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PRODUCTION OF FIBRINOLYTIC ENZYME (NATTOKINASE) FROM *BACILLUS* SP.

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Abstract:

During present study Nattokinase which is a novel fibrinolytic enzyme was produced by Bacillus sp. To screen and extract nattokinase enzyme from Bacillus sp. were isolated from soil of different agricultural field by serial dilution method. Out of 10 isolate, one strain i.e. B3 produced nattokinase on screening medium. B3 was identified by biochemical characterization. The caseinolytic activity of Nattokinase was 0.526 U/ml and the selected isolate Bacillus sp. could produce active nattokinase according to haemolytic activity 200 µl crude enzyme dissolve blood clot sample.

Key Words: *Bacillus sp., Caseinolytic Activity, Fibrinolytic enzyme, Haemolytic activity, Nattokinase.*

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INTRODUCTION:

Nattokinase is a fibrinolytic enzyme, meaning that it breaks down fibrin, an insoluble white protein produced by the conversion of fibrinogen (a protein in the plasma of blood for clotting) by thrombin (a blood clotting enzyme). Nattokinase was discovered in 1980 after testing over 173 natural foods as potential thrombolytic agents, searching for a natural agent that could effectively dissolve thrombus allied with cardiac and cerebral infarction[1][2] .

Nattokinase was discovered in Natto, which is a traditional Japanese food made of soybeans. Natto is considered a very healthy food. To prepare, the beans are cooked and then fermentation occurs by the action of the bacterium ***Bacillus subtilis* sp. *natto*** [3][4]. The botanical source for Nattokinase is *Glycine max (L.) Merr*. It appears as a yellow-white fine powder. Nattokinase is a serine protease with 275 amino acid residues and a molecular weight of 27,728 Daltons. Nattokinase has a high homology with the subtilisin enzyme and DNA sequencing shows 99.5 and 99.3% homology to subtilisin E and amylosacchariticus [5][6] respectively. Nattokinase degrades fibrin clots both directly and indirectly. Nattokinase degrades fibrin directly in clot lysis assays with activity comparable to plasmin. Kinetic assays suggest that it is 6 times more active than plasmin in degrading cross-linked fibrin. Nattokinase degrades fibrin indirectly by affecting plasminogen activator activity. In the main nattokinase works to support healthy blood circulation in two different ways. First off, nattokinase resembles plasmin, so it can break down fibrin directly. Secondly, nattokinase enhances the body's natural production of plasmin, which also helps to break down fibrin[7][8]. Nattokinase help in normal blood circulation, blood flow, and blood viscosity (thickness), it also supports the body's normal blood-clotting mechanism, supports the body's production of plasmin, which reduces fibrin and helps to maintain normal blood pressure level. It is also used for pain, fibromyalgia, chronic fatigue syndrome, endometriosis, uterine fibroids, muscle spasms, tissue oxygen deprivation, infertility and cancer.

Nattokinase is an edible enzyme and is being used as nutrient supplement; it can be used to digest amyloids in body. Nattokinase is used for cardiovascular diseases including heart disease, high blood pressure, stroke, chest pain (angina), deep vein thrombosis "hardening of the arteries" (atherosclerosis), hemorrhoids, varicose veins, poor circulation, and peripheral artery disease[9]. It is also used for pain, fibromyalgia, chronic fatigue syndrome, endometriosis, uterine fibroids, muscle spasms, infertility, cancer, and a Vitamin B1-deficiency disease called Beri-beri. A 2009 study showed that nattokinase may be effective in catabolism of toxic amyloid fibrils associated with Alzheimer's Disease, as well as the insulin fibrils associated with diabetes and the prion peptide fibrils associated with prion diseases [10]. In future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources, it is still the most stable and economic way to obtain protein with fibrinolytic activity by *B. subtilis*.

The ultimate goal of this study was to characterize *Bacillus* sp., production and extraction of a promising nattokinase with potent activity.

