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Optimization and Production of Nattokinase Enzyme from Endophytic Microorganisms

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Abstract

In the present study attention is focused on the microorganisms has potential source of compounds to produce Nattokinase enzyme. The objectives of this study, the microorganisms were screened and optimized for Nattokinase enzyme, production and purification of enzyme, hemolysis and fibrinolytic activity. Totally 3 bacteria such as *Bacillus subtilis, B. licheniformis* and *B. megaterium* and 2 fungi such as *A. fumigatus* and *A. japonicus* were screened from three different mangrove plant leaves like *Avicennia marina*, *Salicornia bracheata* and *Suaeda maritima* at Kattumavadi, Kottaipattinam and Sethubavasathiram – Tamil Nadu, India. The Nattokinase enzyme complex was screened from isolated endophytic bacteria and fungi by plate assay method. In screening, the maximum zone of clearance was recorded in *Bacillus subtilis* (25.0±2.5 mm) and *A. japonicus* (20.3±1.2 mm) than other strains. The optimization and production were analyzed from nattokinase enzyme using endophytic microorganisms. Partial purification of nattokinase enzyme was maximum from selected bacteria and fungi such as *B. subtilis, B. licheniformis and A. japonicus* were analyzed. The hemolysis, fibrinolytic activity, blood clot and lysis rate percentage were recorded. It was concluded that, these findings suggest that thenattokinase enzyme may act as excellent source in pharmaceutical industry. Characterization of the purified enzyme showed promising results for future studies and commercial production.

***Key words*:** Mangrove plants, Nattokinase, Optimization, Purification, Hemolysis, Fibrinolytic activity

Endophytes means microbes that colonize living tissues of plants without causing negative effects [1]. All vascular plant species appear to be inhabited by endophytic microbes, these denoted important components of microbial diversity. The relationship between the host plant and its endophyte bacteria showed symbiotic relationship as the endophytic occupant usually obtains nutrients and protection from the host plant and enhances the fitness of the host by producing certain functional metabolites [2]. During this time, various aspects of endophyte biology were studied including the diversity, taxonomy, reproduction, host ecology and effects on the host [3]. Since natural selection favours the beneficial endophytic strains, several endophytes were found to secrete secondary metabolites that protect plants against insect pests, pathogenic organisms as well as herbivores [4-6] represented endophytes is an important source of biologically active metabolites for industrial, pharmacological and agricultural applications. Enzymes are extracellular nattokinase in character. The processing of enzymes used as drugs play a vital role of today's pharmaceutical industries and these studies mainly focus on the antibacterial, fibrinolytic activity of nattokinase enzyme isolated from marine bacterial strains. [7] Compared to other fibrinolytic enzymes (urokinase, t-PA and streprokinase), nattokinase shows the advantages of having no side effects, low cost and long-life time, and it has the potential to be used as a drug for treating cardiovascular disease and served as a functional food additive. Nattokinase has been developed for the treatment of thrombosis because of its efficiency and stronger affinity to fibrin [8]. The other one is plasmin-like fibrinolytic enzymes, which can directly degrade the fibrin in blood clots, thereby dissolving the thrombi rapidly [9]. Due to these advantages, *B. subtilis* has been used in metabolic engineering, genetic engineering, and other fields until today [10-13]. Nattokinase has a wide range of applications in pharmaceutical industry and food industries. Scientists are researching this enzyme and their further scope for research to be done on nattokinase [14].

MATERIALS AND METHODS

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Sample collection

The marine associated plants *Avicennia marina* were collected from three places of Kattumavadi and Kottaipattinam located in Pudukkottai District and Sethubavachatram located in Thanjavur District. These plants were maintained under controlled environmental conditions in well insulated plastic pots in the green house.

Isolation of endophytic bacteria from Avicennia marina leaves tissue [15]

The samples of leaves were cut into small pieces and macerated separately in phosphate buffer of pH 7.2 with a sterile pestle and mortar. Tissue extract were then prepared for tenfold dilution in sterile saline. Serial dilutions (10^{-4}, 10^{-5} and 10^{-6}) were prepared from this extract. For inoculations 0.1ml of the aliquot was used on nutrient agar medium. These petri plates were then incubated at 37 °C for 72 hrs. Bacterial colonies were differentiated on the basis of morphological colony characters on Yeast Mannitol Agar. The isolation process repeated till monocultures were obtained for further experimentations.

Identification of bacteria [16]

The bacteria were identified by using biochemical test and standard manual of *Bergey's Manual* of Determinative Bacteriology 9th Edn., for identification of bacteria.

