Purification and Characterization of Nattokinase from *Bacillus Subtilis* from Coconut Field Soils

Lakshmaiah. P[1], D. Srinivasa Rao[2], U. Spandana[3]

[1]*Vishwa bharathi College of Pharamaceutical Sciences Perecherla,Guntur,India.*

[2]*Department of Biotechnology, Acharya Nagarjuna University, Guntur, India.*

[3]*Nirmala College of Pharmacy, Mangalagiri, Guntur, Andhra Pradesh, India.*

Abstract: **Nattokinase is an enzyme that finds a wide range of applications in pharmaceutical industry, health care and medicine etc. The main aim of research work is to screen and identification of *Bacillus subtilis* from coconut field soil source and purification of nattokinase. Biochemical test like Methyl red (MR) and the voges-proskauer (V-P) broth opposite result were obtained, in urease test phenol red changes from yellow color to a red or deep pink (cerise) color and catalase test trypticase soy agar slants presence of gas bubbles. Using different nitrogen sources like cow fibrin, sheep fibrin, peptone and tryptone enzyme activity was found to be 6, 26, 28.3 and 38 U/ml respectively. The caseinolytic activity of Nattokinase was found to be 636.73 U/ml. The enzymes were purified to homogeneity by ammonium sulfate precipitation, dialysis and ion exchange chromatography. Characterization studies showed an optimum temperature 40^{0}C, 38 U/ml and 1.0 U/ml of substrate and zinc chloride activator were found to be more when compared to the other.**

Key words: **Natto kinase, Purification and biochemical characterization.**

I. INTRODUCTION

Nattokinase is a plasmin-like fibrinolytic enzyme acts as a plasminogen activator, inactivates fibrin factor directly or indirectly, dissolve blood clots which can directly degrade the fibrin in blood clots and thereby dissolving the thrombi rapidly in thrombosis therapy (Lakshmaiah *et al.,* 2016). It enhance plasmin through active pro-urokinase (endogenous) t-PA (tissue - plasminogen activators) is like urokinase and active plasmin. Nattokinase increases the concentration of t-PA produced from microorganiss (prokaryotic and eukaryotic organisms like *Bacillus subtilis, fusarium* etc. and *Rhizomucor* sp.) and similar kind of enzyme produced from the pancreas of human beings (trypsin and chymotrypsin) (Haritha *et al.,* 2011). The microorganisms used for the production of the nattokinase are *Bacillus subtilis* and *Rhizomucor sp. Bacillus subtilis*, known as bacillus or grass bacillus, gram-positive, obligate aerobe, rod-shaped, catalase-positive bacterium commonly found in soil (Shivasharana and Naik, 2012).

Nattokinase is not a kinase enzyme, but a serine protease of the subtilisin family (Kim and Choi, 2000) with a wide range of applications in pharmaceutical industry, health care products and medicine (Usharani and Muthuraj 2010). It exhibits a strong fibrinolytic action that promote healthy platelet function, contribute to the regular healthy function of the heart and cardiovascular system by maintaining proper blood flow (Ero *et al.,*2013), thinning the blood and preventing blood clots associated with heart attack and stroke. Nattokinase is recommended to take on a regular basis for those who are over 40 years old, stressed-out, have relatively high blood pressure, and have high blood viscosity due to hyperlipidemia or diabetes (Debajit et al., 2012). Nattokinase is found it to be a most promising agent (Kwon *et al.,* 2011) an ideal oral fibrinolytic agent not only reversing clots but in preventing them and modulating the clotting process in ways that help re-establish the coagulation homeostasis

II. MATERIALS AND METHODS

Isolation, identification and screening of organism producing nattokinase

The samples were collected from 5 different coconut field areas at Jaggaiahpet, Vijayawada, Andhrapradesh. After serial dilution, the samples were inoculated on commercially available Hi-Bacillus media and were incubated at 36^{o}C for 24 hrs. The bacterial colonies were sub cultured on nutrient agar slants. *Bacillus subtilis* was identified on the basis of different staining techniques and biochemical tests by Bergey's (Manual of Hsu RL2008) systematic bacteriology, 2nd edition[5] using commercially available HiBacillusTM (KB013) test kits. All the chemicals and the test kits were purchased from HiMedia India Pvt. Ltd

