) Semi- quantitative screening

Results of the study declared that the skim milk agar medium was hydrolyzed by all *S. aureus* isolates by forming variable degrees of hydrolysis halos. The range of halos diameters was between 6 and 22 mm. Isolate S28 (from throat samples) was the superior one in the production of protease with a 22 mm halo diameter of hydrolysis. Adversely, the least efficient one was S21 isolate (from nose samples) which gave a diameter zone of only 6 mm.[17]

B) Quantitative screening

After performing the ability of *S. aureus* for the production of proteases by growing on PB broth, the results showed that in the culture filtrates the protease specific activity ranged between 36.42 and 41.93 U/mg protein. Among these isolates, *S. aureus* S28 isolate was the most efficient by giving the highest specific activity, while the lowest activity was recorded by isolate S21. According to these results, isolate S28 was selected to be used in the determination of the optimum conditions for proteases production. The ability of bacteria for the production of proteases referred to the variations of the genes in the protease production.[18]

3.3 Optimal conditions of protease production

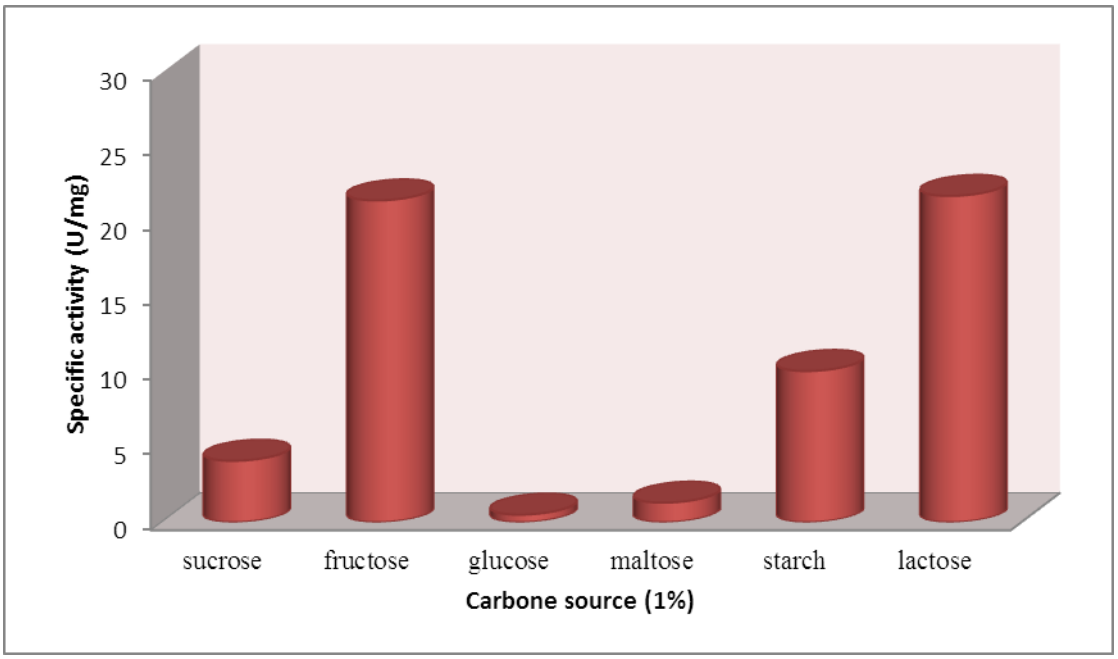

Figure (3-2): Optimization of carbon source for production of proteases by isolate S28 of *S. aureus* after incubation at 37°C in a shaker incubator (150rpm) for 24 h.

