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ISOLATION, PURIFICATION AND CHARACTERIZATION OF NATTOKINASE FROM SOIL ISOLATE

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ABSTRACT

Nattokinase is used for curing the cardiovascular disease, also known as antithrombotic agent. The bacterial culture responsible for nattokinase was isolated from soil sample near to the slaughter house. The production of enzyme was increased by selecting and optimizing the components suitable for the bacterial growth. During the work it was found that yeast is best nitrogen source, dextrose as a carbon source and temperature 37°C for bacterial growth and for the production of nattokinase. Best enzyme activity obtained at pH 11 and 4°C. Where $CaCO_3$, $MgSO_4$ acts as an activators and NaCl , EDTA acts as inhibitors.

Keywords: Nattokinase, oral, antithrombotic agent, cardiovascular disease.

INTRODUCTION

Nattokinase is used for curing the cardiovascular disease, also known as antithrombotic agent [1]. Natto is a fermented product of soybean which consumed as a common food in Japan. Nattokinase is a enzyme which have potential to dissolve the blood clot and used for the treatment of cardiovascular diseases. There are lots of sources for the production of nattokinase but basically natto is produced by the help of bacterium named *Bacillus subtilis, during the* process of soybean fermentation **[2].** Nattokinase is a serine protease purified and extracted from natto. It is also considered as a fibrinolytic miracle food. Hiroyuki Sumi et al. (1990) reported that artificial fibrin can be dissolved by natto. They extracted enzyme from natto and also found that the enzyme have the capability to degrade plasmin substrate, then termed this fibrinolytic enzyme as nattokinase **[3].**

Nattokinase have various pathways for dissolving the blood clot either by such as by hydrolysing plasmin substrate and fibrin, or converting urokinase (uPA) from endogenous pro-urokinase, by degrading plasminogen activator inhibitor-1 (PAI-1), or by increasing the fibrinolytic activity which is supported by the tissue plasminogen activator increasing **[4] .**

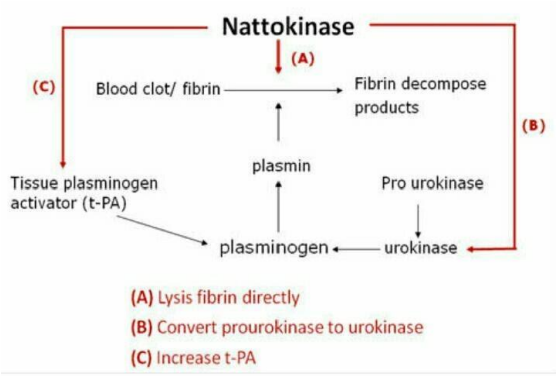


Figure 1: *Showing diagrammatic pathway of nattokinase activity* ***[1]***

The common fibrinolytic proteases uPA& t-PA shows umbers of side effects for example bleeding, whereas nattokinase shows negligible of no side effects.

According to the studies of Fujita et al. 2011 and Fujita M et al. 1995, the nattokinase is absorbed by the intestinal tract by oral observation. It also has resilient fibrinolytic activity after intraduodenal absorption. These properties make nattokinase a useful and potential fibrinolytic enzyme which can be used to conflict blood clots **[5, 6].**

MATERIAL METHODS

Collection of samples

For the following study, soil samples were collected near hospitals and slaughter house in order to find the desired species. The soil is selected from there because blood clotsare present in it **[7].**

Isolation of nattokinase producing bacteria

The samples were spread on nutrient agar plates after serial dilution in 0.85% saline solution [8]. The cultures were selected on the basis of morphological parameters and streaked in continuous quadrant pattern to make pure culture plates [9]. These pure cultures were screened on the basis of zone of hydrolysis presence in skimmed milk agar and blood agar media **[10].**

Biochemical tests for culture C2

The tests are carried out for the identification of culture and it follows the Bergy's manual.