Production of nattokinase from Bacillus subtilis

Different nitrogen source like cow fibrin, sheep fibrin, peptone and tryptone were used in the production media. 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 was collected after centrifugation then it is salting out with 70% ammonium sulphate by pinch by pinch until complete dissolving of ammonium sulphate takes place. After, this it was kept overnight precipitation in the refrigerator the pellet was collected and dissolved in10 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 8 cm of dialysis tube was cut and placed in 100 ml of 2%w/v of sodium-bicarbonate 0.1Mm EDTA was added to chelate any metal ion. It was boiled for 10 min and was again washed in boiling diluted water for 10 minutes. The boiling process was repeated with distill 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 500 ml of 50Mm tris Hcl [pH 7.0] solution on a magnetic stirrer in ice cold condition for 8 hours. The buffer was changed frequently for every hour to avoid equllibriation.

Ion exchange chromatography

The chromatography coloumn packed with 2% DEAE cellulose was washed twice using distilled water and kept sonication for 15 minutes. The matrix was activated using activation buffer (25 mM Hcl and 25 mM Nacl).The dialysed enzyme sample was poured into the column followed by a series if elution buffers containing 25 ml of Tris Hcl and increased concentration (50 Mm,75 Mm till the 150mM) of NaCl solution to elite the enzyme. The elutant 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^oC, 37^oC, 55^oC and 80^oC 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 substrate concentration

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^o 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

Maintenance of Pure culture of Bacillus subtilis

In nature, microbial populations do not segregate themselves by species, but exist in a mixture of many other types. In the laboratory these populations can be separated in a pure culture. These cultures contain one type of organism and are suitable for the study of morphological and biological properties.

Biochemical tests for identification of bacteria

Tryptophan is an essential amino acid, is oxidized by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The methyl red (MR) and the voges-proskauer (V-P) test were performed simultaneously because they are physiologically related and are performed on the same medium MR-VP broth.

Preparation of Simmon's citrate agar:

All constituent were dissolved except phosphate which should be dissolved separately in 100 ml of water, and made the volume to 1 litre. pH was adjusted to 6.9. The medium was poured into the tubes and sterilized by autoclaving at 15 lb pressure for 15 minutes and slants were prepared. Simmon's citrate agar slants were inoculated with pure culture by means of a stab-and streak inoculation. A tube without inoculation (Chen and Chao, 2000) was kept as control. They were incubated at 37^0C for 48 hours. Some organisms have the ability to produce the enzyme urease.

Catalase Test

Trypticase soy agar slants was prepared and poured in culture tubes and flasks and sterilized by autoclaving at 15 lb pressure for 15 minutes. Trypticase soy agar slants were inoculated with pure culture and one without inoculation as control (Pais *et al.,* 2006). The slants were incubated at 30^0 C for 24 -48 hours. While holding the inoculated tube at an angle, 3-4 drops of hydrogen peroxide was allowed to flow over the growth of each slant culture.

III. RESULTS AND DISCUSSIONS

Nattokinase supports normal, healthy blood flow (Milner and Makise, 2002) and circulation. Nattokinase is an enzyme extracted from Natto, a cheese-like food made from fermented soybeans (Morikawa, 2006). Nattokinase may contribute to the regular healthy function of the heart and

cardiovascular system by maintaining proper blood flow (Nakamura *et al.*, 1992), thinning the blood and preventing blood clots (Kim *et al.*, 2008). In the present study *Bacillus subtilis* was isolated from soil source and was screened for the production of protease enzyme on skim milk agar media.

