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ISOLATION, CHARACTERIZATION, PRODUCTION AND PURIFICATION OF FIBRINOLYTIC ENZYME NATTOKINASE FROM *BACILLUS SUBTILIS*

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Keywords:

Bacillus subtilis, Nattokinase, Fermented soybean, Fibrinolytic, Thrombosis

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ABSTRACT: The objective of the present study was characterization, production, and purification of Nattokinase from *Bacillus subtilis*. A fibrinolytic enzyme-producing bacterium was isolated and identified as *Bacillus subtilis* from fermented soybean and soil. *Bacillus subtilis* showed a very strong protease activity by degrading casein skimmed milk agar. Nattokinase was produced on basal medium and then purified from supernatant. The crude enzyme was purified by ammonium sulphate precipitation, followed by dialysis and DEAE cellulose ion-exchange chromatography. Total protein was estimated by Lowry's method (0.58 mg/ml for FS_2 and 0.49 mg/ml for S_1) and enzyme-specific activity was determined by tyrosine standard (0.52 µmole/mg/ml for S_1 and 0.59 µmole/mg/ml for FS_2). The molecular weight of purified enzyme was determined by SDS-PAGE (20-24 kDa for FS_2 and 41-44 kDa for S_1). Finally, the fibrinolytic activity was determined by application of extracted enzyme on pre-existing human blood clots (FS_2 showed greater activity compared to S_1).

INTRODUCTION: Nattokinase, like plasmin, is a potent fibrinolytic enzyme extracted and highly purified from a traditional Japanese food called natto**.** Nattokinase is serine endopeptidases with a molecular weight of 20-28 kDa and a point of ionization (Pi) of 8.6. Natto is a fermented soybean derivative that has been a staple food in the Japanese diet for over 1000 years for its popular taste and as a folk remedy for heart and vascular diseases.

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A fermentation process produces natto by adding *Bacillus* sp. Natto, a beneficial bacteria, boiled soybeans resulting in the production of the nattokinase enzyme.

Nattokinase enhances the body natural ability to fight blood clots and has an advantage over blood thinners because it has a prolonged effect without side effects [1, 2] had long researched thrombolytic enzymes searching for a natural agent that could successfully dissolve thrombus associated with cardiac and cerebral infarction [1] (blood clots associated with heart attacks and stroke). Sumi discovered nattokinase newly discovered enzyme "Nattokinase" which means "enzyme in natto Sumi' commented that nattokinase showed "potency matched by no other enzyme" [1].

Oral administration of the fibrinolytic enzyme nattokinase revealed to be the same as subtilisin NAT and which was produced from *Bacillus* NAT in the traditional Japanese fermented food, Natto, has been reported to enhance fibrinolytic activity in plasma. Intravenous administration of urokinase and streptokinase has been widely used for thrombosis therapy, but these enzymes have a low specificity to fibrin and are expensive [1].

Nattokinase has been developed for the treatment of thrombosis because of its efficiency and stronger affinity to fibrin [2]. Fibrinolytic mechanisms of nattokinase were reported not only to possess plasminogen activator activity [1] but also to directly digest fibrin by limited proteolysis [2, 3]. In addition, nattokinase could cleave plasminogen activator-1 into low molecular weight fragments [1].

Nattokinase has many benefits including its prolonged effects, cost-effectiveness, and its ability to be used preventatively. It is a naturally occurring food-based dietary supplement that has demonstrated stability in the gastrointestinal tract, as well as to changes in pH and temperature [1]. It is definitely a nutritional supplement to consider adding to a cardiovascular health maintenance plan, as well as an anticancerous and antibacterial activity.

Fibrin is protein that when activated forms fibrinogen, which is responsible for blood clotting. Fibrinolytic nattokinase breaks down the fibrin and thrombin besides supporting normal blood pressure and preventing blood clots from forming. It also dissolves existing blood clots and enhances the body's production of plasmin and other clot-dissolving agents, including urokinase.