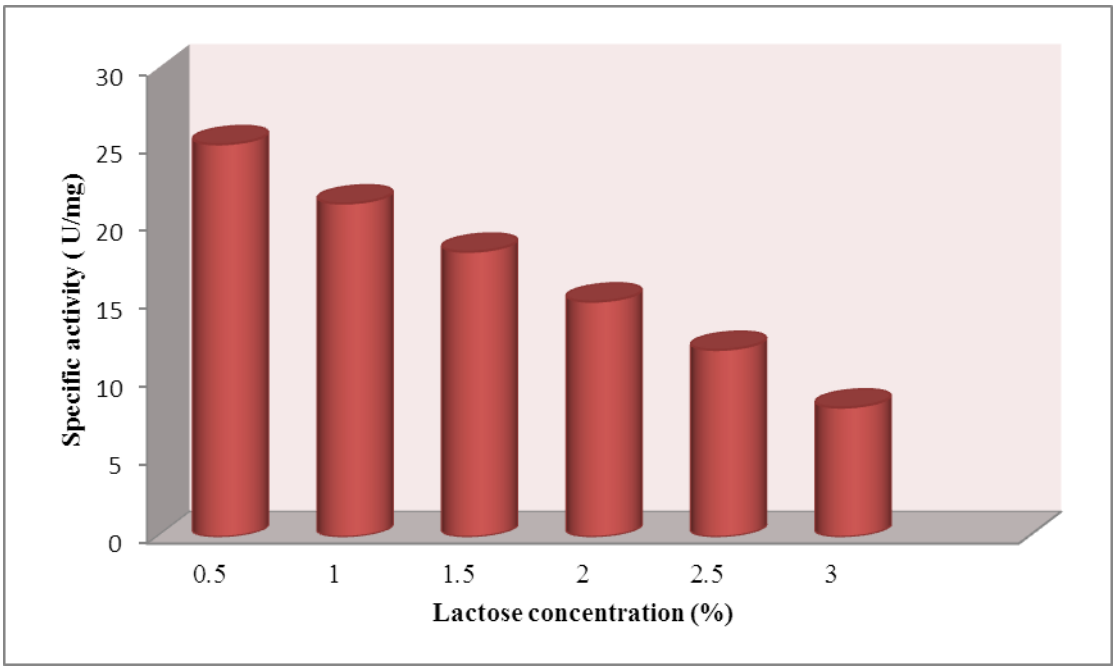

Figure (3-3): Effect of lactose concentration on proteases produced by *S. aureus* isolate S28 after incubation in a shaker incubator with150 rpm at 37°C 24 h.

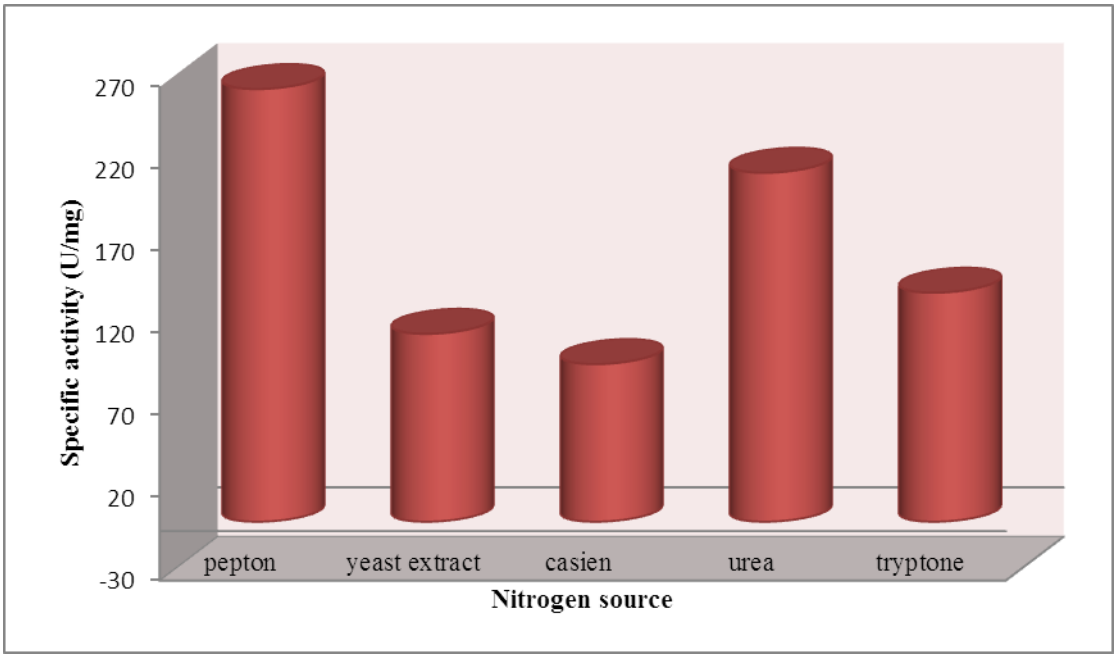

Figure (3-4): Nitrogen source optimization for proteases production by *S. aureus* isolate S28 when incubated for 24 h at 37°C in a shaker incubator (150 rpm).

