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Thrombolytic activity and antibacterial activity optimize staphylokinase enzyme production from *Staphylococcus aureus*

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ABSTRACT Staphylokinase is a virulence factor produced by *Staphylococcus aureus* that enhances its ability to degrade proteins, contributing to tissue damage and increased bacterial invasiveness. This investigation studied staphylokinase production by *S. aureus* isolates obtained from wound and burn patients. Optimal conditions for enhancing staphylokinase production in Sato's component were determined. These conditions included a carbon source (glucose), a nitrogen source (yeast extract), an inoculum size of 1%, an incubation temperature of 37 °C, and a pH of 7. Optimization of the medium components resulted in a significant increase in staphylokinase production (26.4 U/mL), representing a 2.23-fold rise compared to production in the unoptimized Sato's component. The crude staphylokinase enzyme exhibited thrombolytic activity against human blood clots, achieving 42% clot lysis. However, the crude enzyme showed no antibacterial activity against the tested bacteria (*Streptomyces* and *Escherichia coli*). This study represents the first report of optimized media for enhancing staphylokinase production from *S. aureus*. The research is significant because it establishes a method for improving the production of highly active staphylokinase from *S. aureus*, which has potential applications as a thrombolytic agent.

KEYWORDS Clots; Isolation; Optimization; Production; *Staphylococcus aureus*

1. Introduction

Staphylokinase (EC 3.4.99.22) is a member of the staphylococcal protein family. It comprises 136 amino acids and has a molecular weight of approximately 15.5 kilodaltons (kDa) (Vachher et al. 2021). This extracellular protein, secreted by *Staphylococcus aureus* in host tissue, can inactivate the human principal component of fibrinolysis. It is hypothesized that the increased conversion of fibrinogen to fibrin observed during the acute stages of bacterial infection could disrupt hemostasis. Staphylokinase, by activating plasmin, may exert antihemostatic and anti-inflammatory effects. However, clinical use requires that it does not exacerbate inflammation or interfere with antibiotic therapy, which can release toxins potentially leading to fatal sepsis. Therefore, antiendotoxin and antibacterial activities are crucial (Salavati et al. 2024). Thrombotic disorders are a major cause of morbidity and mortality globally. Thrombolysis is a therapeutic approach used to treat conditions such as myocardial infarction (heart attack), pulmonary embolism, stroke, and vascular obstruction (Ren et al. 2021). Heparin, a glycosaminoglycan, is an anticoagulant that enhances antithrombin activity. Administered intravenously (IV) or via injection, heparin is commonly used to treat myocardial infarction and angina, as well as to prevent clotting during specific medical procedures such as diagnostic tests and dialysis.

S. aureus produces staphylokinase primarily during the late exponential (or log) growth phase (Sumera et al. 2018). At this stage, the bacterium likely utilizes staphylokinase to penetrate host tissues by degrading clots that form at the wound, potentially facilitating further dissemination. The discovery of staphylokinase followed the observation of fibrinolytic activity similar to that of streptokinase (Buniya et al. 2023). Plasmin, activated by staphylokinase, breaks down fibrin clots, preventing occlusion of blood vessels (veins and arteries). In certain medical conditions, the physiological breakdown of fibrin within blood vessels is impaired, leading to the accumulation of fibrin, which can contribute to thrombus formation and cardiovascular diseases (Jasim and Ali 2021).

S. aureus is a significant human pathogen responsible for many bacterial infections (Howden et al. 2023). *S. aureus* is a Gram-positive, coccus-shaped bacterium typically forming "grape-like" clusters (staphylococci). Adding salt (NaCl) to growth media can enhance *S. aureus* growth by up to 10% (Mary et al. 2021). *Staphylo-*

coccus aureus utilizes multiple virulence factors to initiate infection, comprising extracellular toxins (such as leukotoxins, hemolysins, and enterotoxins), enzymes (including proteases, staphylokinase, hyaluronidase, and coagulases), and surface proteins (such as adhesins and clumping factors) (Cheung et al. 2021). These elements facilitate tissue colonization, injury, widespread disease, and evasion of the host's immunological response. Staphylokinase has been examined as a possibly more economical alternative thrombolytic drug, especially in light of rising antimicrobial resistance and related mortality (Abdula et al. 2024). This study aimed to isolate and characterize bacteria that produce staphylokinase and to optimize the bioprocess for increased enzyme production. *Staphylococcus aureus* isolates were collected from wound and burn infections.