Many microorganisms are responsible for the production of nattokinase like *Bacillus* sp., *Pseudomonas* sp., *Lactobacillus lactis, Enterobactor sp., Staphylococcus aureus, Cochliobolus lunatus, Fusarium oxysporum, Rhizopus chinensis,* medicinal mushroom (*Cordycepus militaris*), *Streptomyces megasporus, etc*. However, *Bacillus* sp. showed thermophilic, hydrophilic, and strong fibrinolytic activity [1]. Many *Bacilli* such as like *Bacillus subtilis, Bacillus lichenformis, etc.* are able to produce large quantities of fibrinolytic enzymes like nattokinase. Some strains of *E. coli* also produce nattokinase enzyme.

Nattokinase has a wide range of applications in pharmaceutical industry and food industries. Scientists are researching this enzyme and are there is further scope for research to be done on nattokinase. It is seen today that much of our population is suffering from coronary diseases like heart attack, vascular diseases, blood clotting, *etc*. The present study was conducted to isolate, characterize, produce and purify Nattokinase from *Bacillus subtilis* [1].

MATERIALS AND METHODS:

Sample Collection and Isolation of Microorganisms: Soybean sample was collected from Telibag, Lucknow and kept for fermentation for 3-4 days in a jar filled with water. The soil sample was collected from the area nearby Telibag, Lucknow in a sterilize zipper bag and processed immediately. Nattokinase producing micro-organisms were isolated from soil samples using serial dilution method and also from fermented soybean. After fermentation microorganisms were streaked over preformed nutrient agar media Petri plates. The plates were incubated at 37 ºC for 24 h. After incubation for subculture different colonies were selected and streaked over nutrient-agar medium plate. An uninoculated media plate was serving as control. Incubation was done at 37 ºC for 24 h and growth was examined.

Identification of Microorganisms: The identification was done by cultural, morphological and biochemical analysis as per Bergey's manual of Systemic Bacteriology [1, 2].

Screening for Protease Activity (Casein Hydrolysis Test): Casein hydrolysis test was performed on casein agar medium [1, 2].

Production and Extraction of Enzyme: *Bacillus* sp. was grown on basal medium at pH 7.0. For the seed culture, one colony per plate was inoculated into 5ml of basal medium and incubated at 37 ºC in a shaking water bath for 16 h. The seed culture broth (1ml) was then transferred to 1 liter of the basal medium in a Jar fermenter and fermented at 40 ºC at an airflow rate of 1 v/v/min, for 16 h [4].

Extraction of Enzyme: Since the enzyme released is extracellular, centrifugation is the basic technique used to remove all cell debris. Therefore, after fermentation, seed cultures were transferred

into centrifuge tubes and centrifuged at 10,000 rpm for 15 min at 4 ºC. The supernatant fluid was added to 3 volumes of acetone. The mixture of supernatant and acetone was allowed to stand at 4 ºC for 1 day and was used as crude enzyme [4, 9, 10].

Purification of Enzyme:

Ammonium Sulfate Precipitation and Dialysis: About 100 ml of culture filtrate was treated with ammonium sulfate at 10-90% saturation and most of the proteins were precipitated. The precipitate was redissolved in Tris HCl (0.05 M, pH 8.0) buffer. The enzyme was dialyzed three times against the buffer. The enzyme activity was determined in both the precipitated and supernatant. Most of the enzyme activity lies with the precipitated fraction [21].

Purification of Protein by Ion Exchange Chromatography: Preparation of the DEAE cellulose column was done by taking 2.0 g of DEAE cellulose and soaking in D.W. for 1-2 h in a beaker. Soaked materials were mixed with some 0.01N NaOH and left for 30 minutes (repeat the steps twice). Dialyzed enzyme solution was loaded on to a DEAE-cellulose column (2.0 × 2.5 cm) equilibrated with 10mM Tris buffer pH 9.0. The enzyme was eluted with linear gradient of NaCl (5mm, 10mm, 15mm, and 20mm) at flow rate of ml/h. The eluted fractions were assayed for enzyme activity. This partial enzyme solution was used for investigating further processes [22].

Determination of Specific Activity of Enzyme:

Estimation of Protein Concentration using Lowry's Method with BSA (Bovine Serum Albumin): The enzyme assay was done by the method of colorimetry. Bovine serum albumin was taken as standard and the concentration of unknown samples was calculated by taking the O.D at 660nm [1].

