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Production and Purification of Nattokinase from *B. Subtilis*

Shivani Tiwari

MRD Life Sciences Pvt. Ltd. Lucknow, UP, India

Abstract: ***Nattokinase, novel fibrinolytic enzymes which are isolated from Bacillus subtilis isolated from soil samples. The fibrinolytic enzyme Nattokinasewas purified from supernatant of Bacillus subtilis, showed thermophilic, hydrophilic, and strong fibrinolytic activity. The maximum production was carried out in the presence of maltose as carbon source and peptone as nitrogen source. Optimum temperature and pH of Nattokinase, was 37°C and 7 respectively. The fibrinolytic activity of Nattokinase was 576.73 U, respectively.***

Keywords: Anticoagulant Activity, Submerge Fermentation, Fibrinolytic Enzyme Activity, Haemolytic Activity.

1. Introduction

Numerous creatures, such as snakes, earthworms, and bacteria like *Streptococcus pyogenes, Aeromonas hydrophila, Serratia E15, B. natto,* and *Bacillus amyloliquefacens*, as well as fungi like *Fusarium oxysporum, Mucor sp,* and *Armillaria mellea,* have fibrinolytic enzymes that have been identified and studied. [1]. Foods like Korean ChungkookJang soy sauce, edible honey mushrooms, Japanese Natto, and Tofuyo all contain fibrinolytic enzymes. These foods' fibrinolytic enzymes have been isolated, and their physiochemical characteristics have been studied. [2 - 4]. A common Asian spice, fermented shrimp paste, was found to have potent fibrinolytic action. These brand - new fibrinolytic enzymes, which come from conventional Asian meals, are beneficial for thrombolytic therapy. [5 - 6]. Since they can easily and effectively create huge quantities of enzyme, they will serve as a supplement to the expensive fibrinolytic enzymes that are now utilised to manage cardiac disease. Additionally, these enzymes have a great deal of promise for uses in nutraceuticals and food fortification, thus their use could successfully prevent cardiovascular illnesses. [7].

In the Western world, cardiovascular illnesses are now the major cause of mortality [8]. Numerous blood clot - dissolving medications, including tissue plasminogen activator (t - PA), streptokinase, and urokinase, have been used in clinical therapies for cardiovascular disorders. Hemostasis is a difficult process that is achieved by striking the right balance between blood clot formation and haemorrhage [9]. Fibrin clots may not be dissolved in an imbalanced state, leading to thrombosis. Numerous thrombolytic substances have been thoroughly studied. The therapy of thrombosis has traditionally included the administration of enzymes like as plasminogen activators, streptokinase, and urokinase. These enzymes can have negative side effects and are frequently costly and thermolabile [10]. Bacillus subtilis natto produces the serine protease subtilisin nattokinase (NAT), also known as subtilisin BSP, which has been shown to have strong fibrinolytic activity [11]. Numerous in vivo studies had been described [12], in addition to in vitro evaluations of fibrinolytic activity. When Fujita et al. administered nattokinase to dogs orally, the plasma fibrinolytic activity increased and demonstrated that subtilisin NAT could cross the intestinal tract of rats and dissolve chemically generated thrombus. According to research by Suzuki et al., adding natto to the diet decreased intimal thickening and controlled the lysis of mural thrombi following endothelial damage in the rat femoral artery. A comparable impact of dietary Bacillus natto productive protein on in vivo endogenous thrombolysis was previously found by Sumi et al. Similar microorganisms that produce fibrinolytic enzymes have also been found in Chinese douchi, Korean chungkook - jang [13], and Japanese shiokara. However, B. subtilis natto is still the most reliable and cost - effective method for obtaining protein with fibrinolytic activity. Subtilisin NAT provides benefits for commercially utilised treatment for preventative and sustained effects due to its dietary origin, reasonably strong fibrinolytic action, stability in the gastrointestinal system, and simple oral administration [14–15].

2. Materials and Methodology

Isolation of nattokinase producing bacteria and its identification:
An Indian city of Lucknow's soil sample included a bacterial strain that produces nattokinase (NK).2% casein and 2 ml of human serum were added to nutritional agar for the screening of fibrinolytic enzymes. Colony morphology, Gram's staining, a biochemical test (Bergy's Mannual), and selective media were used to identify the culture. The recognised microbes were kept at - 20°C [16 - 17].

Media selection and optimization:
Following the one item at a time approach, the medium were optimised with each component being based on bacterial growth. Chemical parameters including carbon supply, nitrogen source, salt, and physiochemical factors like pH and temperature were investigated during medium optimization [18].

Growth curve study:
The culture was placed in 200 mL of the ideal medium, inoculated with 1% (v/v) inoculum, and grown for 14 hours at 37 °C. As a blank control, the optimised medium devoid of the test bacterial solution was employed, and its OD600 was assessed at the appropriate time intervals [11].