2. Materials and Methods

2.1. Identification of bacteria

Eighteen *S. aureus* isolates were obtained from patients with wound and burn infections between September 2023 and November 2023. These specimens were collected from hospitals in Baghdad. The isolates' identification as *S. aureus* was confirmed using standard biochemical tests, including Gram staining, catalase testing, slide and tube coagulase tests, mannitol fermentation (Masoumeh et al. 2022) , and the VITEK® 2 Compact system (Delpech et al. 2020).

2.2. Screening of isolates for staphylokinase activity

Casein hydrolysis and plasma-heated agar assays were used to assess the proteolytic activity (ability to break down proteins) of the *S. aureus* isolates. The medium used was prepared according to the method described by Shagufta Naseer et al. (2014).

2.3. Production and extraction of staphylokinase

S. aureus was cultivated in Sato's component (containing 10 g/L nutrient broth, 3 g/L yeast extract, 5 g/L NaCl, and 10 mL/L glycerol) and incubated in a shaking incubator at 30 °C and 100 rpm for 24 h. The production broth was then centrifuged at 10,000 rpm for 10 min. The resulting supernatant was filter-sterilized using a 0.22 µm Millipore filter at 4 °C, and the filtrate was collected as the crude enzyme preparation (Chandrappa et al. 2017).

2.4. Staphylokinase activity assay

(Sutar et al. 1986) used the casein digestion technique to measure staphylokinase activity.

2.5. Protein Determine Assay

Bradford assay determined the overall protein content by employing bovine serum albumin (BSA) as the standard (Kruger 2009).

2.6. Optimization of physicochemical parameters for maximum production of Staphylokinase

2.6.1 The optimum source of carbon for staphylokinase enzyme production

Five carbon sources (fructose, lactose, sucrose, maltose, and starch) were tested at 5 g/L, replacing glucose in the production medium. The medium was inoculated with 100 µL of *S. aureus* at a concentration of (5.78×10^8 cells/mL); cultures were then incubated for a day at 37 °C with a pH level of 7.

2.6.2 The optimum nitrogen source for staphylokinase enzyme production

Five nitrogen sources (peptone, soybean, casein, meat extract, and ammonium sulfate) were tested at 10 g/L, replacing yeast extract in the production medium. The medium was inoculated with 100 µL of *S. aureus*, and cultures were then incubated for a day at 37 °C with a pH level of 7.

2.6.3 The optimum source of salt for staphylokinase enzyme production

The best salt source for staphylokinase enzyme production was done by testing different salt sources (5 g/L), which are KNO_3, $ZnSO_4$, $CaCl_2$, $MgCl_2$, $FeSO_4$, and $CuSO_4$, that were added instead of NaCl present in the production medium.

2.6.4 The optimum volume of glycerol for staphylokinase enzyme production

To determine the optimal volume of glycerol for staphylokinase production, five different volumes (1, 5, 15, 20, and 25 mL/L) were tested, replacing the 10 mL/L of glycerol already present in the production medium. The medium was then inoculated with 100 µL of *S. aureus* and day at 37 °C with a pH of 7.

2.6.5 A suitable temperature for staphylokinase enzyme production

The optimal temperature for staphylokinase production was determined by incubating 1,000 µL of *S. aureus* in a production medium at five different temperatures (25, 30, 35, 37, and 40 °C) for a day. Subsequently, staphylokinase enzyme activity was measured.

2.6.6 A suitable pH for staphylokinase staphylokinase enzyme production

The optimal pH for staphylokinase production was determined by incubating 1,000 µL of *S. aureus* in a production medium at five different pH levels (5.5, 6, 6.5, 7, 7.5, and 8) for a day. Subsequently, the staphylokinase enzyme activity was measured.

2.6.7 The optimum incubation time for staphylokinase enzyme production

To determine the optimal incubation time for staphylokinase production, *S. aureus* was inoculated into a production medium (100 µL inoculum) and incubated at 35 °C for 48, 72, and 96 h.