Media selection and optimization

The growth of C2 was checked in three different production media. Media1 (g/l): K_2HPO_4-6, $MgSO_4$-0.2, NaCl-5, Dextrose-8, Peptone-2, Media 2 (g/l): Nutrient broth-13,Media 3 (g/l): Dextrose-8, Peptone-5 and NaCl-5. After the selection of one media the optimization was carried out on the basis of one factor at a time. Further growth curve study was also accomplished **[11].**

Fermentation and downstream processing

The culture was inoculated in optimized media and kept for shake flask fermentation under optimum conditions for 48-72 hours [12]. Further purification was carried out by salt precipitation (40% ammonium sulphate) and dialysis **[13, 14].**

Enzyme assay:

Sodium phosphate buffer (100mM) and casein (1%) was added to the sample. The samples were incubated at 37°C for 15 minutes. After incubation, trichloroaceteicaicd (TCA) was added to stop the reaction, again incubated at 4°C for 30 minutes. 5 ml reagent C (2% Na_2CO_3 + 0.1N NaOH + 0.5% $CuSO_4$ + 1% sodium potassium tartrate) incubated at room temperature for 10 minutes. Further the samples were incubated in dark for 30 min after adding folins reagent to it. Optical density was taken at 680 nm **[15].**

Characterization of enzyme

It was done by changing the following parameters **[16]:**

pH: the pH of enzyme was changed to 4, 7, 9 and 11 then enzyme assay was performed.

Temperature: the enzyme was incubated at 37°C, 4°C, 50°C and room temperature then enzyme assay was done.

Activators and inhibitors: the activators ($CaCO_3$ and $MgSO_4$) and inhibitors (EDTA and NaCl) in 1:10 ratio were added to the enzyme then enzyme assay was performed.

RESULTS

Sample collection

Samples were collected from different places which have the possibility of having nattokinase activity. Following sample was collected.

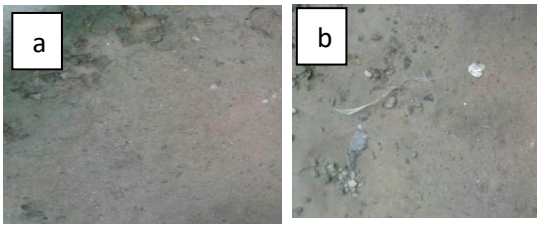


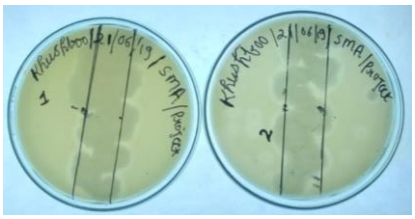
Figure 2: *Sample for isolation of bacteria.*

Isolation of the Nattokinase producing bacteria

Microbes from different samples were isolated by serial dilution and agar plating technique. Mixed cultures were obtained after spreading as shown in fig. below. Further pure culture and then screening of pure cultures were done. Where culture C-2 was found positive.

(a) Spreading plate

(b) Pure culture plate



(c) Screening plates

Figure 3: *a) colonies on nutrient agar plates after serial dilution and spreading, (b) pure cltures after streaking, (c) screeing on casein plate where clear zones indicates the zone of hydrolysis.*

Colony morphology

Table 1: *The morphology of all the selected colonies is listed below.*

Culture	Shape	Margin	Elevation	Texture
C-1	Irregular	Entire	Flat	Hard
C-2	Circular	Entire	Raised	Soft
C-3	Circular	Entire	Raised	Soft
C-4	Irregular	Entire	convex	Gummy
C-5	Irregular	Discrete	Flat	Hard
C-6	Circular	Entire	Raised	Soft
C-7	Circular	Entire	Raised	Hard
C-8	Irregular	Discrete	convex	Gummy

Culture	Pigment	Surface	Opacity
C-1	Off- white	Rough	Opaque
C-2	Off- white	Smooth	Translucent
C-3	Off- white	Smooth	Translucent
C-4	cream	Smooth	Opaque
C-5	Cream	Rough	Translucent
C-6	Off- white	Smooth	Translucent
C-7	Off- white	Rough	Translucent
C-8	cream	Smooth	Opaque

Table 2: *shows the remark of the cultures after primary and secondary screening on blood agar in plates. Secondary cultures showed that Culture2 has maximum core zone of hydrolysis.*

Culture No.	1° Screening	2° Screening
C-1	++	+
C-2	+++	+++
C-3	-	-
C-4	-	-
C-5	-	-
C-6	-	-
C-7	-	-
C-8	-	-

Biochemical tests

From the screening result it became clear that culture-2 has maximum nattokinase activity. Further identification was done by different techniques in accordance with Bergey's manual as mentioned below:

Table 3: *Biochemical tests with results.*

Test	Result
Gram Staining	**+,** *Bacillus*
Catalase Test	**+**
Endospore's staining	**+**
Mannitol test	**-**
Glucose fermentation test	**+**

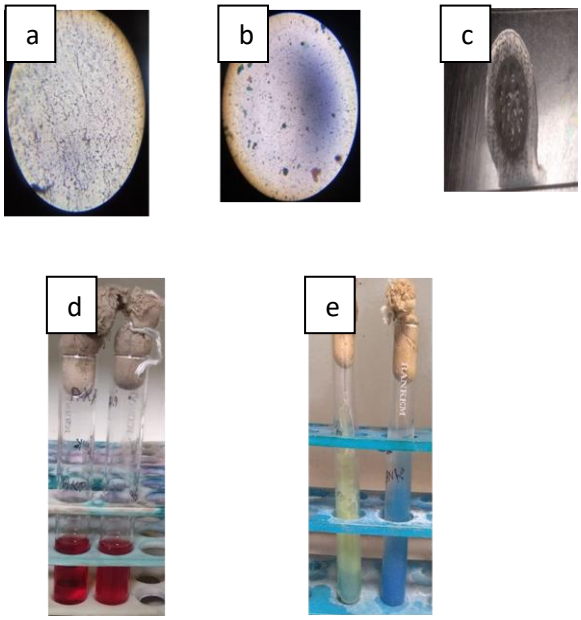


Figure 4:*Above figure shows the results of biochemical tests, a:gram's staining, b:endospore's staining, c:catalase test, d:mannitol test, g: glucose fermentation test.*

Selection and optimization of production media

Three production media was prepared and sterilized then incubated after inoculation of culture. By taking the OD it was found that production media 3 was best for culture. Further optimization was done by changing the factors.

Table 4: *Selection and optimization of production media on the basis of growth.*

Production Media	OD 620 nm
PM- 1	0.19
PM- 2	0.24
PM- 3	0.34
Nitrogen Sources	
Peptone	0.05
Urea	0.06
Yeast	0.19
Carbon Sources	
Dextrose	0.35
Sucrose	0.13
Fructose	0.05
pH	
4	0.01
7	0.29
9	0.24
11	0.20
Temperature	**Growth**
37°C	+++
50°C	-
4°C	-
Room temperature	+

Fermentation and downstream processing

Fermentation is done using shake flask method. The culture 2 is inoculated in the Optimized media under optimum conditions. After fermentation, centrifuge the crude enzyme and collect the supernatant and store it at 4°C. The pure enzyme obtained after salt precipitation and dialysis.

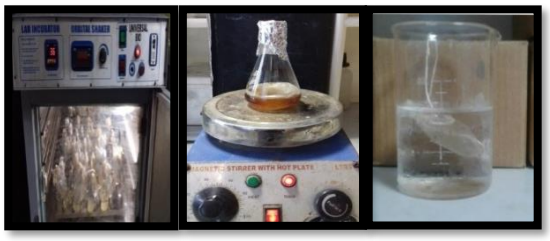


Figure 5: *fermentation of nattokinase and its purification by salt precipitation and dialysis.*

Enzyme assay

Assay is done in order to find out the activity of different form of enzyme like Crude salt precipitate and pure enzyme. The reading is then compared with the standard graph and enzyme activity is calculated.

Table 5*: analysis of crude and pure enzyme.*

Sample	Concentration of enzyme (µg/ml)	Enzyme activity (unit/ml/min)
Crude Enzyme	1.7	0.1
Pure Enzyme	2.1	0.13

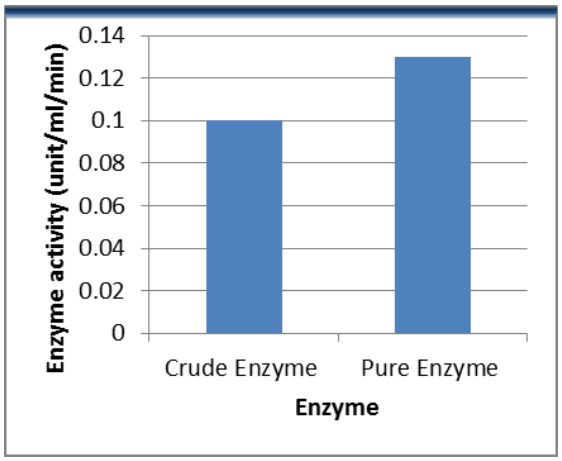


Figure 6: *Graphical analysis of crude and pure enzyme.*

Characterization of pure enzyme

Effects of pH:

Table 6: *Effect of pH on the enzyme:*

Sample	Concentration of enzyme (µg/ml)	Enzyme activity (unit/ml/min)
pH 4	0.37	0.1
pH 7	0.4	0.028
pH 9	0.8	0.05
pH 11	1.1	0.06

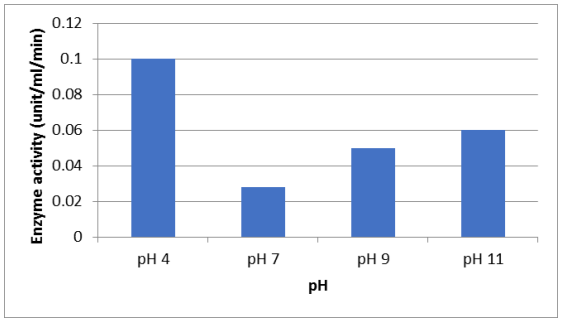


Figure 7: *Graphical representation of effect of pH on the enzyme.*

Effects of temperature:

Table 7: *Effect of temperature on the enzyme:*

Sample	Concentration of enzyme (µg/ml)	Enzyme activity (unit/ml/min)
50 °C	0.8	0.05
37 °C	0.83	0.053
4 °C	1.12	0.06

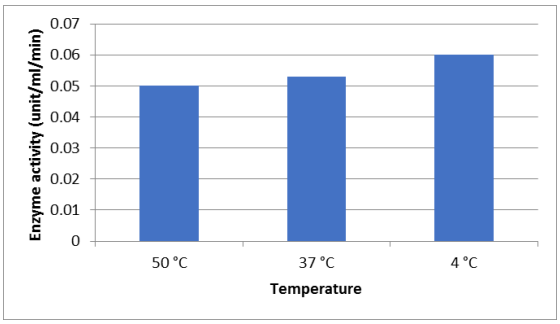


Figure 8: *Graphical representation of effect of temperature on the enzyme.*

Effects of activators and inhibitors:

Table 8: *Effect of activators and inhibitors on the enzyme:*

Sample	Concentration of enzyme (µg/ml)	Enzyme activity (unit/ml/min)
$CaCO_3$	0.83	0.053
$MgSO_4$	1.7	0.1
EDTA	1.12	0.06
NaCl	0.81	0.051

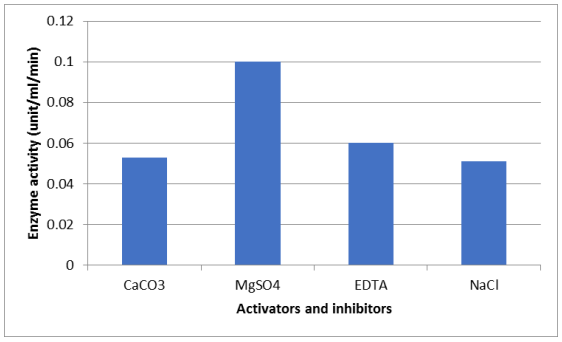


Figure 9: *Effect of activators and inhibitors on the enzyme.*

DISCUSSION AND CONCLUSION

In this project work, nattokinasehas been isolated from *Bacillus*species from soil sample which exhibited fibrinolytic activity. It is concluded from the overall procedure at the laboratory that the maximum activity was obtained after 48 – 72 hrs. The required conditions for the maximum activity and production of nattokinase are pH 7 and temperature 37°C; carbon source- dextrose and nitrogen source- yeast.

The fermentation media was kept for 48 -72 hrs in shaker after inoculation for the growth of bacteria in an efficient manner. The enzyme obtained after the downstream processing was 50-65% pure. The enzyme exhibited good thrombus degrading property and therefore can be considered for further scale up and commercial production. The enzyme has high degree of specificity and hence used as an alternative to treat thrombolytic diseases **[17,18].**

From the results obtained in the present work, the isolated *Bacillus*species was found to be having ability to synthesize economically important fibrinolytic enzyme; nattokinase. Furthermore, the activity and stability profile of the fibrinolytic enzyme from the isolated bacterium suggesting its potential for the further applications in preventive medicines.

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