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Evaluation of Blood Clot Removal Using Immobilized Nattokinase by Carbon Nanotube from *Pseudomonas aeruginosa*

[1]Shahad A. Shwan, [1]Ali J. Reshak

[1]Department of Biotechnology, College of Science, University of Baghdad

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Abstract: Nattokinase (NK) enzyme has been mentioned to have effective fibrinolytic activity and it has amenities over other commercially used medicines in preventative and prolonged effects. Therefore, this study aimed to characterize, immobilize NK from local isolate of *P. aeruginosa* and applies its fibrinolytic activity on blood clot. A previously isolated *P. aeruginosa*, which was obtained from wound burn infection and identified using different examinations, and it diagnosed employing PCR with *16SrRNA* gene (956bp), then previously screened to produce NK enzyme under optimum conditions. The results indicated that partial purified NK has the best activity in pH 7.5, with enzyme activity of 34.33 U/ml. Also, the stability of NK was detected in pH range from 4.0 to 8.5. The best temperature of NK activity was 45°C the NK stability was at temperatures ranging from 25 to 45°C. Casein was the best substrate with high activity (70.6 U/mg protein) of partial purified enzyme, then the activity decrease with bovine (47 U/mg), fibrin (63.36 U/mg), gelatin (31.36 U/mg), collagen(32.22 U/mg). NK has been successfully immobilized covalently on the functionalized carbon nanotube (MWNT). The enzyme loading was 67.14% by using amount of (706 U/mL) NK per 3 mg of MWNT. It was concluded the blood clot degradation by NK was determined by dissolving human blood clot, and the result indicated that the NK display excellent fibrinolytic activities *in vitro*.

Keywords: Nattokinase, Blood clot, Nanotube, *Pseudomonas aeruginosa*.

Corresponding author: (Email: shahadabdullahhhhhhh@gmail.com).

Introduction

Nattokinase (NK) refers to a group of profibrinolytic serine proteases that have significant activity in the breakdown of fibrin. The generation of this enzyme has been accomplished using a diverse range of host strains. NK exemplifies the advantages associated with an extended duration of efficacy, a cost-effective nature, and limited occurrence of adverse effects. Moreover, it exhibits the potential to be employed as a pharmaceutical agent for the management of cardiovascular disorders and as a beneficial dietary supplement, in comparison to other fibrinolytic enzymes (1). NK enhances the endogenous ability of the human body to combat thrombotic events through many mechanisms. Due to its striking similarity to plasmin, it exhibits the ability to immediately lyse fibrin. Moreover, it also enhances the endogenous synthesis of plasmin and other molecules responsible for breaking blood clots, including

urokinase (2). Moreover, when compared to fibrinolytic medications commonly employed in therapy, this particular enzyme presents several notable advantages. These advantages encompass the capacity to be administered orally, a preventive and enduring effect, as well as stability inside the gastrointestinal tract. Moreover, it should be noted that NK has the potential to be absorbed through the gastrointestinal tract, so eliciting the activation of fibrinolysis (3). Furthermore, NK exhibits a diverse range of pharmacological advantages, such as its ability to improve microcirculation and lower blood pressure, prevent atherosclerosis, alleviate retinal angiogenesis, combat cancer, inhibit inflammation and oxidative stress, and provide various other benefits (4). In clot lysis experiments, NK demonstrates activity comparable to that of plasmin and lyses fibrin directly. In terms of breaking down cross-linked fibrin, kinetic experiments suggest that NK is six times more active than plasmin (5). One of the most common species that used to produce NK is *Pseudomonas aeruginosa* (1). This bacterium is ubiquitous microorganism, which may be found in both human-made and natural environment (6). Despite its ability to cause infectious diseases (7), *P. aeruginosa* also have different beneficial activities, including producing useful enzymes (8), such as NK (1).

Many enzymes, including NK, have been reported to be immobilized onto nanoparticles, including Carbon nanotubes (CNTs) (9), which is also called buckytube, nanoscale hollow tubes composed of carbon atoms.

Therefore, this study aimed to characterize, immobilize NK from local isolate of *P. aeruginosa* and applies its fibrinolytic activity on blood clot.

Materials and methods

Identification of NK-producing isolate

A previously isolated *P. aeruginosa* , which was obtained from wound burn infection and identified using different examinations. After that, according to Spilker *et al.* (10) with some modification the genomic DNA was extracted from *P. aeruginosa* by using the HiPurA® bacterial genomic DNA purification kit. The primer that used in this study was for *16SrRNA* gene (956bp), Table (1). The isolate was previously screened to produce NK enzyme and utilized in this study.

Table (1): Primers used in this stud.

Primers' name		Primer's sequence (5'→3')	Product size (bp)	Ref.
16SrRNA	***F***	GGGGGATCTTCGGACCTCA	956	(11)
	R	CCTTAGAGTGCCCACCCG		

Extraction of NK enzyme from *Pseudomonas aeruginosa*

Following 24 hours incubation period, the enzyme from each flask was extracted using centrifugation at a speed of 10,000 rpm for duration of 30 minutes. Subsequently, the supernatant underwent filtration utilizing Whatman filter paper No. 1. The supernatant has been determined to have potential utility as a crude enzyme.