2.6.8 Thrombolytic activity of staphylokinase enzyme

The thrombolytic activity of the staphylokinase enzyme was determined using the Holmström method (Holmström 1965). In this technique, clotted human blood was prepared in eleven Eppendorf tubes. Fresh human blood (1 mL) was collected and dispensed into 1.5 mL Eppendorf tubes. After complete clot formation, the clots were weighed. The weight of each blood clot was determined by subtracting the weight of the empty Eppendorf tube from the weight of the tube containing the clot (Vaithiyalingam et al. 2020).

2.6.9 Antibacterial activity of staphylokinase enzyme

The disk diffusion test was used to determine bacterial susceptibility to antibiotics. A pure bacterial culture was swabbed onto an agar plate, and antibiotic-impregnated disks were placed on the agar surface. After incubation, the diameter of the zone of inhibition around each disk was measured and compared to Clinical and Laboratory Standards Institute (CLSI) guidelines to determine bacterial susceptibility (Kadhim et al. 2024).

3. Results and Discussion

3.1. Staphylokinase production detection

3.1.1 Hydrolysis casein assay

Casein hydrolysis is a well-established method for detecting proteolytic activity (Aminlari 2022). A clear zone surrounding colonies grown on casein agar indicates casein degradation. In this study, 11 of the 18 *S. aureus* isolates (61.11%) exhibited such clear zones (halos). These results suggest that 61.11% of the isolates possess proteolytic activity, potentially including staphylokinase production. This finding is comparable to Devi et al. (2012) study, which reported that 58% of isolates exhibited similar activity. Casein has long been used as a substrate for studying various proteolytic enzymes, including streptokinase, staphylokinase, tryptase, and serum proteases (Devi et al. 2012). Some researchers have even suggested that casein may be a more suitable substrate for plasmin than fibrin or fibrinogen (Sharma et al. 2021).

3.1.2 Plasma heated Assay

The plasma-heated agar assay was primarily used for qualitative assessment of enzyme activity. Clear fibrinolytic halos were observed around the wells, indicating zones of fibrinolysis around the colonies on the plasma agar plates. The diameters of these zones of inhibition were measured in millimeters. Only 13 of 18 isolates (72.22%) exhibited clear halo zones. Plasma is used as a substrate for detecting proteolytic enzymes (Adam et al. 2016).

3.1.3 The optimal isolate for staphylokinase enzyme production

Isolate BS7, selected from 23 screened samples, was confirmed to produce staphylokinase. It exhibited the largest hydrolysis zones in the casein hydrolysis assay (27 mm diameter) and the plasma-heated agar assay (29 mm diameter).

3.1.4 The optimum source of carbon for staphylokinase enzyme production

The availability of metabolizable carbon sources can significantly influence cell growth and the production of essential metabolic substances (Tang et al. 2022). In this study, various carbon sources (fructose, lactose, sucrose, maltose, and starch) were incorporated into the enzyme production medium, which was then incubated at 37 °C and pH 7 for 24 hours to determine the optimal carbon source for staphylokinase production. *S. aureus* yielded the highest enzyme production (13.7 U/mL) when sucrose was used as the carbon source, followed by maltose (10.4 U/mL). The lowest enzyme yields were observed with lactose and starch (7.8 and 5.3 U/mL, respectively), while glucose resulted in an enzyme yield of 11.8 U/mL (Figure 1a). These results align with Alzahrani and El-Shenawy (2020) findings, which demonstrated that sucrose provided the highest enzyme yield. Studies have shown that carbon sources such as glucose, sucrose, and maltose can influence staphylokinase (SAK) production by *S. aureus*. However, the optimal carbon source and concentration can vary depending on the strain, as demonstrated in studies on *S. aureus* ASIA4, where sucrose and maltose resulted in higher SAK production than glucose. Furthermore, the catabolite control protein A (CcpA) can repress *SAK* gene expression in the presence of glucose, further impacting production (Micallef 2008).