MATERIAL AND METHODS:

Isolation and Identification of Bacteria.

In the month of January soil samples were recovered and collected into sterilized plastic bags from different agricultural field i.e. **Sugarcane, Potato and Banana field**. Soil samples were taken from 15-20 cm depth after removing approximately 3 cm of earth surface. Isolation of the samples was performed by the serial dilution plate technique [11]. Different aqueous dilutions (10-7) of the soil suspension were applied separately into sterilized petri-dishes containing sterilized nutrient agar medium and incubated for 24 hr at 37^{o}C. Identification of the selected microorganism was determined on the basis of cultural, microscopic and biochemical characteristics and according to the directions given by the Bergey's Manual of Systematic Bacteriology (Table 1)[12][13] .

Extraction of the crude Nattokinase

Bacillus was grown on basal medium containing (g/L) Soya Peptone 10, K2HPO4 2, MgSO4 1, Maltose 20, Yeast extract 10, Glucose 2 and pH was adjusted at 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 of *Bacillus* sp. was used as inoculum and incubated at 37°C and 150 rpm in an orbital shaker. After 7 days of the fermentation of the production medium, cells were removed by centrifugation at - 4^{o}C and 12000 rpm for 15 min. The supernatant was considered as extracellular crude nattokinase [14][15].

Blood haemolytic assay

Nattokinase shows fibrinolytic activity which can dissolve blood clot, because of its capability to digest fibrin in blood vessels. 100 mg of coagulated blood

was dissolved by adding 200 µl of the extract within 2 h at 37°C temperature [16].

Caseinolytic activity:
A mixture (1ml) of 0.7 ml of 0.1 M sodium phosphate buffer (pH7.5), 0.1 ml of 2% casein, and 0.1 ml of enzyme solution was incubated for 5 min at 270C, 370C, 470C and 570C mixed with 0.1 ml of1.5 M tri-chloro acetic acid, allowed to stand at 4°C for 30 min and then centrifuged at room temperature. The absorbance at 560 nm for the supernatant was measured and converted to the amount of tyrosine equivalent. One unit of Caseinolytic activity (CU) was defined as the amount of enzyme releasing1µ mole of tyrosine equivalent/min (Table 2, graph 1) [14].

RESULTS AND DISCUSSION:

Isolation and Identification of Bacteria.

10 different types of bacteria were streaked on nutrient agar plates. After 48 hours of incubation time cultural characteristics were studied and then B3 isolate was identified as *Bacillus* sp. according to microscopical and biochemical characteristics (Table 1). *Bacillus* is a Gram positive, rod shaped, aerobic and endospore forming bacteria. B3 isolate was positive for starch hydrolysis, catalase, casein hydrolysis, nitrate reduction, methyl red and fermentation (sucrose, lactose and dextrose). Culture B3 showed fermentation in all (sucrose, lactose and dextrose) with acid production without gas formation and was negative for indole, urease, mannitol fermentation, Voges Proskauer and citrate utilization. *Bacillus subtilis* RJAS19 was found to be having ability to synthesize economically important fibrinolytic enzyme [17]. Bacillus subtilis producing fibrinolytic enzyme were isolated from soil obtained from various regions of Kolkata [14].