Determination of Enzyme Activity (Enzyme Assay): Activity of extracted enzyme was determined by the tyrosine standard after each step of purification of enzyme. According to the procedure, tyrosine was used in different concentrations. The maximum concentration of tyrosine gave maximum OD, and it was continuously decreased according to the tyrosine concentration. According to the procedure determined the OD of own working extracted enzyme from isolates of S1and FS2. The statistical analysis was done and find out correlation relationship between variables of OD and protein concentration. The correlation coefficient was rxy=0.982+ve [1].

Characterization of Purified Enzyme (Fibrin Plate Assay): Plasma was obtained by centrifugation in the presence of EDTA at 2500 rpm for 20 min at 4 ºC. Fibrin was clotted by adding Ca^{2+} in the plasma by the following process *i.e.*, 2 ml of 10% anhydrous $CaCl_2$ for 10 ml of 3.8% sodium citrate, 12.5 ml of 2% EDTA and 16.6 ml of oxalate. Serum was collected by centrifugation at 15000 rpm for 20 min at 4 ºC add 25 ml phosphate buffer pH 7.4 and 1% agarose gel [8]. The fibrinolytic activity of nattokinase was determined by using standard bovine fibrin plate. For each sample 0.01 cm was loaded into agar wells and incubated at 37 ºC for 16 h. A commercially produced nattokinase, 'Nattokinase Fungi 60,000' was used as standard (0.1-200 U cm3) for fibrinolytic activity [2, 5].

Determination of Molecular Weight of Nattokinase using SDS-PAGE: Separating gel mixture was prepared to determine the molecular weight. The gel solution was poured into chamber between the glass plates and kept for 30-60 min. The stacking gel mixture was prepared and poured then comb was place into the stacking gel. After the stacking gel has polymerized the comb was removed without disturbing the shapes of wells. The gel was installed into the electrophoresis apparatus. The tank was filled with electrode buffer. The sample was prepared for electrophoresis. The sample solution micropipette and carefully poured into wells through the electrode buffer. D.C. current was applied and allowed the electrophoresis unit to run for about 3 h. The gel was transformed into a suitable container with at least 100 ml of distaining solution and shacked gently continuously. The unbound dye was removed and observed the gel bands [2].

Determination of Fibrinolytic Activity: The enzyme nattokinase was believed to possess fibrinolytic properties *i.e.* it functions as 'clot busters' when it was applied on clotted human blood, the enzyme successfully dissolved the thrombus [1].

RESULTS AND DISCUSSION: Screening for Protease Activity: Nattokinase enzyme is a type of protease enzyme isolated from *Bacillus subtilis*. *Bacillus subtilis* has the ability to degrade the casein by producing proteolytic enzyme protease (nattokinase).

Casein hydrolysis was done by these isolated organisms and gave positive results with S1, FS2, and FS3 by formation of a cleared zone adjacent to the bacterial growth **Plate 7, 8** and **9**.

The result was comparable with an earlier report of [21, 14] and [21] by uses of *Bacillus subtilis* (**Table 1**; **Plate 1, 2, 3, 4, 5** and **6**).

TABLE 1: SCREENING THE PROTEASE ACTIVITY

S. no.	Isolates	Protease Activity
1	S_1	+
2	S_5	-
3	FS_2	+
4	FS_3	+

S = isolates from soil, FS = isolate from fermented soybean

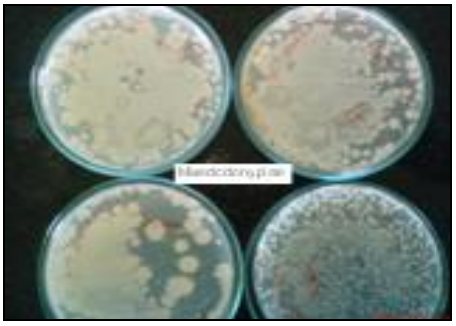

Plate 1: Mixed colonies

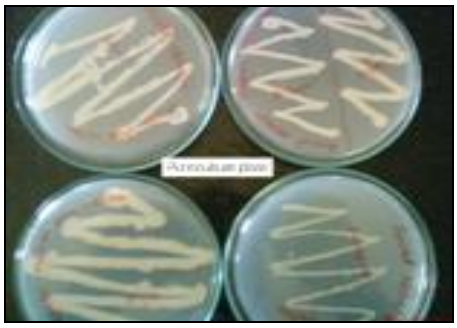

Plate 2: Pure culture

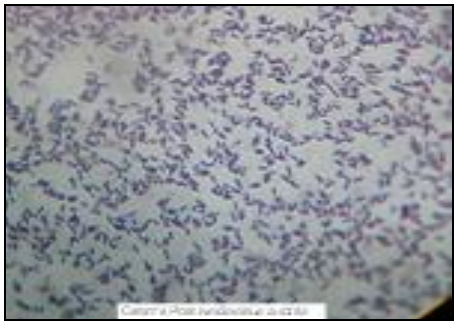

Plate 3: staining of S_1 (100X)