Figure – 1: Enzyme activity differences at a) temperature b) substrate concentration c) activator

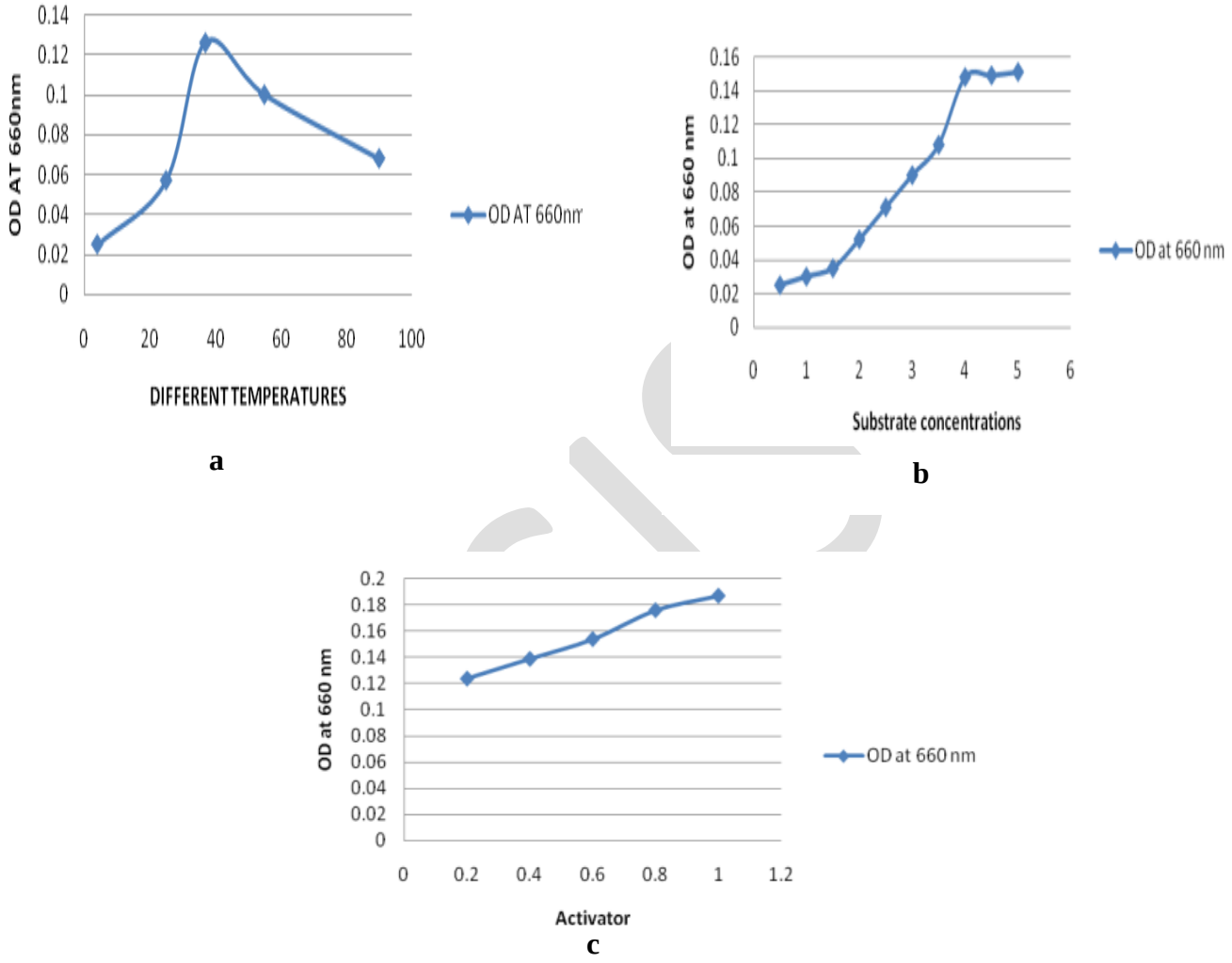

Figure 1 illustrated that effect of enzyme activity at different temperatures, substrate concentrations and activators. The activity of the purified enzyme was determined at different temperatures ranging from 0^{o}C-100^{o}C. The enzyme activity gradually declined at temperature beyond 70^{o}C, but at 40^{0} C enzyme showed maximum activity when compared to the other temperatures, similarly increased enzyme activity were observed by increasing concentration of substrate concentrations and activators and similar results also reported by Sumi *et al.*, (1995). Different nitrogen source like cow fibrin, sheep fibrin, peptone and tryptone were used in the media. The production media containing 0.3% beef extract, 1% milk casein was used along with different nitrogen source as mentioned above. The pH was adjusted to 7.0 with 1 M glacial acetic acid or 1 M NaOH and incubated at 37^{0}C in orbital shaker for 48 hrs at 86 rpm. Using different nitrogen sources like cow fibrin, sheep fibrin, peptone and tryptone enzyme activity was found to be 6, 26, 28.3 and 38 U/ml respectively. The caseinolytic activity of Nattokinase was found to be 636.73 U/ml. Sahelian *et al.*, (1998) also stated that nattokinase activity at different nitrogen sources.