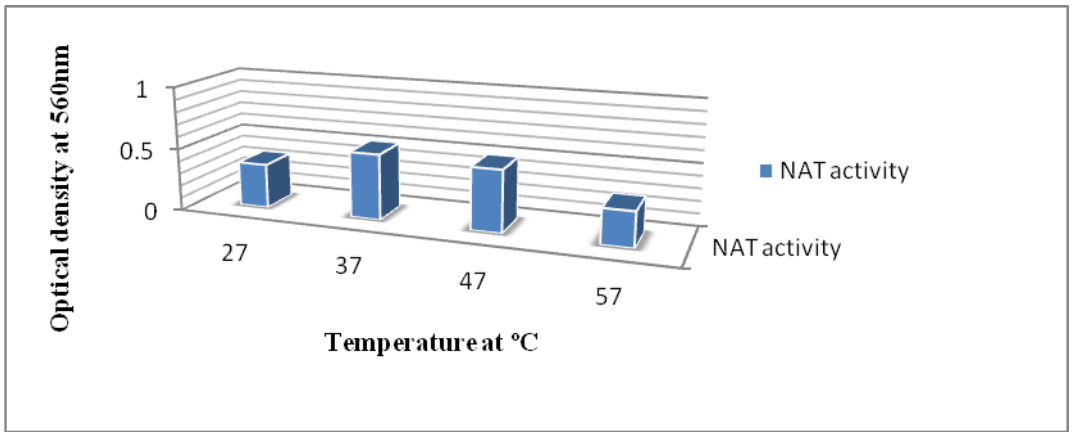
Table 1: Chemical and morphological characteristic of the isolate

S.no	Biochemical Test	*Bacillus subtilis* (B3)
1.	Lactose Fermentation	+ve
2.	Sucrose Fermentation	+ve , A
3.	Mannitol Fermentation	-ve
4.	Dextrose Fermentation	+ve, A
5.	Casein hydrolysis	+ve
6.	Catalase test	+ve
7.	Urease test	-ve
8.	Nitrate reduction test	+ve
9.	Indole test	-ve
10.	Methyl red (MR)	+ve
11.	VogesProskauer test	-ve
12.	Citrate utilization test	-ve
13.	Starch hydrolysis test	+ve
14.	Gram's reaction	+ve

-ve = negative, +ve = positive, A = Acid production

Table 2: Caseinolytic activity at different temperature of NAT enzyme (average of triplicate)

S.No.	Temperature (ºC)	Optical density at 560 nm
1	27	0.352
2.	37	0.526
3.	47	0.499
4.	57	0.280



Graph 1: Caseinolytic activity at different temperature of NAT (enzyme assay)

Detection of Nattokinase by haemolytic activity.

The extracted crude supernatant sample was tested for the presence of nattokinase by haemolytic activity. The blood clots were mixed with 200µl of crude enzyme and were incubated for 2 hours at 37ºC, the clots were nearly dissolved showing presence of nattokinase. The presence of nattokinase in the selected bacteria was determined by dissolving human blood clots. Since enzymes overcomes difficulties of the conventional methods in the breakdown of complex matters [18]. Nattokinase degrades fibrin directly in clot lysis assay with activity comparable to plasmin. Nattokinase possess various applications including anti-hypertension, blood thinning, thrombolytic and digestive capability [19]. In addition, fibrinolytic enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases [20].

Caseinolytic activity of NAT enzyme.

B3 isolate was screened by caseinolytic activity. For screening the absorbance was recorded at 560nm. Caseinolytic activity was done at different temperature. The optimum temperature was 37ºC at which activity was observed maximum i.e. 0.526 U/ml. *Bacillus* sp. showed both haemolytic and caseinolytic activity and is a competent bacteria as it is capable to produce significant enzymes like amylase, protease, etc.[21-23]

CONCLUSION:

Fibrinolytic enzyme such as Nattokinase, Streptokinase and Urokinase used as thrombolytic agent but very expensive, needs large scale production by some alternative method with high purity. So isolation, production, assay characterization of fibrinolytic enzyme from bacterial sources are very effective and useful. The main objective of our study was to characterize nattokinase producing bacteria and to check their ability for enzyme production especially nattokinase. *Bacillus* strain B3was isolated. It has potent source of nattokinase as it show high nattokinase activity. B3 strain produce nattokinase was novel and make it potential for medical sciences as it can be used in heart diseases, Alzihmer's and thrombolytic therapy. Hence it is concluded that the microorganism which are present in soil can produce nattokinase enzyme are cost effective in production and can easily be manipulated.

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