Determination of NK activity

Method described by (12) was utilized to determine the NK enzyme activity as shown below: 1.8 milliliters of casein solution was placed in water

bath for 5 min at 45°C., then 0.2 milliliters of the crude enzyme was added to the substrate solution and incubated for 30 min at 37°C. After adding three milliliters of a trichloroacetic acid (TCA) solution with a concentration of ten percent, the reaction was stopped, and the pellet was separated by centrifuging the mixture at ten thousand revolutions per minute for ten minutes. A blank was made by following the identical protocols as the test sample, with the exception that a 10% TCA solution was mixed with the casein solution before the addition of 0.2 milliliters of enzyme. A UV-VIS spectrophotometer was used in order to determine the absorbance of the supernatant at 280 nm. The enzyme activity and protein concentration were each measured twice (13), and the findings were reported based on the average of the two readings. According to the formula that follows, one unit of enzyme activity was defined as the quantity of enzyme that induced a rise of 0.01 in the absorbance at 280 nm during one minute under the experimental progression (14):

$$\text{Enzyme activity}\left(\frac{\text{U}}{\text{milliliters}}\right) = \frac{\text{Absorbance at 280 nm.}}{0.01 \times 30 \times 0.2}$$

Where: 0.01: Constant, 30: Reactive time (min.) and 0.2 : Enzyme volume

Characterization of NK

Effect of different pH values on activity of NK

Different buffers were prepared with ionic power of 0.05 M and pH range of 3-9 including sodium acetate buffer pH (3, 3.5, 4, 4.5, 5, 5.5, 6 and 6.5), potassium phosphate buffer pH (7, 7.5), and Tris-HCl buffer pH (8, 8.5 and 9). In order to determine the effect of pH on activity of partial purified NK, solutions of substrate (casein 1%), were prepared at different pH values. The enzyme activity was estimated and the relation between enzyme activity toward pH values was plotted to determine the optimum pH of NK activity (12).

Effect of different pH values on stability of NK

Different buffers were prepared with ionic power of 0.05 M and pH range of 3-9 including sodium acetate buffer pH (3, 3.5, 4, 4.5, 5, 5.5, 6 and 6.5), potassium phosphate buffer pH (7, 7.5), and Tris-HCl buffer pH (8, 8.5 and 9). Equal volume of partial purified enzyme was mixed with various buffers at a ratio of 1:1 (v:v), and the mixture was incubated in a water bath for 30 min. at 45°C, then the samples were transferred directly to ice bath. The enzyme activity was calculated, and the optimal pH for NK stability was found by plotting the residual activity percentage against pH (12).

Effect of different temperatures on NK activity

Different temperature ranges (25-50)°C were used to evaluate NK activity, and a graph of enzyme activity vs temperature was created to establish the optimum temperature of enzymatic activity (12).

Effect of different temperatures on NK stability

The NK was partially purified by incubating it at various temperatures between 25 and 50 degrees Celsius for 30 minutes before chilling it in an ice bath. The optimal temperature for NK stability was calculated by estimating NK activity and then plotting the remaining activity % versus temperatures (12).

Effect of different substrates on partial purified NK

Partial purified NK was incubated with different substrate including (bovine, fibrine, gelatin, collagen and casein) to determine the best substrate on NK activity (12).

Functionalization of carbon nanotube (MWCNTs)

Carbon nanotubes (MWCNTs) can be made functional by an acid oxidation treatment process. One thousand milligrams of carbon nanotubes are combined with one hundred milliliters of a 3:1 concentration of H_2SO_4 and HNO_3 (15). Functionalization will take place while the combination sits in a water bath at 40 °C for 4.5 hours and then in an ultrasonic bath for 15 minutes. After being dried in a vacuum oven at 60 °C for 48 hours, the functionalized carbon nanotube (MWCNT) is ready for collection. The multi-wall carbon nanotube (MWCNT) is used after being dried and cooled for 24 hours.

Immobilization of NK enzyme on functionalized carbon nanotube (MWCNT)

Physical means were used to immobilize the NK enzyme. Dissolving 30 mg of multi-walled carbon nanotubes (MWCNTs) in 10 mL of NK solution (706 U/mL). After a thorough mixing, a sample of the produced solution is incubated in an incubator shaker at 30 ^{o}C, 140 rpm speed, for 2.5 hours. The functionalized carbon nanotube (MWCNT)-NK conjugate is separated from the composite mixture by centrifugation at 3800 rpm for 15 minutes following incubation. Subsequently, the supernatant is decanted with great care to prevent conjugation loss. After that, the MWCNT-NK composite is washed in a new phosphate buffer solution (pH 7) before being re-dispersed. In order to eliminate the unbound functionalized MWCNT, the procedure of washing and centrifugation must be performed at least three to four times. Supernatant activity of functionalized MWCNT- NK was measured and preserved for future experiments (15).

Blood clot degradation tests

The ability of NK to degrade fake blood clots was measured in two ways: Slides were prepared with human blood (blood groups A, B, AB, and O) that had been drawn without an anticoagulant. After 45 minutes of clot formation in room temperature, a drop of blood (2 ml) was treated with 100 µl of free and immobilized enzyme. At room temperature, the clot disintegration was tracked for 5 minutes (14). Another blood clot destruction test was performed according to the method of Vijayaraghavan *et al*. (17) with slight modification. The clot formed naturally after the blood sample (2 ml) was placed in a glass test tube and allowed to sit at room temperature for 60 minutes. The synthetic blood clot was then given immobilized partially pure NK and a free partial purified enzyme. The control experiment was carried out at 37°C. Blood clot destruction was measured after a 5-minute incubation period.