3.1.5 The optimum source of nitrogen for staphylokinase enzyme production

The carbon source was held constant while different nitrogen sources (peptone, casein, meat extract, soybean, and ammonium sulfate) were tested to determine the optimal nitrogen source for staphylokinase production. The media were incubated at 37 °C and pH 7 for 24 hours. Nitrogen sources influence bacterial growth and cell production (Wang et al. 2016) significantly. In this study, *S. aureus* yielded the highest enzyme production (16.4 U/mL), with soybean as the nitrogen source. Lower yields were obtained with casein (12.9 U/mL), peptone (12.1 U/mL), ammonium sulfate (11.7 U/mL), and meat extract (9.8 U/mL). Yeast extract, used as a control, resulted in a yield of 13.7

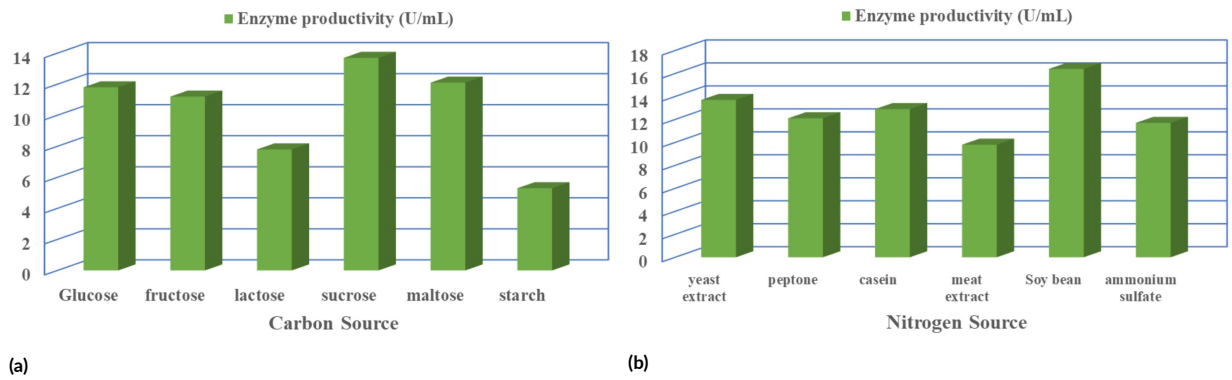

FIGURE 1 (a) Staphylokinase enzyme productivity with optimum carbon source (b) Staphylokinase enzyme productivity with optimum nitrogen source.

U/mL (Figure 1b). These results contrast with Deepa et al. (2019) findings, which identified yeast extract as a primary nitrogen source for staphylokinase synthesis, with optimal efficacy at a concentration of 2.65% (w/v). It is established that nitrogen sources such as yeast extract, beef extract, and peptone can influence staphylokinase (SAK) production by *S. aureus* and that the optimal nitrogen source and concentration can vary depending on the strain (Kotra et al. 2012).

3.1.6 The optimum source of salt for staphylokinase enzyme production

To determine the optimal salt source for staphylokinase production, various salts (KNO_3, $ZnSO_4$, $CaCl_2$, $MgCl_2$, $FeSO_4$, and $CuSO_4$) were individually added to the enzyme production medium. The mixtures were then incubated for 24 hours at 37 °C and pH 7. The carbon and nitrogen sources were carefully controlled to ensure optimal conditions. The results indicated that enzyme productivity was lower when using ($CaCl_2$, $MgCl_2$, $FeSO_4$, KNO_3, and $CuSO_4$) compared to NaCl. When NaCl was used as the sole salt source, enzyme productivity reached 16.7 U/mL (Figure 2a). These findings demonstrate that the salt source can significantly influence staphylokinase (SAK) production by *S. aureus*. While NaCl is commonly used, studies have shown that its replacement with other salts, such as ($CaCl_2$, $MgCl_2$, KNO_3, $ZnSO_4$, and $CuSO_4$), can significantly reduce SAK production (Deepa et al. 2019).

3.1.7 The optimum concentration of glycerol for staphylokinase enzyme production

Different glycerol concentrations (1, 5, 10, 15, 20, and 25 mL/L) were added to the production medium to determine the optimal glycerol concentration for staphylokinase production. The media were incubated for 24 hours at 37 °C and pH 7, with optimal carbon, nitrogen, and salt sources maintained. *S. aureus* yielded the highest enzyme production (19.6 U/mL) at a 15 mL/L glycerol concentration. Lower enzyme yields were observed at 1 mL (7.6 U/mL), 5 mL (9.9 U/mL), 10 mL (13.2 U/mL), 20 mL (12.6 U/mL), and 25 mL/L (12.6 U/mL) (Figure 2b). Glycerol is commonly used as a carbon source in microbial fermentation processes, providing energy and carbon for cell growth and metabolism (Patil et al. 2017). However, excessive glycerol concentrations can induce osmotic stress, inhibiting cell growth and metabolism and consequently affecting the production of secondary metabolites such as staphylokinase (Nevoigt and Stahl 1997).