Production and purification of Nattokinase:
On an improved basal medium made up of (g/lit.) Peptone, 10. K2HPO4, 2. MgSO4, 1. Maltose, 20. and 1000 ml distilled water, B. subtilis was cultivated.1N HCl and 1N NaOH were used to bring the pH down to 7.2. The medium was autoclaved at 121°C for 35 minutes, and it was then cooled to room temperature. The inoculum was 1 ml of uniformly produced B. subtilis suspension, which was incubated at 37 °C and 150 rpm in an orbital shaker. Cells were collected by centrifugation (10, 000 RPM, 10 min, 4°C) after 3 days of fermentation, and the purification process involved salt precipitation (40% ammonium sulphate) and dialysis [19 - 22].

Assay for fibrinolytic activity
A total of 0.4 mL of 0.72% (w/v) fibrinogen solution was mixed with 1.4 mL of 50 mM Tris - HCl (pH 8.0) buffer, and was incubated at 37 °C for 5 min. Then, 0.1 mL of thrombin solution (20 U/mL) was added to the above reaction system and was incubated at 37 °C for 10 min, then 0.1 mL of diluted enzyme solution was added to fully homogenize the mixture, and it was incubated in a 37 °C water bath for 60 min. After the reaction was stopped by adding 2 mL of 0.2 mM trichloroacetic acid and centrifuged at low speed, the absorbance at 275 nm of the supernatant was measured. One unit of enzyme activity (FU) is defined as the amount of enzyme required to change the absorbance at 275 nm by 0.01 per minute at 37 °C, pH 8.0 [23 - 24].

3. Results & Discussions

Traditional antithrombotic medications have drawbacks, including ineffective thrombolytic effects, a propensity for bleeding, and a single manner of administration. Nattokinase has emerged as a promising novel thrombolytic medication due to its high thrombolytic efficacy, few side effects, and oral administration [12]. Nattokinase is mostly produced by the fermentation of *Bacillus*, however *Pseudomonas* [19] and some marine *Bacillus subtilis* [18] have also been discovered to produce nattokinase.

In this study, soil samples from slaughter house were subjected to serial dilution in 0.85% NaCl solution and on the basis of their morphological parameters and streaked on nutrient agar plates, and a total of 41 cultures were isolated and purified. While screening these cultures only five strains were found to grow normally, namely S1C2, S1C4, S2C2, S3C5, S4C1 respectively. Among these, only the S2C2 strain produced colonies after 24 h of culture, and the other four strains produced colonies after 48 h. Using the fibirnolytic assay, it was determined that the S2C2 strain had the greatest fibrinolytic activity. Therefore, we selected the S2C2 strain for follow - up experiments (figure 1).

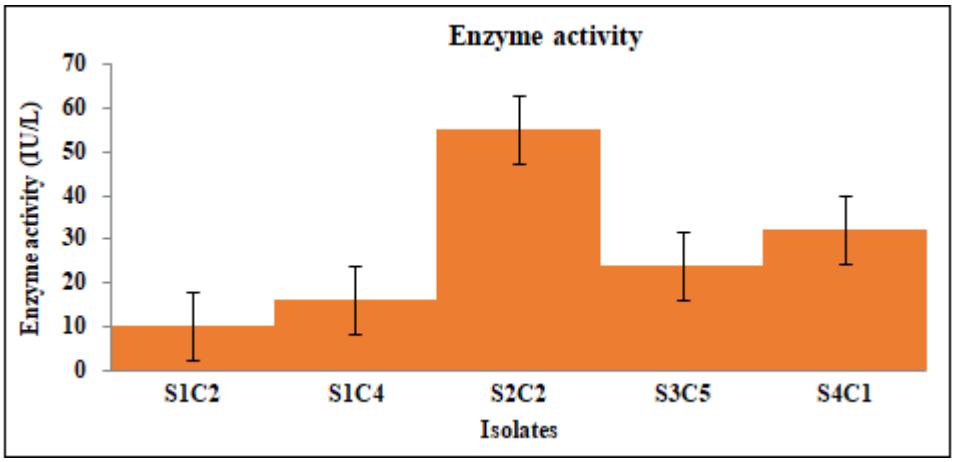

Figure 1: Screening of the bacterial cultures for fibrinolytic activity

We carried out morphological observation, Gram staining, and molecular biological identification of the S2C2 strain to determine the species of this strain. The colonies of S2C2 were flat and rough, with irregular edges, and could be stained purple - red with Gram dyes (Figure 2). BY following bergy's manual the culture determined as *Bacillus subtilis*.

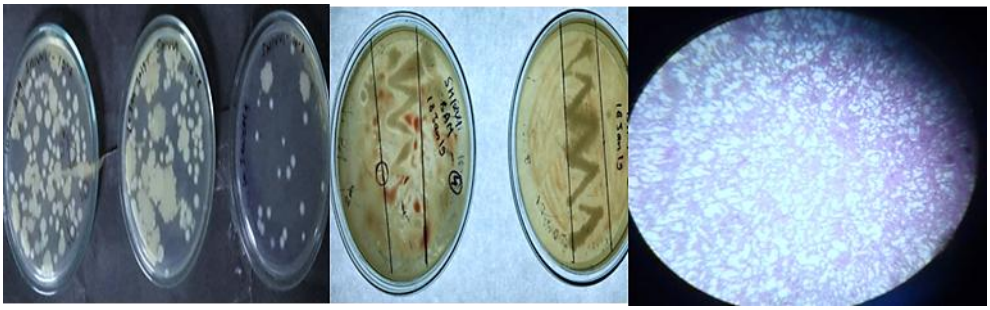

Figure 2: Morphological observation, enzymatic activity and gram staining of B. subtilis