Screening of Nattokinase enzyme [17]

Casein agar medium used for the screening of nattokinase enzymes from endophytic bacteria by plate assay method. The petri plates were inoculated the endophytic bacteria and above the medium for simple streak and incubated at 37°C for 24 hrs. The petri plates were flooded on grams iodine to observed clear zone of casein hydrolysis around the streaking was an indicated nattokinase enzyme.

Optimization of nattokinase enzyme production [18]

The effect of liquid substrate on nattokinase production, three types of substrates was tested in soybean meal, rice husk and wheat bran. One percentage of each agricultural waste were prepared and then inoculated with 1.0 ml of the selected bacterial and fungal inoculum containing 2.5×107 cell/ml and incubated at 30- 37 ºC for 48 hrs. After filtration, the volume of crude extract (clear supernatant) of each flask was measured and it was applied for nattokinase productivity.

Production of nattokinase enzyme [19]

The nattokinase enzyme producing bacteria and fungi individually was grown on basal medium containing (g/lit.) The pH was adjusted to 7.2 with 2 M acetic acid and 2 M NaOH. Medium was sterilized by autoclaving at 121°C for 35 min and cooled to room temperature. One ml of uniformly prepared suspension was used as inoculum and incubated at 37°C and 150 rpm in an orbital shaker. After 7 days of fermentation to estimate the nattokinase enzyme production.

Extraction and purification of enzyme [20-21]

The enzyme released is extracellular, centrifugation is the basic technique used to remove all cell debris. Purification of Enzymes using TCA Precipitation, Ammonium Sulfate Precipitation and Dialysis and also determine Specific Activity and Total Protein content of Nattokinase Enzyme.

TCA precipitation of total protein

One ml of TCA was added to 4ml of enzymes and incubated for 10 minutes at 4ºC. The precipitate obtained was separated by centrifugation at 14,000 rpm for 5 minutes at 4ºC. Pellet was washed with 200µl of chilled acetone. Pellet was collected by centrifugation. Again, the pellet was washed with 2 volumes of acetone and centrifugation was done at 14000rpm for 5 minutes. Pellet was dried at 37ºC for 10 minutes and dissolved in 50mM Tris HCl.

Ammonium sulfate precipitation

Solid ammonium sulphate was added to the culture filtrate at 50% saturation for nattokinase enzyme and stirred for 24hrs at 4ºC. The precipitate obtained was separated by centrifugation at 10000 rpm for 20 minutes at 4ºC and the resultant supernatant was treated with solid ammonium sulphate at 60% saturation. The precipitate was collected by centrifugation. The supernatant was similarly treated with ammonium sulphate at 70% and 80% saturation and the precipitate obtained was solubilized in 50mM Tris HCl buffer (pH 8.0) and dialysis was performed.

Dialysis

The pre-treatment of the dialysis membrane was carried out by boiling the membrane in 2% sodium carbonate solution containing 0.1 mM EDTA for 20 min and kept in distilled water for 10 min. The dialysis membrane was then transferred to a solution of 0.1 mM EDTA and boiled again for 10 min. The activated dialysis bag was kept in 0.1 mM EDTA and stored at 4°C until use. The protein obtained after ammonium sulfate saturation was filled in the dialysis membrane and then dialyzed against 50 mM Tris-HCl buffer (pH-7.8) for 48 h at 4°C.

In vitro hemolysis assay [22]

1 ml blood was collected from healthy adult individuals in sterile tubes containing sodium citrate and centrifuged at 3000 rpm for 15 min. The plasma obtained was discarded and the pellet was washed thrice with ice-cold PBS buffer (pH-7.4) and red blood cell (RBC) suspension was prepared by mixing RBC with PBS buffer in 1:1 ratio. Fibrinolytic enzymes (100 µl) were added to 1 ml RBC suspension and incubated at 37°C for 1 h. After incubation, the reaction mixture was centrifuged at 3000 rpm for 15 min. The hemoglobulin released into the supernatant was determined spectrophotometrically at 540 nm. 1 ml PBS was used as the negative control and 1% Triton X-100 was used as the positive control.

$$\text{Rate of hemolysis (\%)} = (ODt - ODnc) / (ODpc - ODnc) \times 100$$

ODt was the absorbance of the test. ODnc was the absorbance of the negative control and ODpc was the absorbance of the positive control.