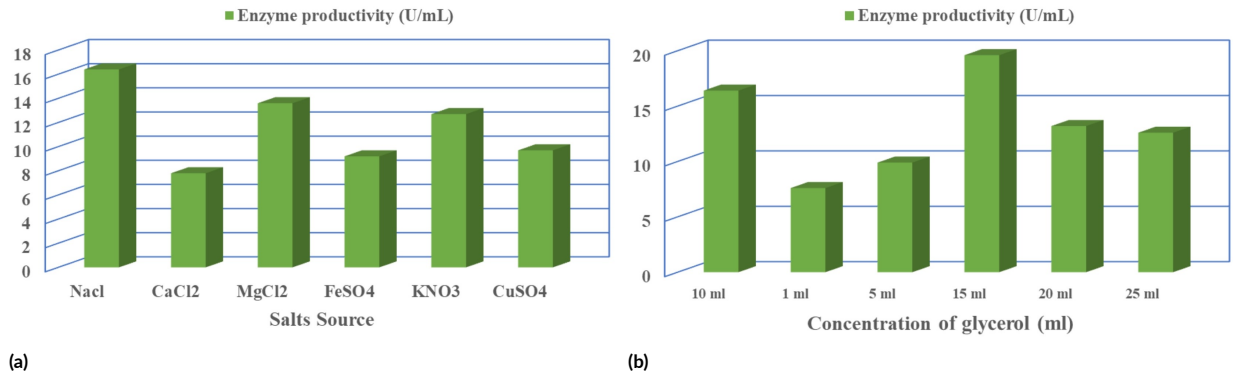

FIGURE 2 (a) Staphylokinase enzyme productivity with optimum salts source (b) Staphylokinase enzyme productivity with optimum glycerol concentration.

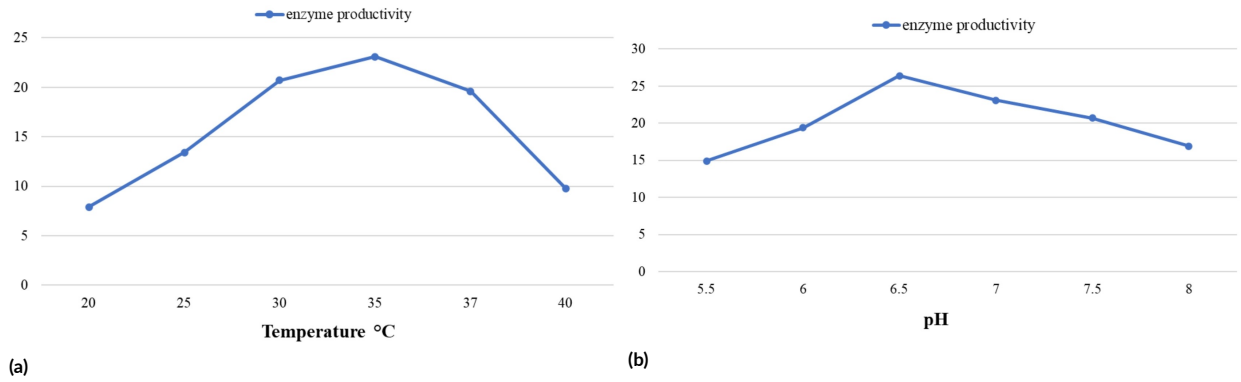

FIGURE 3 (a) Staphylokinase enzyme productivity with optimum temperature (b) Staphylokinase enzyme productivity with optimum pH.

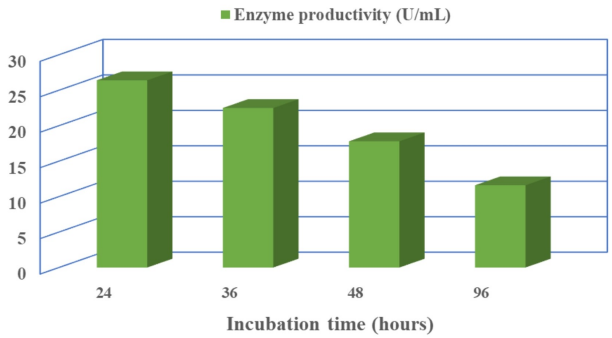

FIGURE 4 Staphylokinase enzyme productivity with optimum incubation time.