Results and discussion

Diagnosis on *P. aeruginosa* using PCR

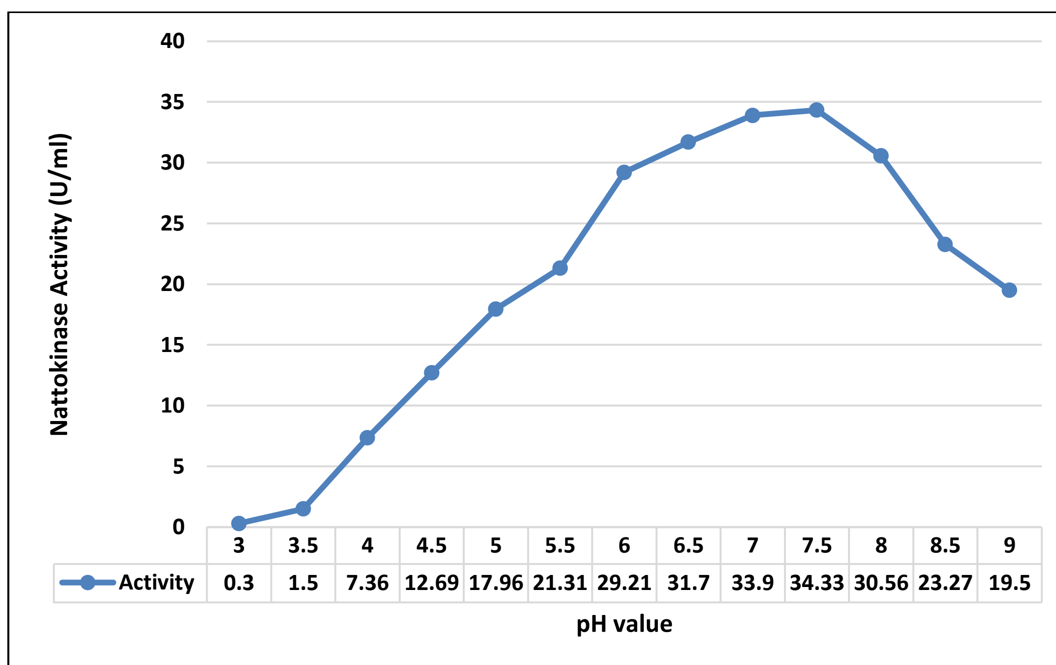
The current findings showed that *16SrRNA* gene (956bp) was present in *P. aeruginosa* isolates.

Characterization of partial purified NK

Optimal pH for NK activity

The results, in (figure 1), demonstrated that NK has the best activity in pH ranged between

(6.5 to 8), with maximum enzyme activity at pH 7.5 with enzyme activity of 34.33 U/ml. Whereas reduced activity was observed at alkaline pH (pH 8,8.5 and 9) and at nearly acidic pH (pH 5.5, 6 and 6.5).



	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
Activity	0.3	1.5	7.36	12.69	17.96	21.31	29.21	31.7	33.9	34.33	30.56	23.27	19.5

Figure (1): Effect of different pH values on partial purified NK activity produced by *P.* aeruginosa using casein as a substrate.

Based on the results, the activity of NK was gradually increased with increasing in pH until reach to 7.5, then the activity start to reduced. Possible mechanisms by which pH affects enzyme activity include changes in enzyme or substrate shape, ionization of groups in the substrate and ionization of groups in the enzyme active site (18).

A previous study has recorded that the amino acids composition of NK plays an important role in its optimum reaction pH value (17). Hence, it is speculated that, in this study, the NK enzyme may contain more basic amino acids than acidic amino acids. The findings of this work was compatible with the result obtained by Lin *et al.* (20), who found that purified NK activity from *Bacillus subtilis* N1 has an optimal pH of 8.0. Other studies also mentioned that the optimum pH of purified fibrinolytic enzymes from different *Bacillus* spp. was 8.0 (19,20). Also, Moidutty *et al.* (23) found that the optimum pH of NK activity purified from *B. subtilis* was 7.0.

Stability of NK at different pH values

The results in (Figure 2) indicated that the stability of NK was detected in pH range (4.0 – 8.5). Out of this range, the activity was decreased, specifically, at nearly acidic pH, whereas approximately half of activity was lost at pH 3. The enzyme was retained 90% of its activity in pH 8.0 while retained 86% and 64%of its activity in pH 8.5 and pH 9.0, respectively.