Evaluation of the fibrinolytic activity [23]

In vitro fibrinolytic activity was observed by artificial blood clot degradation method. An artificial blood clot was formed by spontaneous coagulation in a glass test tube using freshly collected venous blood. After 1 hr, the artificial blood clot was rinsed out thoroughly and dipped in 4 mL of phosphate-buffered saline (PBS, pH 7.0) containing nattokinase solution at room temperature. As a control, normal saline was used without nattokinase solution.

Blood clot and lysis analysis of NT enzyme [24]

Venous blood drawn from healthy volunteers were transferred to different pre weighed sterile micro-centrifuge tubes (500µL/tube) and incubated at 37 °C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube with clot was again weighed to determine the clot weight (clot weight was

equal to weight of clot containing tube minus weight of tube alone). Each micro centrifuge tube, containing clot, was properly labelled and 100 µL of crude enzyme and ammonium sulfate precipitated enzyme was added to the clots. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the obtained fluid was removed and the tubes were again weighed to observe the difference in weight after clot disruption. The obtained difference in weight taken before and after clot lysis was expressed as percentage of clot lysis:

$$\% \text{ of clot lysis} = \frac{\text{Clot weight before lysis} - \text{Clot weight after lysis}}{\text{Clot weight before lysis}} \times 100$$

RESULTS AND DISCUSSION

Nattokinase is an enzyme considered to be a promising remedy for thrombosis healing due to its potent fibrinolytic activity. In this report three bacterial strains were isolated from leaf tissues of *Avicennia marina.* According to the biochemical characterization performed, the bacterial isolates were identified as *Bacillus subtilis, Bacillus megaterium* and *Bacillus licheniformis*.

Table 1 Screening of Nattokinase enzyme from endophytic microorganisms

Name of the endophytic microorganisms	Zone of clearance (mm)
Bacillus megaterium	19.4±1.2
B. subtilis	25.0±2.5
B. licheniformis	21.2±1.4
A. fumigatus	16.0±1.0
A. japonicus	20.3±1.2

The values are expressed in terms of (Mean ± Standard deviation)

These organisms showed gram positive, aerobic and endospore forming bacteria. *Bacillus subtilis* is a model organism for studying endospore formation in bacteria [25] also showed positive result for casein hydrolysis, starch hydrolysis and even motility test. But negative results for oxidase and indole production test. For all the 5 isolates, Nattokinase assays were done. Screening of Nattokinase enzyme from *Bacillus subtilis, Bacillus lichenifromis, Bacillus megaterium, Aspergillus japonicas* and *A. fumigatus* and zone measurement were recorded with plate assay for (25.0±2.5, 21.2±1.4, 19.4±1.2, 20.3±1.2 and 16.0±1.0 mm). The maximum screening of microbes is analyzed with *Bacillus subtilis* compared with others. The best isolate produced a maximum amount of nattokinase enzyme was identified and it was subjected to further studies (Table 1).

The enzymes production started in an exponential phase, and its activity showed linear growth with the tested strain. At 72 hours, nattokinase production reached its maximum. The culture medium is essential for the production of enzymes because it should provide the organism all the essential nutritional requirements for enzyme production [26]. The production medium must have cheap sources of carbon, nitrogen, growth promoters, and trace elements in order to reduce production costs. In order to increase the production of enzymes, the growth conditions, including pH, incubation time and temperature were also kept at their optimum levels. The cultural and media conditions have to be optimized before the vaccination. In the present study, Optimization of nattokinase enzyme production with different parameters like substrates, incubation periods, temperature, pH, carbon source, nitrogen source and salinity source were altered with microbial treatment like *Bacillus subtilis, Bacillus lichenifromis, B. megaterium, Aspergillus japonicas* and *A. fumigatus* were performed. The nattokinase enzyme optimized by selected microorganisms produced three substrates by soybean, wheat bran, rice husk in (1%), four different incubation period at 2, 4, 6 and 8 days, temperature are recorded with 25, 30, 35, 40 and 45 °C, pH were observed at 5 to 9, carbon source of glucose, maltose and sucrose were analyzed, different nitrogen source such as beef, yeast and malt extract were obtained. The maximum parameters like substrates were best result for all selected microorganisms were noted wheat bran, 6th day of incubation period gives best enzyme production, the temperature 35˚C produces high level of enzymes, pH 7 produce high rate of enzyme, carbon and nitrogen source such as glucose and beef extract were maximum optimized nattokinase enzyme production (Table 2).