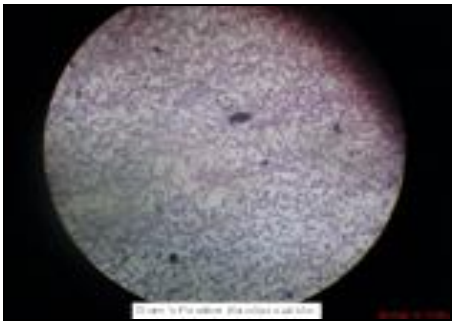

Plate 4: Staining of FS_2 (100X)

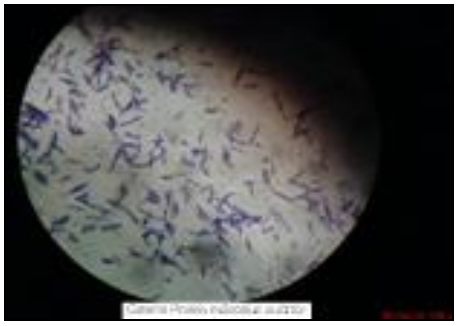

Plate 5: Staining of FS_3 (40X)

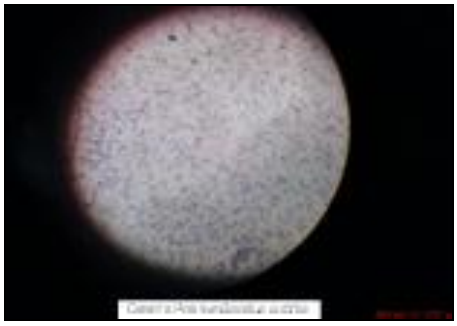

Plate 6: Staining of S_5 (40X)

Casein Hydrolysis Test:

Plate 7: (FS_2)

Plate 8: test (FS_3)

Plate 9: test (S_1)

Purification of Nattokinase from the Culture Supernatant of *Bacillus subtilis*: The extracellular nattokinase of Bacillus subtilis was produced. The culture supernatant was obtained by centrifugation at 10,000 rpm for 15 min. Ammonium sulfate was added into the culture supernatant and the precipitate obtained at (55 to 60%) of S1 and (70 to 75%) of FS2. The supernatant was collected; the precipitate was dissolved in potassium phosphate buffer [22]. It was dialyzed under vacuum (cut off, 20 kDa) dialyzed against the same buffer overnight. The dialyzed material was then applied to carboxyl methylcellulose (CMC) column (2.0×2.5 cm) with the same buffer. The unbound proteins were eluted

with the same buffer and 5 ml fractions were collected [23, 24]. The partially purified enzyme was further purified by DEAE cellulose column chromatography. Generally, nattokinase is the positively charged and it is not bound to anion exchangers, therefore, cation exchangers are the methods of choice. The matrices for IEC containing ionizable functional groups such as diethyl aminoethyl (DEAE) get associated with changed protein molecule thereby absorbing the protein to the matrices. The series as purification steps were very effective and yielded overall purification. The protein estimation (mg/ml) was done following the method of Lowry's [17, 25] enzyme activity (µmole/min) was determined following the method of Charles *et al.,* (2008) [26]. Therefore, through protein estimation and enzyme activity we were determined the specific activity (µmole/mg/ml) based on previously published reports [27, 28].

Estimation of Protein Concentration using Lowry's Method:

BSA (Bovine Serum Albumin) Standard: BSA used as a substrate. The concentration of purified enzyme (protein estimation) was estimated by BSA standard after each step of purification. According to procedure, BSA was used in different concentration, and OD was taken at 660 nm. Maximum concentration gave maximum OD means products formed, protein concentration µg/ml **Fig. 1** showed the standard by use of BSA.