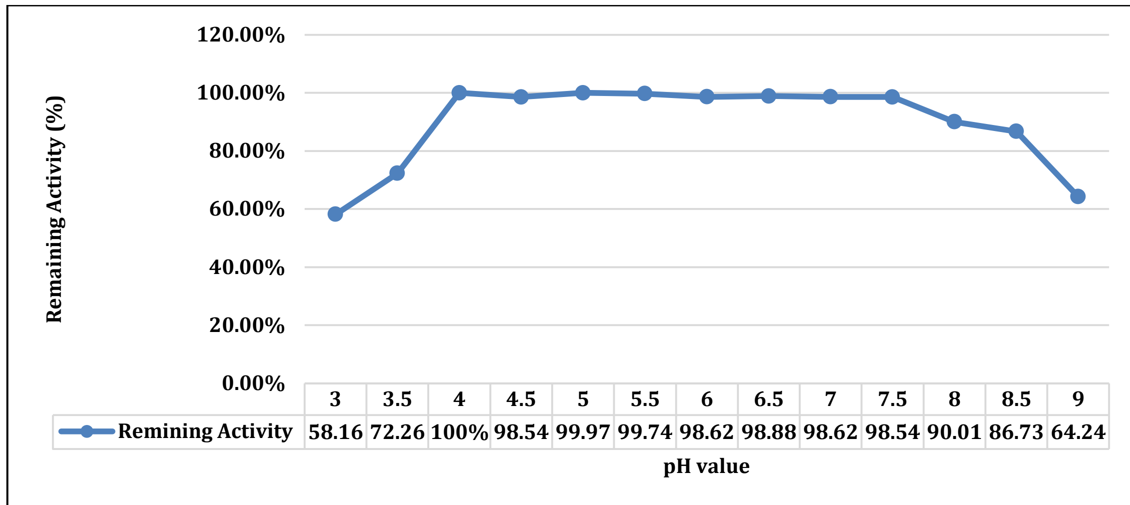


Figure (2): Effect of different pH values on partial purified NK stability using casein as a substrate.

The enzyme's activity decreases as the pH of the buffer solution moves away from its optimal value because the enzyme's secondary and tertiary structures are altered, and the enzyme's active site and substrate undergo ionic changes (24). Furthermore, most enzymes are susceptible to irreversible denaturation in solutions that are either highly acidic or highly basic (22). Multiple studies have shown a specific pH range where fibrinolytic enzymes are most stable. Lin *et al*. (20), found that P.P.NK from *B. subtilis* has the highest stability at a pH of 5.0 to 9.0, whereas Bajaj *et al*. (21) showed that purified fibrinolytic protease from *B. subtilis* has a pH sweet spot between 7 and 10, also Purified fibrinolytic enzyme from *Bacillus cereus* is most stable between a pH range of 7.0 and 9.0 (20). NK enzyme activity was found to be rather stable at neutral pH but to be easily inhibited by both high and low pH (25).

Optimal temperature for NK activity

By incubating the partially purified enzyme and substrate mixture at temperatures between 25 and 50°C, the optimal temperature for NK activity was identified. (Figure 3) shows that at 45 degrees Celsius, NK activity is at its highest at 66.9U/ml, and that it gradually decreases to 33.03U/ml at 50°C.

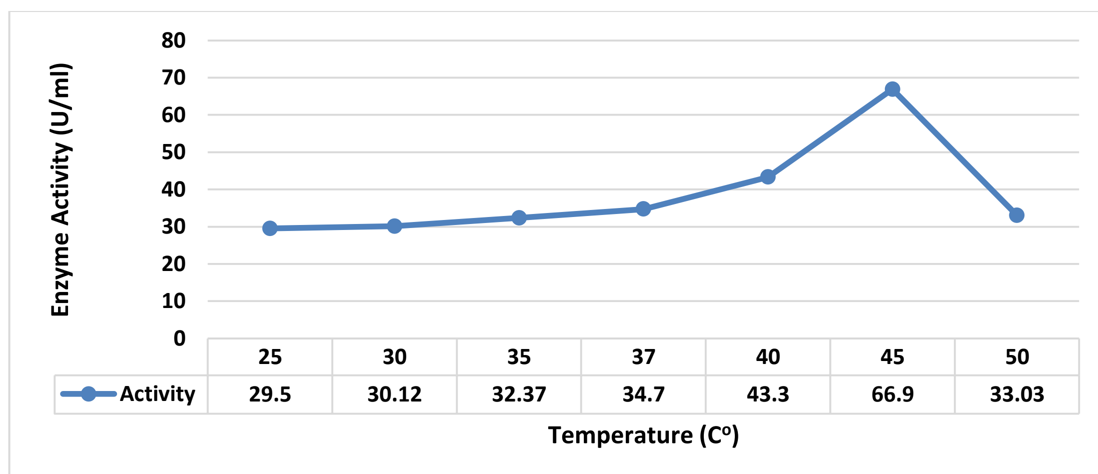


Figure (3): Effect of different temperatures on NK activity at pH 7.5 using casein as a substrate.

Multiple aspects of an enzymatic reaction, including pH, enzyme substrate affinity, and ionization of the prosthetic group, are affected by changes in temperature (26). The data revealed a rise in reaction speed up to 45°C, after which it began to fall; this may be because the movement energy of the enzyme molecules sharing in the reaction with the substrate increased, leading to an increase in the clash between them and the substrate (27). In agreement with the findings, several studies indicated that the optimum temperature of purified NK was 40 ^{o}C, which produced by *Bacillus subtilis* A26 (26), *Bacillus cereus* (22) and *Streptomyces* sp. CS624 (27). Nevertheless, it was higher than of Moidutty *et al*. (23), where they found that the NK maximum activity obtained from *B.* subtilis was 37 °C.