Table 2 Optimization of nattokinase enzyme production

Parameters	Concentration	Enzyme assay (U/mg)				
		Bacillus subtilis	*B. licheniformis*	*B. megaterium*	*Aspergillus japonicus*	*A. fumigatus*
Substrates (1%)	Soybean	90.8±2.5	86.5±2.2	88.2±5.9	85.6±1.8	70.3±6.4
	Wheat bran	190.9±5.8	176.5±3.6	163.7±12.3	152.6±2.4	149.2±19.9
	Rice husk	22.15±2.4	19.5±2.1	17.6±2.8	21.5±1.6	16.9±1.3
Incubation period (days)	2	20.5±0.9	12.3±0.9	14.5±1.2	13.5±1.6	9.24±1.4
	4	39.4±2.4	35.6±1.2	31.2±3.5	33.6±1.8	29.5±1.9
	6	92.8±2.6	87.2±2.5	78.1±3.9	86.3±2.4	82.4±1.1
	8	81.4±4.1	75.3±1.6	71.8±2.7	67.3±4.3	60.5±9.4
	10	51.6±2.1	46.3±2.2	39.4±2.8	43.8±1.4	35.5±9.9
Temperature (°C)	25	46.2±2.5	43.9±3.8	38.6±2.4	58.7±6.1	42.6±3.4
	30	52.7±1.7	50.6±1.6	44.1±2.1	67.3±5.3	55.4±5.9
	35	79.8±2.4	69.5±1.6	52.7±1.2	71.5±1.2	62.7±4.8
	40	61.4±1.7	49.5±0.4	46.3±1.8	65.3±1.5	58.3±7.1
	45	53.8±2.6	46.3±5.4	37.1±3.5	61.6±1.0	53.5±0.0
pH	5	59.8±1.7	52.6±1.2	46.9±1.8	53.6±1.5	41.3±6.1
	6	65.2±2.6	59.3±2.4	52.6±2.6	73.8±5.9	48.5±6.4
	7	73.8±2.5	68.9±2.9	64.7±2.4	97.9±1.5	55.3±9.9
	8	61.1±0.7	46.7±5.2	58.5±3.5	67.5±4.7	52.7±0.5
	9	45.6±1.8	34.3±0.9	49.6±5.6	39.2±1.2	47.2±2.7
Carbon source (mg/100ml)	Glucose	59.5±1.0	51.2±1.6	48.9±5.4	61.2±2.5	55.2±3.7
	Maltose	48.6±1.4	43.6±2.0	39.5±2.6	50.6±1.8	48.3±3.6

	Sucrose	32.6±1.7	31.2±1.1	30.5±1.2	32.5±1.4	31.1±3.5.
Nitrogen	Beef extract	79.8±1.2	78.9±1.8	75.4±5.2	67.5±1.5	65.5±1.3
source	Yeast extract	76.2±0.9	75.6±1.0	71.2±2.3	79.5±1.8	72.4±1.5
(mg/100ml)	Malt extract	58.6±1.2	57.6±1.3	52.4 ±3.7	69.5±1.7	64. 2±2.4
Salinity (ppt)	80	55.3±5.8	53.9±1.9	51.5±5.2	35.5±0.9	32.3±0.8
	90	63.7±3.5	59.7±2.7	53.2±3.2	46.7±2.8	44.5±2.8
	100	78.4±1.6	76.5±1.3	71.9±1.4	67.8±1.3	63.6±1.1
	110	65.3±2.1	63.6±2.5	61.8±2.2	59.2±1.4	57.1±1.6
	120	59.3±1.3	58.9±2.3	55.3±2.5	41.2±2.1	39.3±1.9

The values are expressed in terms of (Mean ± Standard deviation)

Table 3 Production of nattokinase enzyme

Name of the endophytic microorganisms	Quantity (U/mg)
Bacillus subtilis	15.58±1.8
B. licheniformis	14.32±2.4
B. megaterium	11.51±1.9
Aspergillus japonicus	14.15±1.7
A. fumigatus	10.84±2.1

The values are expressed in terms of (Mean ± Standard deviation)

Nattokinase enzymes are also known as subtilism NAT is one of the most considerable extracellular enzymes produced by bacteria. The protein estimation (mg/ml) was done following the method of Lowry's enzyme activity (µmole/min) was determined following the method of [27]. In the present study, Production of nattokinase enzymes from *Bacillus subtilis, B. licheniformis, B. megaterium, Aspergillus japonicas* and *A. fumigatus* with 15.58±1.8, 14.32±2.4, 11.51±1.9, 14.15±1.7 and 10.84±2.1 IU/mg quantity recorded respectively and triplicates also maintained. According to the production of NK enzyme from *Bacillus subtilis* followed by *B. lichenifroms, Bacillus megaterium*. *Aspergillus japonicas* and *A. fumigatus.* Nattokinase was produced on basal medium and then purified from supernatant. The maximum production of nattokinase enzyme of microorganisms such as *Bacillus subtilis, B. lichenifroms* and *Aspergillus japonicas* are observed (Table 3).