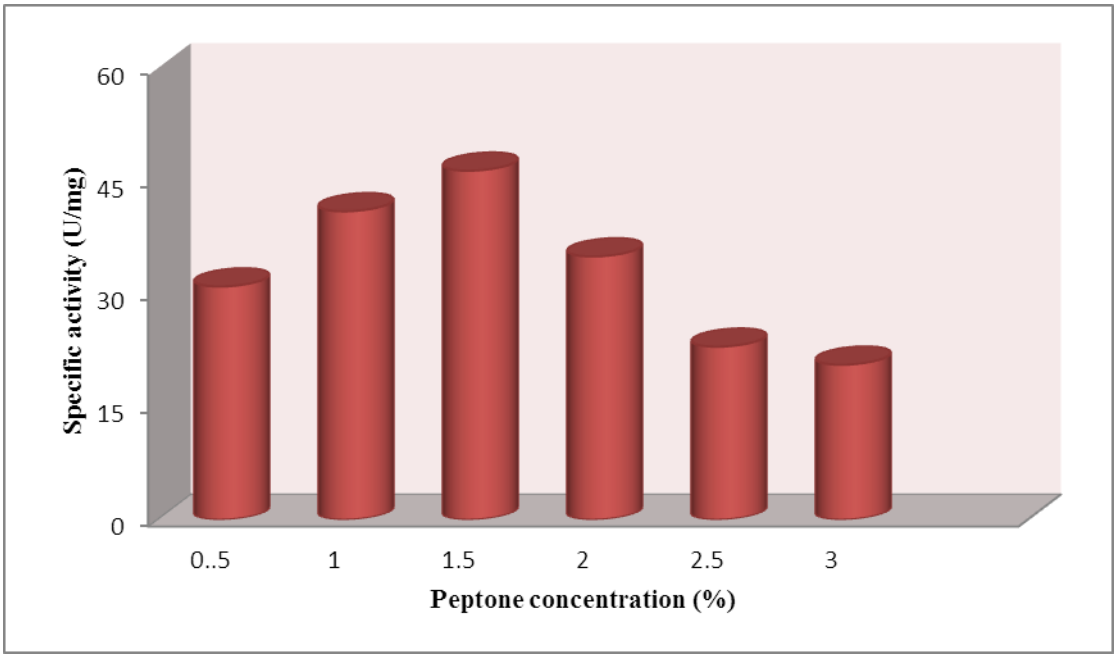

Figure (3-5): Optimization of peptone concentration for production of proteases by isolate S28 of *S. aureus* which incubated at 37°C in a shaker incubator with 150 rpm for 24 h.