Media optimization:
We evaluated the ability of different carbon and nitrogen sources to ferment the Bacillus subtilis S2C2 strain to produce nattokinase and found that maltose and peptone as was the best carbon and nitrogen sources. Various types of carbon sources such as shrimp shell wastes, maltose, lactose, and galactose have been reported to be the best carbon sources for the fermentation of nattokinase by different Bacillus species, and some low - cost carbon sources (tapioca starch) and even industrial wastes (tofu processing wastewater) can also be used as carbon sources for the fermentation of nattokinase [16, 26]. While optimizing the pH and temperature it was found that the culture shows its best growth and nattokinase activity at pH 7 and temperature 37°C (Figure 3, table 1).

Table 1: Media optimization for *B. subtilis*

Production media	Factors	Enzyme activity (IU/L)
PM1	Peptone	59
PM2	Yeast	10
PM3	Malt	14
PM4	Sucrose	17
PM5	Dextrose	13
PM6	Maltose	68
PM7	pH4	55
PM8	pH7	98
PM9	pH12	68
PM10	10 C	28
PM11	37 C	158
PM12	60 C	87

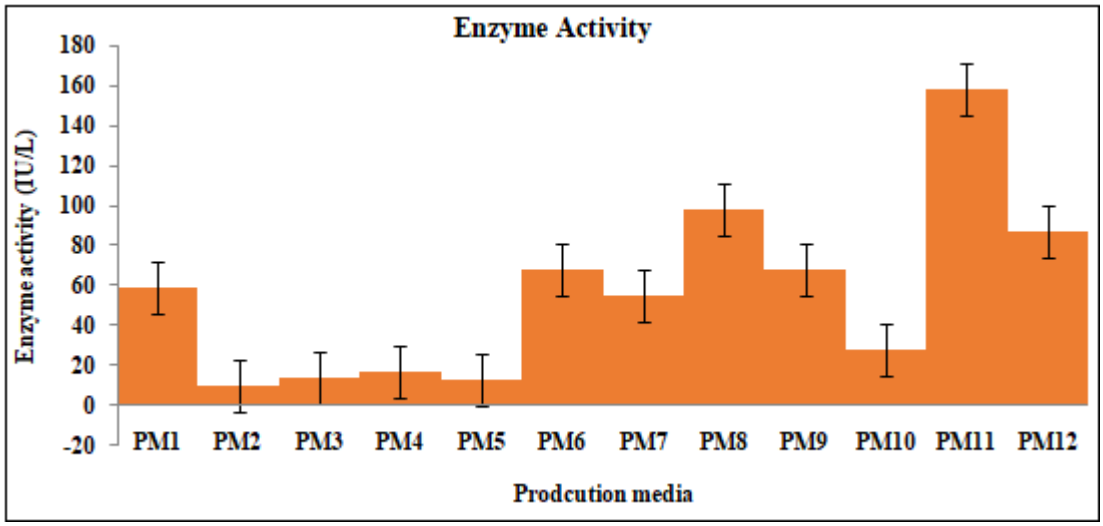

Figure 3: Optimization of production media

Production and purification:
The activity of the crude and purified enzyme after precipitation through 40% (NH4) 2SO4 and dialysis were studied for the activity and found.

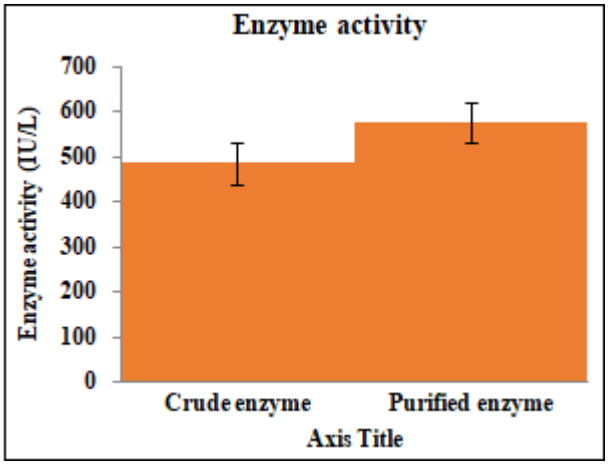

Figure 4: Estimation of purified enzyme

4. Conclusion

By optimising the nutritional contents and culture circumstances, the nattokinase activity of crude enzyme from B. subtilis was 576.63IU/mg. In comparison to certain other nattokinases, it demonstrates comparatively high thermostability and alkali tolerance, according to earlier investigations. Our findings suggest that metal ions, nutritional makeup, and culture conditions all play significant roles in the fibrinolytic activity of nattokinase from B. subtilis. The biochemistry techniques for enhancing nattokinase's fibrinolytic activity, as well as the molecular structure, including the amino acid sequence and secondary and tertiary structures, will be the subject of further research.

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