The crude enzyme was purified by ammonium sulphate precipitation, followed by dialysis and DEAE cellulose ion-exchange chromatography. Total protein was estimated by Lowry's method (0.58 mg/ml for FS2 and 0.49 mg/ml for S1) and enzyme-specific activity was determined by tyrosine standard (0.52 µmole/mg/ml for S1 and 0.59 µmole/mg/ml for FS2) (Table 3). Similar results were reported earlier [28]. In the current study, the partial purification of nattokinase enzyme were analyzed selected three microbes such as *Bacillus subtilis, B. licheniformis* and *A. japonicas* from wild strain were performed cell free supernatant, TCA precipitation, $(NH_4)_2SO_4$ precipitation and dialysis. The total protein is mostly presented at TCA precipitation in all microbes then followed with cell-free supernatant. The total activity was recorded *B. subtilis* in TCA precipitation, *B. licheniformis* in cell-free supernatant and *A. japonicas* in TCA precipitation when compared with other purification steps. The specific activity was analysed with maximum for all microbes in common $(NH_4)_2SO_4$ precipitation followed by dialysis. The maximum yield factors are observed at cell-free supernatant in all microbes. The purification fold is maximum at $(NH_4)_2SO_4$ precipitation for *B. subtilis*, TCA precipitation and dialysis for *B. licheniformis* and dialysis for *A. japonicas* were analyzed (Table 4).

Table 4 Partial purification of nattokinase enzyme

Name of the endophytes	Purification step	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Yield (%)	Purification fold
Bacillus subtilis	Cell-free supernatant	5.10±1.4	124.4±15.7	33.4±2.0	97	1
	TCA precipitation	6.15±0.2	212.7±16.1	45.7±8.6	67	3
	$(NH_4)_2SO_4$ Precipitation	2.25±0.5	122.4±4.1	122.0±12.6	63	4
	Dialysis	2.17±0.7	52.2±4.4	122.2±23.1	65	3
Bacillus licheniformis	Cell-free supernatant	4.17±0.9	206.7±14.5	20.4±3.4	97	1
	TCA precipitation	5.4±1.0	181.4±8.4	29.6±2.8	72	2
	$(NH_4)_2SO_4$ Precipitation	1.8±0.9	145.1±8.7	128.7±10.8	65	1
	Dialysis	2.1±1.1	34.1±5.7	98.5±7.1	65	2
Aspergillus japonicus	Cell-free supernatant	6.1±1.5	145.3±9.7	56.2±3.6	93	1
	TCA precipitation	8.2±1.7	205.2±17.1	45.4±6.0	63	2
	$(NH_4)_2SO_4$ Precipitation	2.7±1.0	125.4±7.5	173.4±6.4	69	2
	Dialysis	2.1±1.4	29.2±1.8	102.2±5.4	43	3

The values are expressed in terms of (Mean ± Standard deviation)

In another study [29] reported that when nattokinase was given to human subject by oral administration, fibrinolytic activity and the amount of tPA and fibrin degradation product in plasma increased about two folds. On the basis of these reports, *B. subtilis* producing fibrinolytic enzyme were isolated from soil obtained from various regions of Sudan. Among them *B. subtilis* showed strongest fibrinolytic, activity. In view of this report, it can be suggested that our fibrinolytic enzymes isolated from *B. subtilis* can be given orally for use as a thrombolytic agent. Due to its presence in food and relatively robust fibrinolytic activity, nattokinase has benefits over other available commercially used drugs in prophylactic and extended effects, particularly due to stability in the GI tract and comfortable oral administration [30]. In the present study, the hemolytic activity and fibrinolytic enzymes were analyzed with different concentration of control, 1, 10, 100 and1000 µg/ml of nattokinase enzymes were performed. 1000µg/ml was maximum activity of 45.5±3.1, 42.7±3.4 and 58.4±4.5% in *B. subtilis*, 45.4±2.6, 49.5±2.3 and 51.2±1.8% in *B. licheniformis* and 45.6±2.7, 51.2±3.4 and 53.6±1.9% in *Aspergillus japonicus* than other concentration. So, assay and characteristics of hemolysis and fibrinolytic activity from *Bacillus subtilis* are very effective and useful for cardiovascular increase of human being (Table 5).