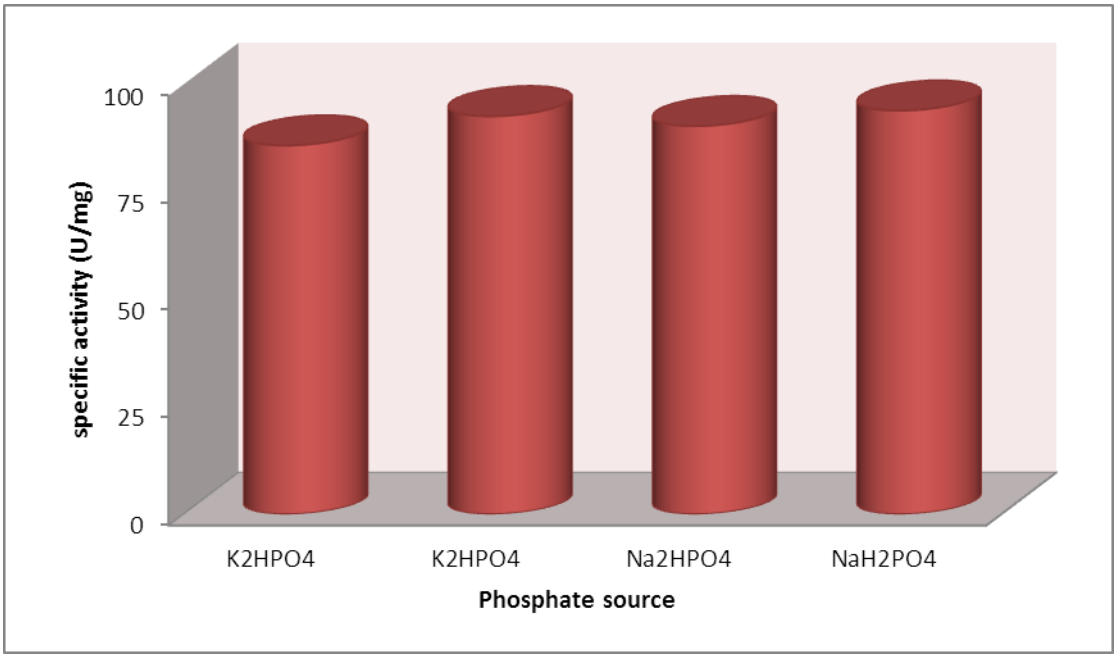

Figure (3-6): Influence of various phosphate sources on proteases production by the *S. aureus* isolate S28 which incubated at 37ºC in a shaker incubator (150 rpm) for 24 h.

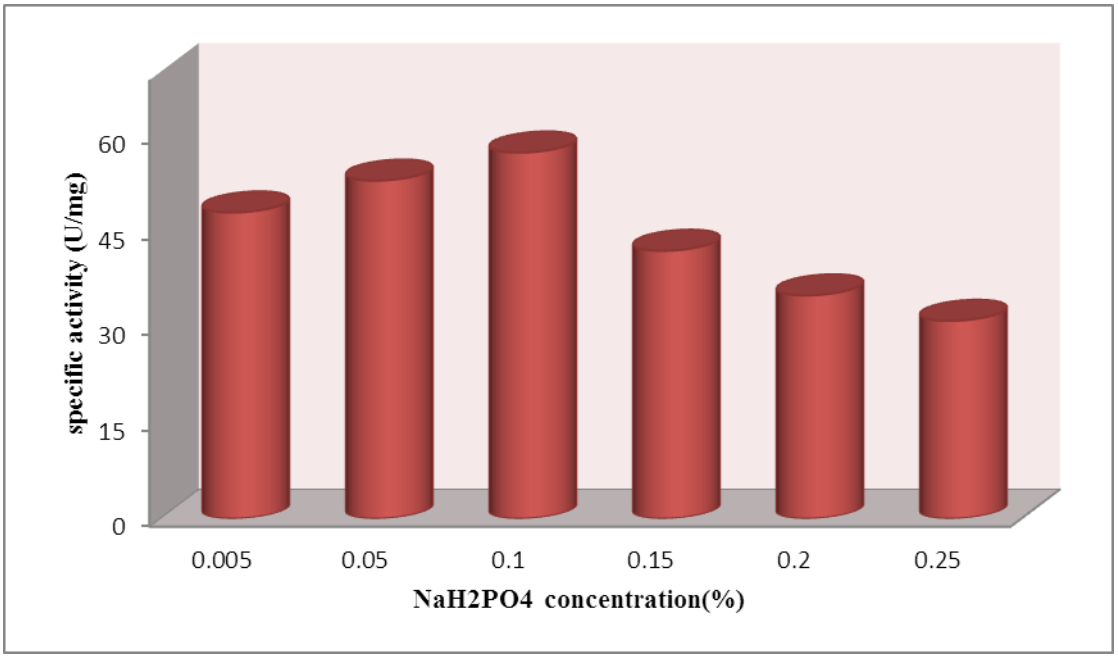

Figure (3-7): Optimizing phosphate concentration for proteases production by S28 isolate of *S. aureus* after shaking incubation (150 rpm) at 37ºC for 24 h