3.1.8 A suitable temperature for staphylokinase enzyme production

The optimal temperature for staphylokinase production was determined by incubating the production medium with *S. aureus* (100 µL inoculum) at temperatures ranging from 25 to 45 °C, replacing the standard 37 °C, for 24 hours at pH 7. The highest enzyme productivity (23.1 U/mL) was observed at 35 °C, while the lowest (9.8 U/mL) was recorded at 25 °C (Figure 3a). Fluctuations in incubation temperature and pH can significantly influence staphylokinase production by *Staphylococcus* sp. (Vijayaraghavan and Prakash Vincent 2015). The *S. aureus* strain used in this study demonstrated robust growth and enzyme synthesis across a range of temperatures, suggesting its adaptability for enzyme production under varying temperature conditions. Temperature is a known factor influencing bacterial enzyme production (Saravanan et al. 2021), and specifically, temperature significantly influences staphylokinase (SAK) production by *S. aureus*, with optimal temperatures typically ranging from 30 to 37 °C. Temperatures outside this range can reduce SAK production (Salman and Tajaldeen 2018).

3.1.9 A suitable pH for staphylokinase enzyme production

To determine the optimal pH for staphylokinase production, *S. aureus* was incubated in a production medium at five different pH levels (5.5, 6, 6.5, 7.5, and 8) for 24 hours at 37 °C, replacing the standard pH of 7. The highest enzyme productivity (26.4 U/mL) was observed at pH 6.5, which was identified as the optimal pH. Conversely, the lowest enzyme productivity (10.6 U/mL) was observed at pH 8 (Figure 3b). This finding is consistent with Deepa et al. (2019) study, which also reported an optimal pH of 6.5. In this study, enzyme-specific activity decreased with increasing pH. pH is a crucial factor influencing bacterial enzyme production. It affects microbial growth by controlling protein synthesis and inhibition and cellular reduction-oxidation reactions. pH also impacts cellular energy production and utilization, which is essential for bacterial biological functions (Arzoo et al. 2020).

3.1.10 The optimum incubation time for staphylokinase enzyme production

To determine the optimal incubation time for staphylokinase production, the production medium was inoculated with 100 µL of *S. aureus* and incubated at 35 °C for various durations (48, 72, and 96 h). The highest enzyme productivity (26.4 U/mL) was observed after 24 h. When incubation was extended to 48, 72, and 96 h, enzyme productivity decreased to 22.5, 17.8, and 11.6 U/mL, respectively (Figure 4). This decrease in enzyme productivity with longer incubation times may be attributed to changes in culture conditions, such as depletion of nutrients and oxygen and the accumulation of toxic metabolites that can inhibit bacterial growth (Gonzalez and Aranda 2023). Staphylokinase production is typically highest during the logarithmic (log) growth phase, which correlates with optimal enzyme production. A more consistent, albeit lower, level of enzyme production during the stationary phase suggests that staphylokinase production may play a nutritionally significant role for this strain. In contrast, Khursade et al. (2019) observed increased growth and enzyme production with increasing incubation time in *Stenotrophomonas maltophilia* Gd2, suggesting that enzyme production in that strain is directly linked to growth.

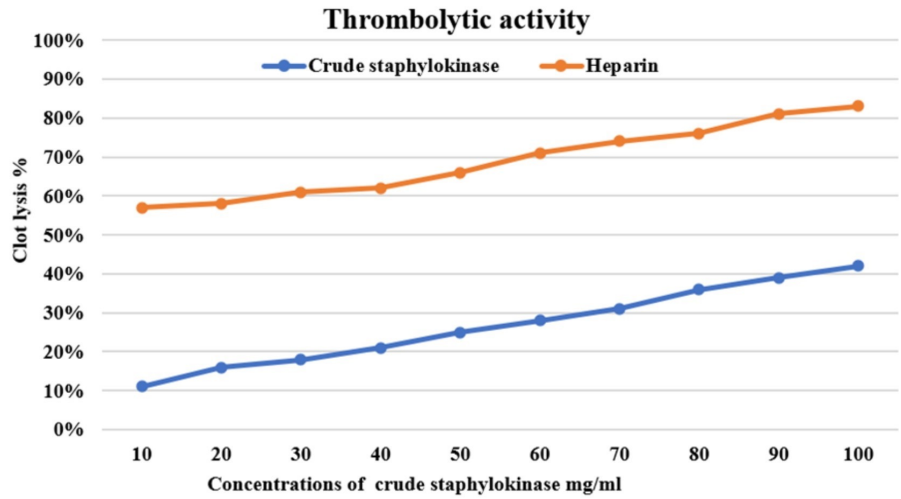

FIGURE 5 Thrombolytic activity of crude staphylokinase enzyme and heparin on human blood clots.