Effect of temperatures on NK stability

The temperature stability of NK was investigated via incubating P.P.NK at temperatures ranging from 25 to 50°C and measuring remaining activity after 30 minutes at 45°C. According to the results shown in (Figure 4), the enzyme maintained its activity at temperatures ranging from 25 to 45°C, after which the activity began to decrease with rising temperature, with around 92% of the activity remaining at 45°C. Higher temperatures resulted in a dramatic fall in stability; at 50°C, the enzyme preserved 52% of its initial activity.

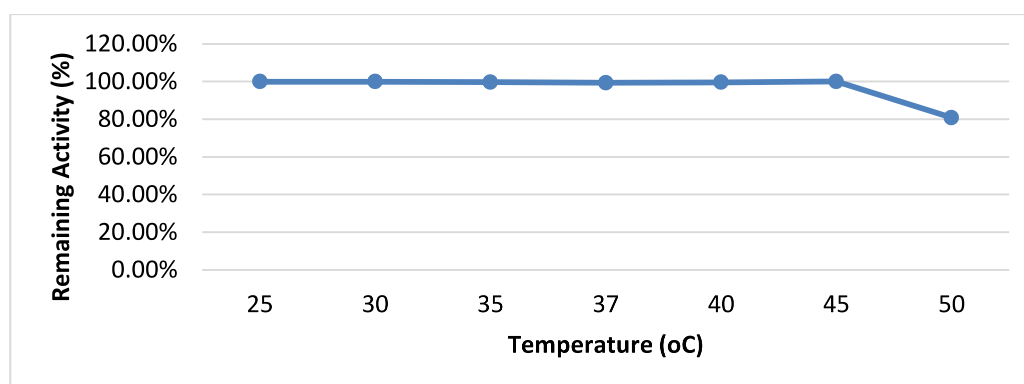


Figure (4): Effect of different ranges of temperature on NK stability using casein as a substrate

Enzymes are typically kept in a refrigerator since they are more stable at lower temperatures. NK is sensitive to high temperatures, as evidenced by the fact that its activity decreases when the temperature rises beyond 45 ^{o}C. This is because heat disrupts the protein's three-dimensional structure by destroying the R-groups of its amino acids, leading to denaturation and the loss of its biological function (30). Nguyen *et al.* (31) found that the *B. subtilis* NK was shown to be thermophilic, as it was able to maintain over 85% of its activity after being heated to 50 ^{o}C for 1 hour.

Impact of different substrate on partial purified NK

The stability of NK at various substrate was assessed by incubating the partial purified enzyme at optimum temperature 45°C and assaying residual activity after 30 min. The results in (Figure 5) showed that casein was the best substrate with higher activity (70.6 U/mg protein) of partial purified enzyme, then the activity decrease with bovine (47 U/mg), fibrin (63.36 U/mg), gelatin (31.36 U/mg), collagen (32.22 U/mg). In study conducted by (31), the best production medium with containing

casein (0.75%). It has been demonstrated that casein can serve as a source of nitrogen and amino acids, which are essential for the growth and metabolic activities of bacterium. This can lead to increased biomass production and subsequently higher nattokinase yields (38).

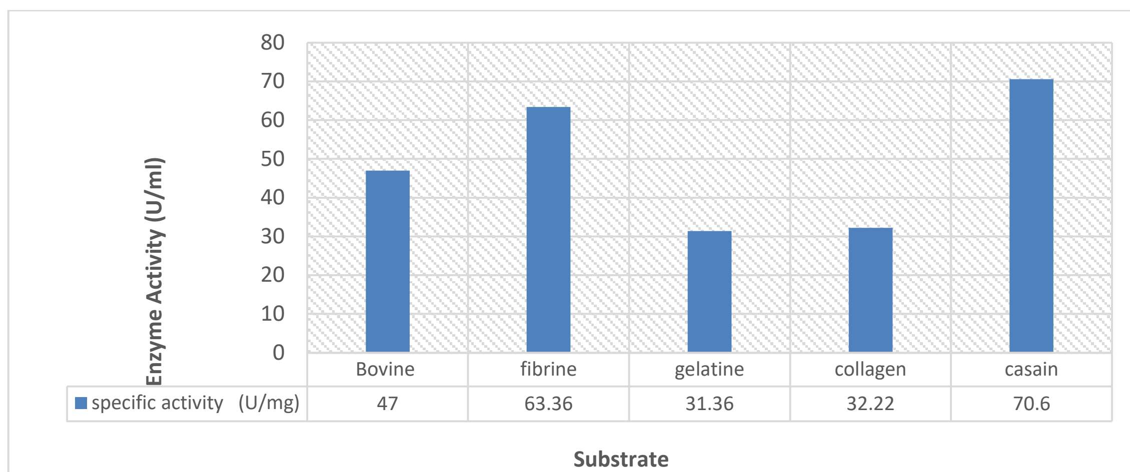


Figure (5): Effect of different substrates on NK activity at pH 7.5.