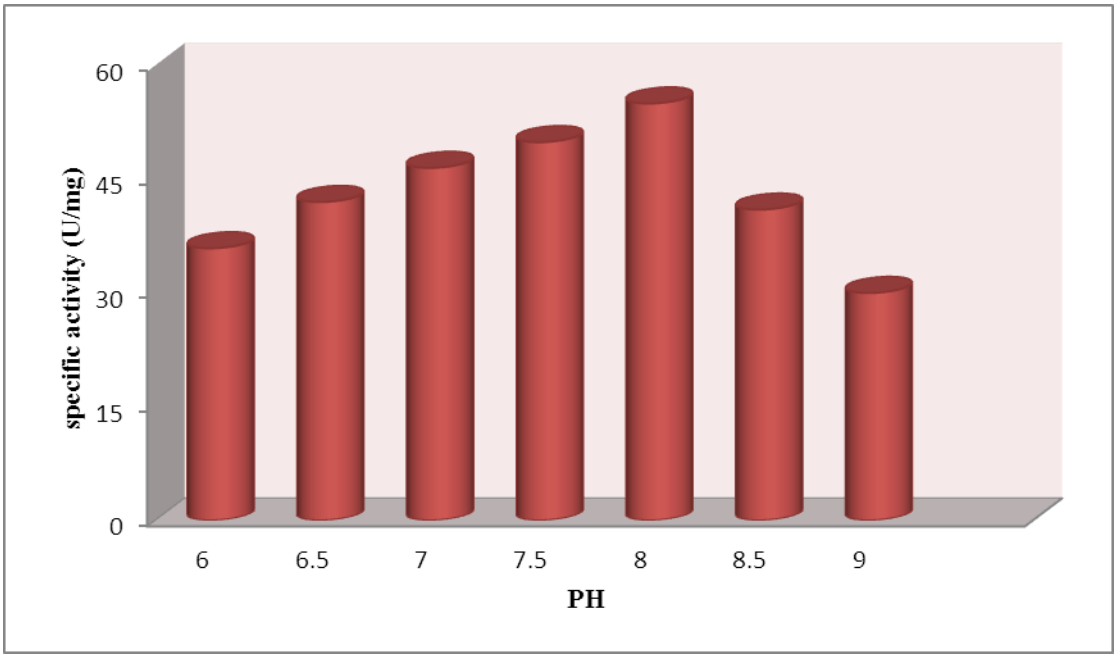

Figure (3-8): Effect of medium pH on proteases produced by the *S. aureus* isolate S28 after incubation at 37ºC in shaker incubator at 150 rpm for 24 h.

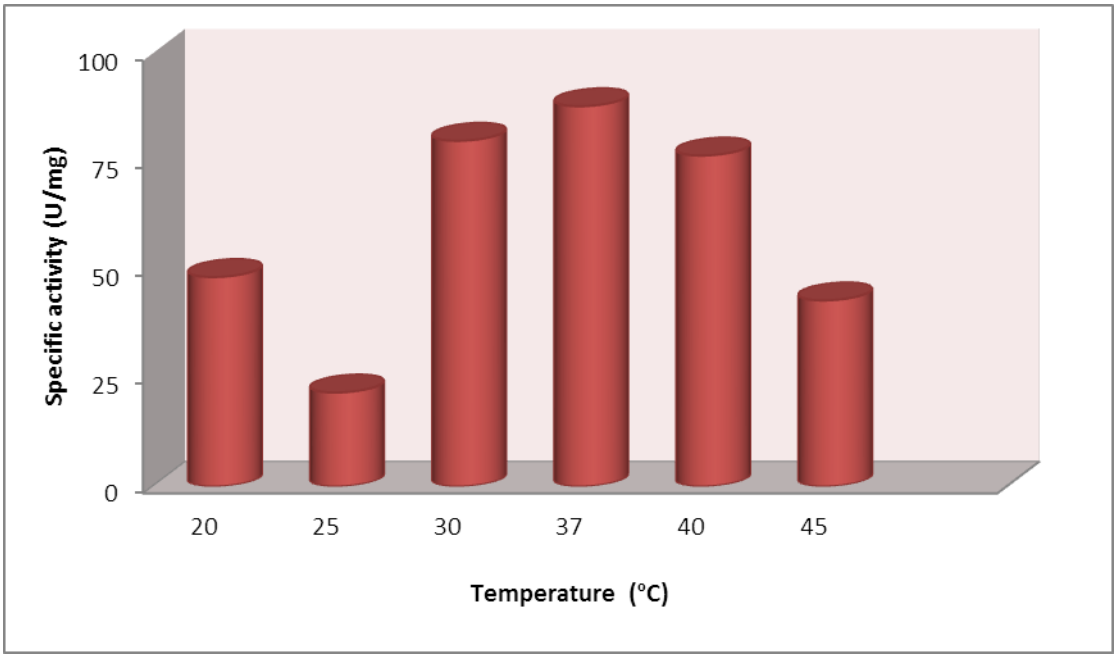

Figure (3-9): Optimal temperatures on proteases secreted by the *Staphylococcus aureus* isolate S28 in shaker incubator at 150 rpm for 24 h.