3.2. *Thrombolytic activity of staphylokinase enzyme*

The thrombolytic activity of crude staphylokinase and heparin was tested on human blood clots. At a 100 mg/mL concentration, crude staphylokinase and heparin exhibited maximum clot lysis. Heparin achieved 83% clot lysis within one hour, while crude staphylokinase achieved 42% (Figure 5). Staphylokinase (SAK) is a potent thrombolytic enzyme that effectively dissolves blood clots by activating plasminogen to plasmin, degrading fibrin. Numerous studies have demonstrated its efficacy in both *in vitro* and *in vivo* models, including clinical trials showing promising results in clot dissolution and reduced mortality rates (Szarka et al. 1999).

3.3. *Antibacterial activity of staphylokinase enzyme*

The antibacterial activity of staphylokinase was tested using the agar well diffusion method (Omran et al. 2024). The results showed that crude staphylokinase, at concentrations of 50, 100, 150, and 200 mg/mL, did not exhibit antibacterial activity against any tested bacteria (*Streptomyces* and *Escherichia coli*) (Figure 6). Staphylokinase (SAK) primarily functions as a thrombolytic agent, dissolving blood clots by activating plasminogen to plasmin. While some studies suggest that SAK may indirectly influence bacterial infections by modulating the host immune response, its direct antibacterial activity is limited. Investigating the potential antibacterial activity of staphylokinase is relevant due to its dual nature as both a potential therapeutic agent and a virulence factor. Although primarily known for its thrombolytic properties, some studies suggest that staphylokinase may indirectly affect bacterial infections by modulating the host immune response. However, direct antibacterial activity remains limited. Understanding any potential antibacterial effects of staphylokinase could provide further insights into its role in bacterial pathogenesis and inform its therapeutic applications (Wang et al. 2022).

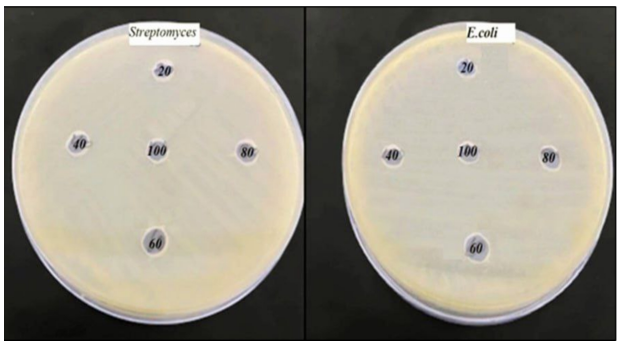

FIGURE 6 Antibacterial activity of Staphylokinase on *Streptomyces* (left) and *Escherichia coli* (right).

3.4. *Significance of the study*

S. aureus produces staphylokinase, a virulence factor contributing to tissue damage and increased bacterial invasiveness. Optimization of the production conditions in Sato's component resulted in a significant 2.23-fold increase in enzyme yield, reaching 26.4 U/mL compared to the standard medium. This study represents the first report of media optimization for staphylokinase production in Iraq and has potential implications for therapeutic thrombolysis.

4. Conclusions

Optimizing culture media conditions and modifying media composition enhanced staphylokinase production. A one-factor-at-a-time approach identified the independent variables influencing enzyme production and their optimal levels for further optimization. This study contributes to reducing the time and cost associated with staphylokinase production. The crude staphylokinase enzyme exhibited significant thrombolytic activity against human blood clots, suggesting its potential for development as a therapeutic thrombolytic agent.

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Authors' contributions

Literature search: BHJ. Data acquisition: MSJ and RJS. Data analysis & interpretation: BHJ and MDK. Manuscript preparation: BHJ and EHA. Manuscript editing & review: BHJ, MHS, BSM, ZAF.

Competing interests

The authors declare have no competing interest.

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