Immobilization of NK

The NK has been successfully covalently immobilized on functionalized carbon nanotubes (MWNT). With 706 U/mL of NK per 3 mg of MWNT, the enzyme loading was 67.14%. The purpose of this investigation was to identify a suitable support for NK immobilization that would improve its stability in response to variations in temperature and pH. Increased application of NK in clot degradation may result from this method's application to MWNT, which improves the enzyme's physicochemical qualities. Optimizing reaction conditions to maximize enzyme loading efficiency is now of paramount importance, as is maintaining enzyme activity. Nanotubes, particularly carbon nanotubes, have garnered interest in various biomedical and biotechnological applications due to their unique properties such as high surface area, mechanical strength, and biocompatibility. These characteristics make them suitable candidates for immobilizing enzymes like nattokinase (33). Immobilizing nattokinase using nanotubes offers several advantages. Firstly, it can enhance the stability of the enzyme, protecting it from denaturation and degradation. This increased stability can prolong the enzyme's activity and lifespan, making it more practical for industrial and therapeutic applications. Furthermore, nanotube immobilization can facilitate the reusability of nattokinase, reducing production costs and minimizing waste. The high surface area of nanotubes also provides ample space for enzyme binding, potentially leading to higher catalytic efficiency (34).

Application of NK on blood clot

The ability of NK to degrade human blood clots was tested by dissolving the clots. One hundred microliters of partly purified NK was used to break down a coagulated drop at 37°C. Blood clot digestion with 0.7U/100µl enzyme was shown after 5 minutes in Figure 6. The glass test tubes were also used to evaluate the blood clot lytic. The results indicated that clot lysis was only observed in the treatment group when immobilized NK was administered (Figure 6). NK isolated

from *P. aeruginosa* utilizing casein as substrate has outstanding fibrinolytic activity in vitro, according to the findings of the current investigation. NK's capacity to convert plasminogen into active plasmin and hence boost t-PA levels gives it powerful thrombus lysing activity (30). In agreement with this study, Chandrasekaran *et al.* (33) reported that NK produced from *P. aeruginosa* was found to have more tendency to lyse blood clot. Also, they reported that the efficiency of NK to lysis of clot was differed according to the species of produced isolates. The activity of fibrinolytic enzymes purified from *Bacillus subtilis* LD- 8547 and *Bacillus cereus* was reported (20,32). Similar results were also reported the fibrinolytic activity of NK from other microorganisms like *Bacillus* subtilis (35), *Bacillus* subtilis DC33 (36), *Bacillus ubtilis* (37, 38). Also, in study conducted by AL-Fridawy *et al.*, (39) and Shawkat (40), nattokinase produced by *Bacillus* spp. Nattokinase supplementation led to a significant decrease in plasma levels of fibrinogen, a protein involved in blood clot formation (39). Also, NK may possess anticoagulant properties by influencing various factors related to blood clotting (36).

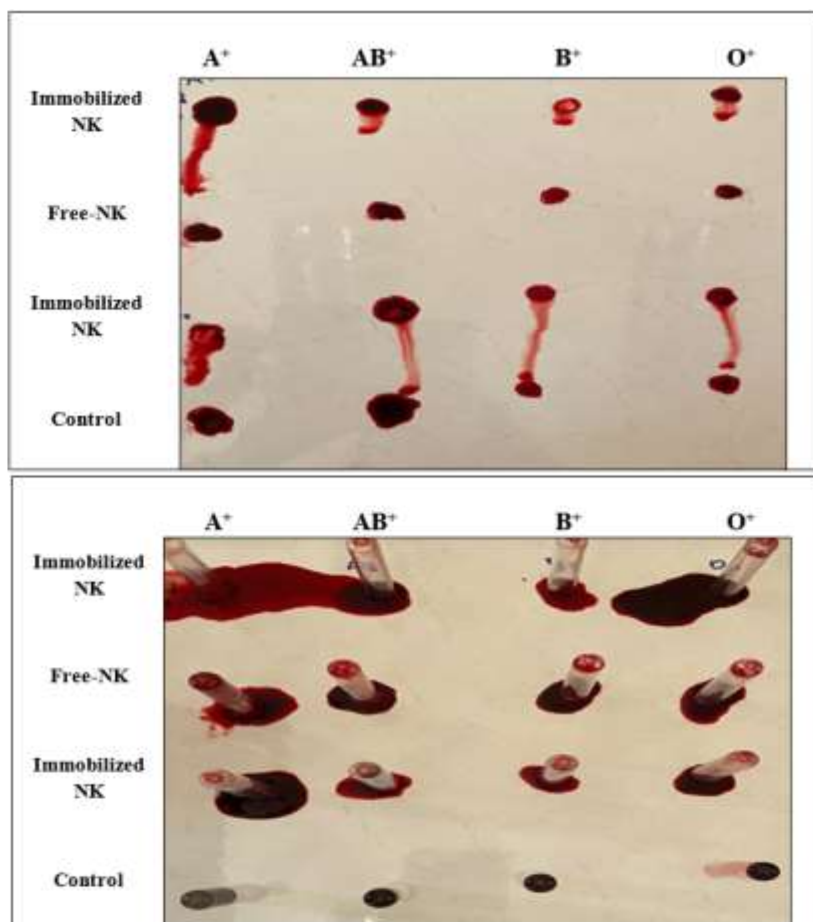


Figure (6): Effect of partial purified NK enzyme on human blood clot.