Table 5 Invitro hemolysis assay and fibrinolytic activity

Nattokinase enzymes	Percentage of activity (%)			
	Concentration (µg/ml)	Hemolysis	Fibrinolysis	Control (Asprin)
Bacillus subtilis	1	19.5±2.0	15.6±3.1	12.2±1.8
	10	28.2±1.9	30.2±3.7	19.0±3.1
	100	38.9±2.3	46.8±4.5	24.4±2.4
	1000	42.7±3.4	58.4±4.5	45.5±3.1
Bacillus licheniformis	1	15.2±2.8	14.5±4.0	12.6±2.4
	10	24.8±2.4	25.6±3.0	19.5±2.1
	100	37.1±1.6	35.6±2.9	24.2±3.2
	1000	49.5±2.3	51.2±1.8	45.4±2.6
Aspergillus japonicus	1	18.5±2.1	14.2±2.8	12.4±1.9
	10	29.5±1.9	26.8±4.4	19.3±2.1
	100	37.8±4.5	42.0±2.4	24.3±1.9
	1000	51.2±3.4	53.6±1.9	45.6±2.7

The values are expressed in terms of (Mean ± Standard deviation)

Blood clot lysis activity was observed for the crude enzyme and ammonium sulphate precipitates of 20%, 40%, 60% and 70% NK enzyme from mutant strain UV60 and UV90. Furthermore, 94% clot lysis was visually observed after 10 minutes in the tube that received 70% precipitate of mutant UV60 strain while 77% clot lysis was observed in the tube that received70% precipitate of mutant UV90 strain. Fibrinolytic activity was determined by the in vitro fibrin plate method. The partially purified 70% precipitate NK enzyme from mutant UV60 strain showed maximum hydrolysis fibrin clot liquefaction when compared to NK enzyme from mutant UV90 strain, which was visually observed after two hours of incubation at 37˚C [31]. In the current study, the blood clot and lysis rate were analyzed with different concentration of control, 1, 10, 100, 1000, 1100 and 1200 µg/ml of nattokinase enzymes were performed. The 1100 µg/ml was maximum percentage activity of blood clot and lysis rate were 39.5±0.5 and 54.8±1.9% in *B. subtilis*, 41.8±0.9 and 49.9±2.1% in *B. licheniformis* and 40.4±2.7 and 50.2±3.0 % in *Aspergillus japonicus* recorded than the other concentration (Table 6).

Table 6 Blood clot and lysis analysis of NT enzyme

Nattokinase enzymes	Concentration (µg/ml)	Percentage of activity (%)		
		Blood clot	Lysis rate	Control (Tris. HCl)
Bacillus subtilis	1	24.3±2.1	32.5±2.4	-
	10	35.2±1.9	51.2±3.1	-
	100	38.4±1.4	59.4±3.5	-
	1000	42.5±1.6	61.4±3.3	-
Bacillus licheniformis	1	19.6±1.8	26.9±1.9	-
	10	17.2±1.6	31.8±3.4	-
	100	25.3±2.1	42.6±2.7	-
	1000	38.0±2.6	52.6±3.1	-
Aspergillus japonicus	1	22.5±1.8	24.5±1.9	-
	10	25.2±1.7	38.2±2.4	-
	100	34.6±2.3	41.5±3.1	-
	1000	40.4±2.7	50.2±3.0	-

The values are expressed in terms of (Mean ± Standard deviation)

CONCLUSION

In the current research, with increasing trends in health issues and diets that include fast food, cardiovascular diseases around the world, Nattokinase will provide as a best supplement in the daily diet. Nattokinase can now be produced by recombinant means and in batch culture, rather than relying on extraction from Natto. The process of enzyme production can be further improved by knowing the metabolic pathways of the enzyme and hence bringing cellular kinetics into account, will improve the quality of production. The main interest about this enzyme is due to its direct fibrinolytic activity. Being stable enough in the gastrointestinal tract makes this enzyme a useful agent for the oral thrombolytic therapy. It implies this fibrinolytic strain, with significant potential for dietary supplement and functional food, as well as the therapy of thromboembolic diseases, which deserves further investigation, including the nattokinase-coding gene analysis and modification, could be used for the development of liquid or solid batch fermentation in high-capacity bioreactor for NK production.

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