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Research Article

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF NATTOKINASE FROM *BACILLUS SUBTILIS*, ISOLATED FROM TEA GARDEN SOIL SAMPLES OF DIBRUGARH, ASSAM

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ABSTRACT

Nattokinase is an enzyme that finds a wide range of applications in Pharmaceutical industry, health care and Medicine etc. Nattokinase is a naturally-occurring proteolytic enzyme derived from natto, a traditional Japanese food produced from the fermentation of soybeans with *Bacillus subtilis* natto. It is an enzyme that digests fibrin both directly and indirectly. Indirectly, it activates pro-urokinase and tissue plasminogen activator (t-PA), supporting the fibrinolytic activity of plasmin. These combined actions promote healthy platelet function, circulation and blood flow. Our aim was to isolate and characterize nattokinase from *Bacillus subtilis* isolated from tea garden soil. In this study, nattokinase producing *Bacillus subtillis* was isolated from soil samples collected from 5 different tea gardens located in Dibrugarh district of Assam. The characterization results of the purified enzyme showed its optimum conditions at 37°C, pH 7, at 5% substrate concentration and at 1 ml of 10% zinc chloride activator. The enzyme activity found to be gradually declined at temperature beyond 70°C. The purified enzyme was characterized and the results were found to be promising for future studies and commercial production.

Keywords: Nattokinase, *Bacillus subtilis*, tea garden soil.

INTRODUCTION

Nattokinase supports normal, healthy blood flow and circulation. Nattokinase is an enzyme extracted from Natto, a cheese-like food made from fermented soybeans [1]. Nattokinase may contribute to the regular healthy function of the heart and cardiovascular system by maintaining proper blood flow, thinning the blood and preventing blood clots [2]. Nattokinase can hydrolyze fibrin in blood clots as fibrinogen acts as a very good substrate which hastens the production in the media [3]. Fibrinolytic enzymes such as Nattokinase used as thrombolytic agent but too costly and also used through intravenous instillation, needs large scale production by some alternative methods and high purity [2]. So, isolation, production, purification, assay and characterization of fibrinolytic enzymes from bacterial sources are very effective and useful. In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources [4].

In our present study, our aim is to isolate and characterize nattokinase from *Bacillus subtilis* isolated from tea garden soil.

MATERIALS AND METHODS

Isolation and identification of *Bacillus subtilis* from tea garden soil samples

Tea garden soil samples were collected from 5 different tea estates located in Dibrugarh district of Assam (India). After serial dilution, the samples were inoculated on commercially available Hi-Bacillus media and were incubated at 36°C for 24 hrs. The bacterial colonies were subcultured on nutrient agar slants. *Bacillus subtilis* was identified on the basis of different staining techniques and biochemical tests by Bergey's Manual of systematic bacteriology, 2nd edition [5] using commercially available HiBacillus™ (KB013) test kits. All the chemicals and the test kits were purchased from HiMedia India Pvt. Ltd.

Production of nattokinase from *Bacillus substilis*

The production media containing 0.3% beef extract, 1% milk casein was used along with different nitrogen sources (cow fibrin, sheep fibrin, peptone and tryptone). The pH was adjusted to 7.0 with 1 M glacial acetic acid and 1 M NaOH and inoculated with *Bacillus subtilis* and incubated at 37º C in orbital shaker for 48 hrs at 86 rpm.

Purification of nattokinase

Modified method of Peng, *et. al.,* (2003) was used for the enzyme production and purification. The Production media was centrifuged at 10,000 rpm for 12 minutes to obtain the supernatant. The supernatant after centrifugation was collected and the volume of it was measured and accordingly 70% ammonium sulphate was measured in order to conduct the salting out procedure. The supernatant was transferred into a conical flask and this flask was placed in ice cold condition on the magnetic stirrer, and the ammonium sulphate powder was added pinch by pinch until complete dissolving of ammonium sulphate takes place. After this, it was kept for overnight precipitation in the refrigerator. The pellet was collected and dissolved in 10 ml of 50 mM Tris hydrochloric acid solution. This sample was subjected to dialysis followed by ion-exchange chromatography.

Dialysis of the purified enzyme

About 8cm of the dialysis tube was cut and placed in 100 ml of 2% w/v sodium bicarbonate. 1 mM EDTA was added to chelate any metal ions. It was boiled for 10 min and was again washed in boiling distilled water for 10 minutes. The boiling process was repeated with distilled water again. The activated dialysis bag was filled with the enzymes and sealed from the both sides without any air bubbles. The bag was kept in 500ml of 50 mM Tris HCl (pH 7.0) solution on a magnetic stirrer in ice cold condition for 8 hrs. The buffer was changed frequently for every hour to avoid equilibration.

Ion Exchange Chromatography

The chromatography column packed with 2% DEAE cellulose was washed twice using distilled water and kept for sonication for 15 minutes. The matrix was activated using activation buffer (25 mM HCl and 25 mM NaCl). The dialyzed enzyme sample was poured into the column followed by a series of elution buffers containing 25 mM Tris HCl and increased concentration (50 mM, 75 mM…150 mM) of NaCl solution to elute the enzyme. The elutants were collected in the same test tube.