Conclusion

According to the present study, the enzyme nattokinase had the ability to remove blood clots. This indicates that the enzyme nattokinase has the ability to break down the protein fibrinogen, which leads to the non-formation of fibrin and the other

components which responsible on blood clot. Additionally, the results showed that the immobilized enzyme on carbon nanotube was more efficient in destroying the blood clot from free enzyme, and this indicates that the immobilized process produced of the enzyme with more efficient and more stable for temperature and pH. For that, it also made the enzyme more active than free enzyme. This also indicates that the immobilization of enzyme process was successful.

References

1. Cai, D.; Zhu, C. and Chen, S. (2017). Microbial production of nattokinase: current progress, challenge and prospect. World Journal of Microbiology Biotechnology, 33: 1–7.
2. Wang C, Chen J, Tian W, Han Y, Xu X, Ren T, (2023). Natto: A medicinal and edible food with health function. Chin HerbMed.
3. Pagnoncelli, M.G.B.; Fernandes, M.J.; Rodrigues, C. and Soccol, C.R. (2017). Nattokinases. In: Current Developments in Biotechnology and Bioengineering. Elsevier; 509–26.
4. Yuan, L.; Liangqi, C.; Xiyu, T. and Jinyao, L. (2022). Biotechnology, bioengineering and applications of *Bacillus* nattokinase. Biomolecules, 12(7): 980.
5. Zhou, X.; Liu, L. and Zeng, X. (2021). Research progress on the utilisation of embedding technology and suitable delivery systems for improving the bioavailability of nattokinase: a review. Food Structure, 30:100219.
6. Jayaprakashvel, M.; Sami, M. and Subramani, R. (2020). Antibiofilm, antifouling, and anticorrosive biomaterials and nanomaterials for marine applications. Nanostructures for Antimicrobial and Antibiofilm Applications, 233–72.
7. Wood, S.J.; Kuzel, T.M. and Shafikhani, S.H. (2023). *Pseudomonas aeruginosa*: Infections, Animal Modeling, and Therapeutics. Cells, 12(1): 199.
8. Morihara, K. and Homma, J.Y. (2018). Pseudomonas proteases. Bacterial enzymes and Virulence, 41–80.
9. Heba, K.; Hamid, T.M. and Al-Mathkhury, H.J.F. (2021). The Prevalence of *Pseudomonas aeruginosa* among Baghdad Hospitalised Patients. Medico-legal Update, 21(2): 603-605.
10. Spilker, T.; Coenye, T.; Vandamme P. and LiPuma J.J. (2004). PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients. Journal of Clinical Microbiology. 42(5):2074-2079.
11. Zhu, R.; Wang, D.; Liu, Y.; Liu, M. and Fu, S. (2022). Bifunctional superwetting carbon nanotubes/cellulose composite membrane for solar desalination and oily seawater purification. Chemical Engineering Journal, 433: 133510.
12. Senior, B.W. (1999). Investigation of the types and characteristics of the proteolytic enzymes formed by diverse strains of Proteus species. Journal of Medical Microbiology, 48(7): 623–8.
13. Al-Sa’ady, A.J.R. (2020). Comparsion Between Ppo from Plant Sources And Differ-Ent Chemicals In Tattoo Dyes Decolorization. Iraqi Journal of Agricultural Sciences, 51(2): 550–5.
14. AL-Sa’ady, A.J.R. and Hilal, M.H. (2020). Determination of the optimum conditions for extracting polyphenol ox-idase and laccase enzymes from malva parviflora and their role in the decolorization of some dyes. Iraqi Journal of Science, 306–13.
15. Liese, A. and Hilterhaus, L. (2013). Evaluation of immobilized enzymes for industrial applications. Chemical Society Reviews journal, 42(15): 6236–49.
16. Lu, C.L. and Chen, S.N. (2012). Fibrinolytic enzymes from medicinal mushrooms. Protein structure, 15:338–62.
17. Vijayaraghavan P, Raj SRF, Vincent SGP (2015). Purification and characterization of fibrinolytic enzyme from Pseudoalteromonas sp., IND11 and its in vitro activity on blood clot. International Journal of Biological Chemistry, 9:11–20.
18. Chimbekujwo, K.I.; Ja’afaru, M.I. and Adeyemo, O.M. (2020). Purification, characterization and optimization conditions of protease produced by *Aspergillus brasiliensis* strain BCW2. Scientific African, 8: e00398.
19. Wong, A.H.K. and Mine, Y. (2004). Novel fibrinolytic enzyme in fermented shrimp paste, a traditional Asian fermented seasoning. Journal of Agricultural and Food Chemistry, 52(4): 980–986.
20. Lin, H.T.V.; Wu, G.J.; Hsieh, M.C.; Chang, S.H. and Tsai, G.J. (2015). Purification and characterization of Nattokinase from cultural filtrate of red

alga porphyra dentata fermented by *Bacillus subtilis* N1. Journal of Marine Science and Technology, 23(2): 13.