Biochemical testing is powerful tool for the identification of the genus and species of a particular bacterium. Since each different species of bacterium has different genetic code, they are able to synthesize different protein enzymes. These enzymes catalyze all the various chemical reactions of which the organism is capable (Desai, 2009). Therefore the different species of bacteria carry out

different and unique sets of biochemical reactions. The type of biochemical reactions each organism undergoes acts as a thumb print for its identification.

Figure -1: Identification of Nattokinase a) Indole Production Test i) un-inoculated ii) negative iii) positive, b) Methyl Red and Voges-Proskauer test (MRVP) i) positive ii) negative, c) Citrate utilization test i) negative ii) positive d)Urease Test:

The indole test is performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction is detected by adding Kovac's reagent (dimethylaminobenzaldehyde) which produces a cherry-red reagent layer. The methyl red (MR) and the voges-proskauer (V-P) broth opposite result were obtained (Nakano MM, Zuber P 1998) for the methyl red and voges-proskauer tests, i.e., MR+, VP- or MR-, VP+. In these tests, if an organism produces large amount of organic acids: formic, acetic, lactic and succinic (end product) from glucose, the medium will (Juan *et al.*, 2010) remain red (a positive test) after the addition of the methyl red a pH indicator (i.e. pH remaining below 4.4). In other organis (Mc Carty, 2006) ms, methyl red will turn yellow (a negative test) due to the elevation of pH above 6.0 because of the enzymatic conversion of the organic acids (produced during the glucose fermentation) to non-acidic end product such as ethanol and acetoin (acetylmethylcarbinol). The urease is a hydrolytic enzyme which attacks the carbon and nitrogen bond amide compounds (e.g. Urea) with the liberation of ammonia (Wittkowsky *et al.*, 2008). The organisms are grown on urea broth or agar medium containing the pH indicator phenol red (pH 6.8). Microorganisms possessing urease will produce ammonia that raise the pH of the medium. As the pH become higher the phenol red changes from yellow color to a red or deep pink (cerise) color. Catalase test trypticase soy agar slants were examined for presence (positive) or absence of gas bubbles (negative) (Chongqing *et al.*, 2000).

IV. CONCLUSION

The enzyme Nattokinase has powerful antithrombic properties which surpasses even those of urokinase giving it vast therapeutic potential. Hence there is a need for cost effective, good quantity and large scale production of the enzyme. Isolation, production, purification, assay and characterization of nattokinase from bacterial sources is very effective and useful.

In the present study *Bacillus subtilis* was isolated from soil source and screened for the production of nattokinase. Different nitrogen sources like cow fibrin, sheep fibrin, peptone and tryptone were used in the media to achieve maximum production of enzyme using different nitrogen sources as mentioned above, maximum enzyme activity was achieved using tryptone. The enzyme was purified to homogeneity by ammonium sulfate precipitation, dialysis and ion exchange chromatography. DEAE-Cellulose was used for purification of enzyme. The enzyme activity was checked at each and every step of purification. Characterization studies showed optimum pH 9 and the optimum temperature was found to be 37^0C at which enzyme showed higher activity.

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REFERENCES

[1]. Shivasharana CT, Naik GR, 2012. Production of alkaline protease from a thermoalkalophilic *Bacillus* sp.jb-99 under solid state fermentation. Int j pharm bio sci, 3(4): (b) 571 – 587.