Characterization of the purified enzyme

Effect of temperature

The enzyme was assayed in the reaction mixture containing 2.0ml of 0.5% casein solution in 0.1M carbonate buffer (pH 9.3) and 0.1ml of enzyme solution in the total volume of 2.1 ml. After incubation at 4°C, 37°C, 55°C and 80°C for 5mins, the reaction was stopped by adding 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 min. The reading was taken at 660 nm in UV- Spectrophotometer to monitor the enzyme activity.

Effect of pH

The above method was repeated using 0.1M carbonate buffer of different pH (2, 4…10) and the enzyme activity was monitored spectrophotometrically.

Effect of substrate

The enzyme was assayed in the reaction mixture containing different concentration 0.5% casein solution in 0.1M carbonate buffer (pH 9) and 0.1ml of enzyme solution in the total volume of 3.0ml. After incubation at 37° C for 5 min and the reaction was stopped by adding of 3.0 ml of 10% ice cold TCA (Trichloroacetic acid) and centrifuged at 10,000 rpm for 5 min. The absorbance was taken at 660 nm.

Effect of activator

The activator 10% zinc chloride was added to the enzyme and the enzyme assay was carried out to check the activation and the above mentioned method was again repeated and the absorbance was taken at 660 nm.

Effect of inhibitor

The inhibitor 10% EDTA (Ethylene diamine tetra acetate) was added to the enzyme and the enzyme assay was carried out to check the activity of inhibitor. The reaction mixture was prepared as mentioned above and the absorbance was taken at 660 nm.

RESULTS AND DISCUSSION

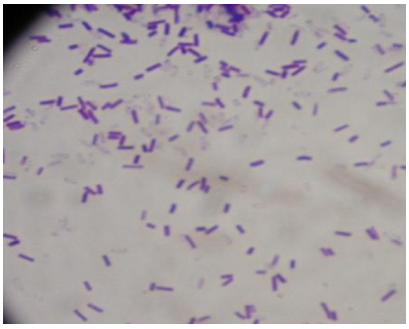

Fig. 1: Bacillus subtilis under 40X (Photograph taken under Leica ATC 2700 Microscope)

Nattokinase producing *Bacillus subtilis* (Fig 1) was isolated and was identified on the basis of biochemical characteristics (Table 1). The characterization results of the purified enzyme showed its optimum absorbance of 0.126 at 37°C (Fig 2), 0.875 at pH 7 (Fig 2), 0.151 at 5% substrate concentration (Fig 3), 0.187 at 1 ml of 10% zinc chloride activator (Fig 4) and enzyme activity found to be decreasing gradually with the increase in the volume of the inhibitor (Fig 5).

Table 1: Biochemical test results of *Bacillus subtilis*

Tests	Malonate	VP	Citrate	ONPG	Nitrate reduction	Catalase	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalose
Results	-	+	+	+	+	+	-	+	+	+	+	+

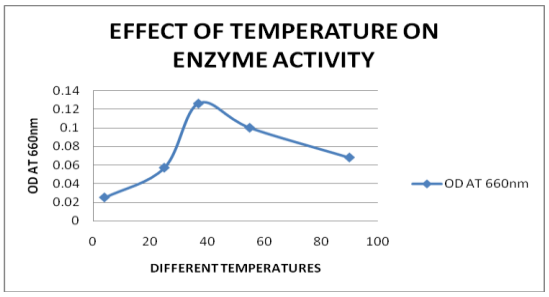

Fig. 2: Effect of temperature

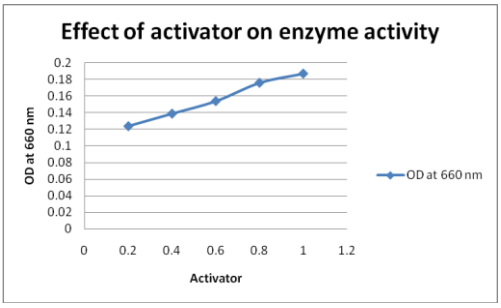

Fig. 3: Effect of pH

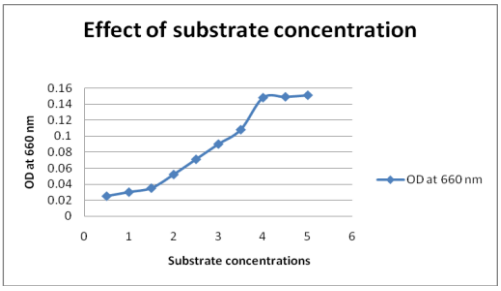

Fig. 4: Effect of Substrate Concentration

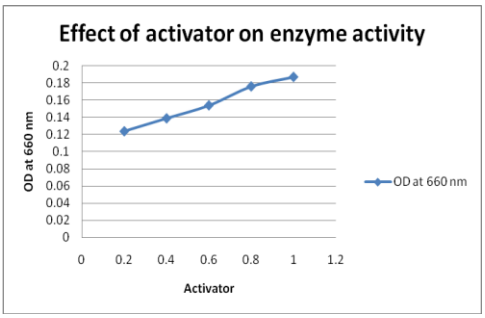

Fig. 5: Effect of inhibitor

The activity of the purified enzyme was determined at different temperatures ranging from 0°C-100°C. The enzyme activity gradually declined at temperature beyond 70°C. Considering all the above findings, nattokinase found to be promising for industrial production and for further research.

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