21. Bajaj, B.K.; Singh, S.; Khullar, M.; Singh, K. and Bhardwaj, S. (2014). Optimization of fibrinolytic protease production from Bacillus subtilis I-2 using agro-residues. Brazilian Archives of Biology and Technology, 57: 653–662.
22. Vijayaraghavan, P. and Vincent, S.G.P. (2014). Statistical optimization of fibrinolytic enzyme production by Pseudoalteromonas sp. IND11 using cow dung substrate by response surface methodology. Springerplus, 3(1): 1–10.
23. Moidutty, A.; Balasubramanian, T.M. and OM FR, P. (2015). Purification and Characterization of Fibrinolytic Enzyme Nattokinase from Bacillus subtilis. International Journal of Pharmacy and Pharmaceutical Sciences, 4(1): 80–8.
24. Wu, X.; Hou, M. and Ge, J. (2015). Metal–organic frameworks and inorganic nanoflowers: a type of emerging inorganic crystal nanocarrier for enzyme immobilization. Catalysis Science and Technology Journal, 5(12): 5077–85.
25. Ju, S.; Cao, Z.; Wong, C.; Liu, Y.; Foda, M.F. and Zhang, Z. (2019). Isolation and optimal fermentation condition of the Bacillus subtilis subsp. natto strain WTC016 for nattokinase production. Fermentation, 5(4): 92.
26. M-Ridha, M.J. (2023). Characterization the urease enzyme extracted from some local plants under optimum conditions. Iraqi Journal of Agricultural Sciences, 54(3): 647–56.
27. Pokharel, P.; Ma, Z. and Chang, S.X. (2020). Biochar increases soil microbial biomass with changes in extra-and intracellular enzyme activities: a global meta-analysis. Biochar, 2: 65–79.
28. Agrebi, R.; Haddar, A.; Hmidet, N.; Jellouli, K.; Manni, L. and Nasri, M. (2009). BSF1 fibrinolytic enzyme from a marine bacterium Bacillus subtilis A26: purification, biochemical and molecular characterization. Process Biochemistry, 44(11): 1252–1259.
29. Mander, P.; Cho, S.S.; Simkhada, J.R.; Choi, Y.H. and Yoo, J.C. (2011). A low molecular weight chymotrypsin-like novel fibrinolytic enzyme from Streptomyces sp. CS624. Process Biochemistry, 46(7): 1449–55.
30. Roshni, K.G. (2021). Protein folding, misfolding, and coping mechanism of cells–A short discussion. Open Journal of Cell and Protein Science, 4(1): 001-004.
31. Nguyen, T.T.; Quyen, T.D. and Le, H.T. (2013). Cloning and enhancing production of a detergent-and organic-solvent-resistant nattokinase from Bacillus subtilis VTCC-DVN-12-01 by using an eight-protease-gene-deficient Bacillus subtilis WB800. Microbial Cell Factories, 12:1–11.
32. Selvarajan, E. and Bhatnagar, N. (2013). Nattokinase: an updated critical review on challenges and perspectives. Cardiovascular & Hematological Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Cardiovascular and Hematological Agents), 15(2): 128–135.
33. Chandrasekaran, S.D.; Vaithilingam, M.; Shanker, R.; Kumar, S.; Thiyur, S. and Babu, V. (2015). Exploring the in vitro thrombolytic activity of nattokinase from a new strain *Pseudomonas aeruginosa* CMSS. Jundishapur Journal of Microbiology, 8(10).
34. Yuan, J.; Yang, J.; Zhuang, Z.; Yang, Y.; Lin, L. and Wang, S. (2012). Thrombolytic effects of Douchi Fibrinolytic enzyme from Bacillus subtilis LD-8547 in vitro and in vivo. BMC Biotechnology, 12(1): 1–9.
35. Jeong, Y.K.; Park, J.U.; Baek, H.; Park, S.H.; Kong, I.S. and Kim, D.W. (2001). Purification and biochemical characterization of a fibrinolytic enzyme from Bacillus subtilis BK-17. World Journal Microbiol Biotechnology, 17: 89–92.
36. Wang, C.T.; Ji, B.P.; Li, B.; Nout, R.; Li, P.L. and Ji, H. (2006). Purification and characterization of a fibrinolytic enzyme of Bacillus subtilis DC33, isolated from Chinese traditional Douchi. Journal of Industrial Microbiology and Biotechnology, 33(9): 750–8.
37. Deepak, V.; Kalishwaralal, K.; Ramkumarpandian, S.; Babu, S.V.; Senthilkumar, S.R. and Sangiliyandi, G. (2008). Optimization of media composition for Nattokinase production by Bacillus subtilis using response surface methodology. Bioresource Technology, 99(17): 8170–8174.
38. Hmood, S.A. and Aziz, G.M. (2016). Purification and characterization of nattokinase produced by local isolate of *Bacillus* sp. B24. Iraqi Journal of Biotechnology, 15(2).

39. AL-Fridawy, R.A.K.; Al-Daraghi, W.A.H. and Alkhafaji, M.H. (2020). Isolation and Identification of Multidrug Resistance Among Clinical and Environmental *Pseudomonas aeruginosa* Isolates. Iraqi Journal of Biotechnology, 19(2).
40. Shawkat, A.A.K.M.S. (2022). Primary and Secondary Screening of *Pseudomonas aeruginosa* for Protease Production. Iraqi Journal of Biotechnology, 21(2).