[2]. Usharani B, Muthuraj M, 2010. Production and characterization of protease enzyme from *Bacillus laterosporous.* Afr. J. Microbial. Res, 4(11): 1057-1063.
[3]. Iba, T., Saitoh, D., Gando, S. & Thachil, J. 2015. The usefulness of antithrombin activity monitoring during antithrombin supplementation in patients with sepsis-associated disseminated intravascular coagulation. *Thromb Res.* pii:S0049-3848(15)00113-9.
[4]. Ero, M. P., Ng, C. M., Mihailovski, T., Harvey, N. R. & Lewis, B. H. 2013.A pilot study on the serum pharmacokinetics of nattokinase in humans following a single, oral, daily dose. *Altern Ther Health Med*. **19**, 16–19.
[5]. Kim, S. H. Choi, N. S. 2000. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus sp.* strain DJ-4 screened from Doen-Jang. Biosci. Biotechnol. Biochem. 64: 1722-1725.
[6]. Kwon, E. Y. Kim, K. M. Kim, M. K. Lee, I. Y. Kim, B. S. 2011. Production of nattokinase by high cell density fed-batch culture of *Bacillus subtilis*". Bioprocess and Biosystems Engineering, 34(7): 789–793.
[7]. Morikawa, M. 2006. Beneficial Biofilm Formation by Industrial Bacteria *Bacillus subtilis* and Related Species. Journal of Bioscience and Bioengineering, 101(1): 1-8.
[8]. Nakamura, T. Yamagata, Y. Lchishima, E. 1992. Nucleotide sequence of the subtilisin NAT gene, aprN, of *Bacillus subtilis* (natto). Biosci. Biotechnol. Biochem. 56: 1869-1871.
[9]. Sumi H, Nakajima N, Yatagai C. A.1995. Unique strong fibrinolytic enzyme (katsuwokinase) in skipjack. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology; 112(3):543–547.
[10]. Sahelian R, Borken S. 1998. Dehydroepiandrosterone and cardiac arrhythmia. Annals of internal medicine. ; 129(7):588.
[11]. Desai AJ. 2009 Isolation and Characterization of *Halophilic Archaea*: Production, Characterization and Application of Extracellular Protease from Halobacterium sp. SP1 (1).
[12]. Nakano MM, Zuber P. 1998. Anaerobic growth of a strict aerobe"(*Bacillus subtilis*). Annual Reviews in Microbiology ; 129(7):588
[13]. McCarty MF, Block KI. 2006Toward a core nutraceutical program for cancer management. Integrative Cancer Therapies; 5(2):150.
[14]. Wittkowsky AK. 2008. Dietary supplements, herbs and oral anticoagulants: the nature of the evidence. Journal of Thrombosis and Thrombolysis. ;25(1):72–77.
[15]. Chongqing W, Meimei H, Shuguang GUO, Binggen RU. 2000. Isolation and Purification of Liver Metallothioneins of Rabbit. Acta Scicentiarum Naturalum Universitis Pekinesis.
[16]. Kim JY, Gum SN, Paik JK, Lim HH, Kim K, Ogasawara K, et al 2008. Effects of nattokinase on blood pressure: a randomized, controlled trial. Hypertension Research.;31(8):1583–1588.
[17]. Peng Y, Huang Q, Zhang R, Zhang Y. 2003, Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi, a traditional Chinese soybean food. Comparative biochemistry and physiology part b: Biochemistry and Molecular Biology;134(1):45– 52.
[18]. Juan MY, Wu CH, Chou CC. 2010. Fermentation with *Bacillus* sp. as a bioprocess to enhance anthocyanin content, the angiotensin converting enzyme inhibitory effect, and the reducing activity of black soybeans. Food Microbiology.
[19]. Milner M, Makise K. 2002.Natto and its active ingredient nattokinase: A potent and safe thrombolytic agent. Alternative & complementary therapies; 8 (3) : 157–164
[20]. Chen PT, Chao YP. 2006, Enhanced production of recombinant nattokinase in *Bacillus subtilis* by the elimination of limiting factors. Biotechnology Letters; 28(19):1595–1600.
[21]. Hsu RL, Lee KT, Wang JH, Lee LYL, Chen RPY. 2008. Amyloid-degrading ability of nattokinase from *Bacillus subtilis natto.* Journal of agricultural and food chemistry, 57(2): 503–508.
[22]. Pais E, Alexy T, Holsworth J, Meiselman HJ 2006Effects of nattokinase, a pro-fibrinolytic enzyme, on red blood cell aggregation and whole blood viscosity. Clinical hemorheology and microcirculation; 35 (1):139–142.