3.4 Antibiotic susceptibility of *S. aureus*

Isolate S28 of *S. aureus* was selected to study, its susceptibility toward antibiotics. The results showed that the isolate was completely sensitive to ciprofloxacin, chloramphenicol, novobiocin and rifampicin. On the other hand, it was highly resistant (83%) to clindamycin,

gentamicin and penicillin, but totally (100%) resistance to cephalothin, methicillin, tetracycline and vancomycin.

3.5 Ability of *S. aureus* isolates to form biofilm

Biofilm-forming ability has been increasingly recognized as an important virulence factor in *Staphylococcus*.[19] In addition, testing for biofilm formation could be a useful marker for the pathogenicity of *Staphylococcus*.[20]

Table (3-2): ELISA reader value detected the biofilm producer *Staphylococcus aureus* isolates by TCP method.

Mean OD values	Biofilm formation	*Staphylococcus aureus* isolate		
		TSB	TSBglu	BHIsuc
< 0.120	Non or weak	1	1	2
0.120 – 0.240	Moderate	14	13	13
0.240	High	Non	1	Non

3.6 Effect of protease on biofilm integrity

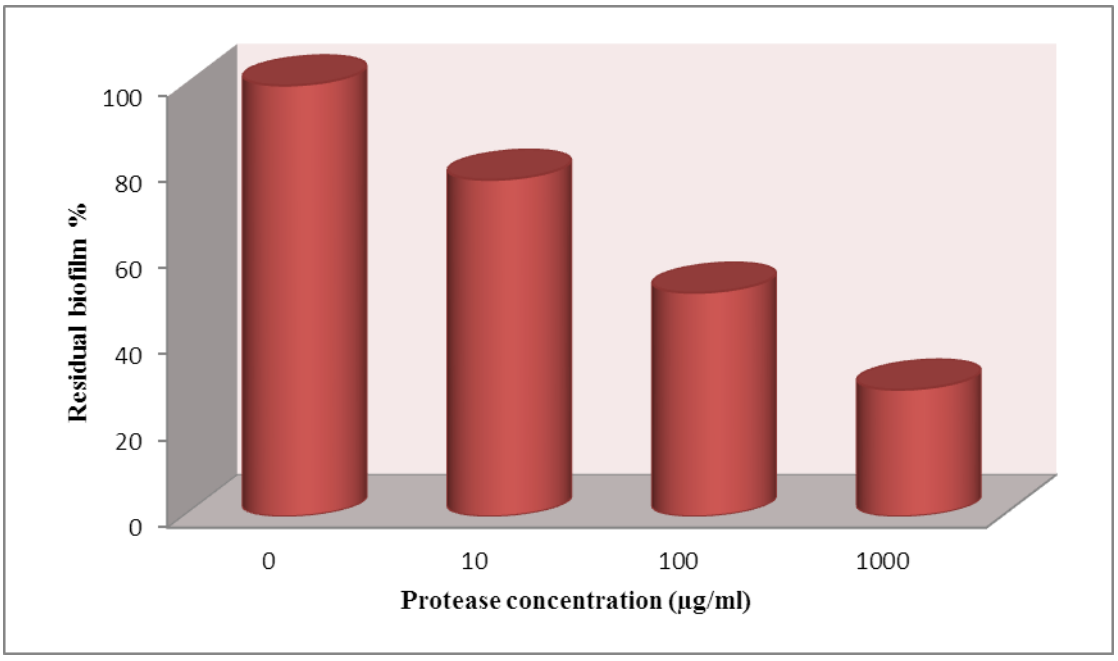

Figure (3-10): *Staphylococcus aureus* disintegration the biofilm by protease enzyme.

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