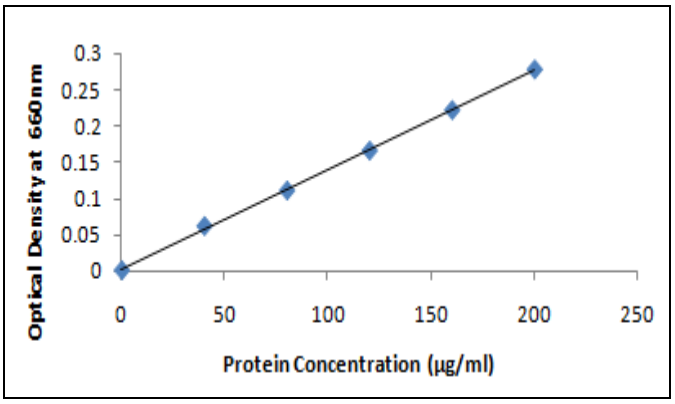

FIG. 1: PROTEIN ESTIMATION WITH BSA STANDARD

The standard graph was compared with present findings enzyme and estimated the concentration of protein. BSA concentration increased the OD and protein concentration and results were found correlation coefficient $r_{xy}=0.982^{+ve}$ Fisher and Yates, (1968) [18]. By the Lowry's method determined the concentration of protein of the extracted enzyme. Compared BSA found standard total protein in crude enzyme (492.21 µg/ml of S_1) and (583.23 µg/ml of FS_2). With the help of this protein, estimation we were determined the specific activity, **Fig. 2, 3** purity and recovery of extracted enzyme Lowry's [17].

Estimation of Protein with Working Enzyme from FS_1

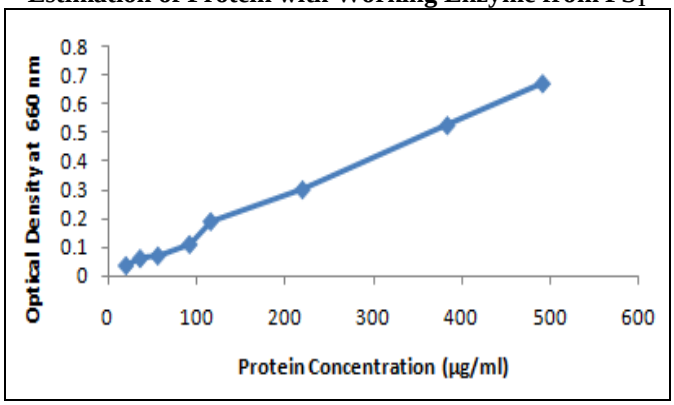

FIG. 2: PROTEIN ESTIMATION WITH ENZYME FROM S_1

Estimation of Protein with Working Enzyme from FS_2

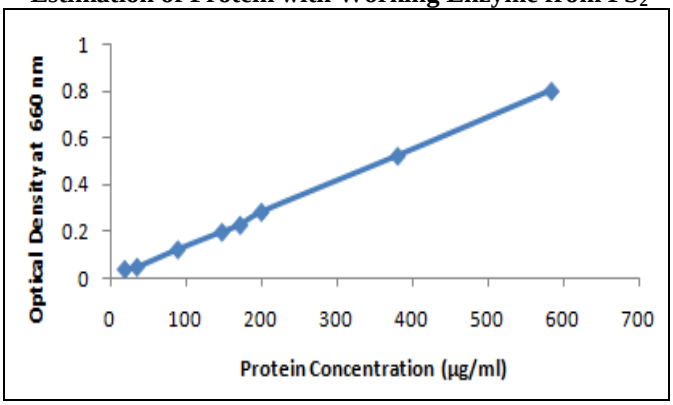

FIG. 3: PROTEIN ESTIMATION WITH ENZYME FROM FS_2

Determination of Enzyme Activity (Enzyme Assay):

Tyrosine Standard for Enzyme Assay (Tyrosine used as Substrate): Activity of extracted enzyme was determined by the tyrosine standard after each step of purification of the enzyme. According to the procedure, tyrosine was used in different concentrations. The maximum concentration of tyrosine gave maximum OD which continuously decreased according to the tyrosine concentration **Fig. 4-6**. According to the procedure the OD of extracted enzyme from isolates of S1and FS2was determined. The statistical analysis was done to find out the correlation relationship between variables of OD and protein concentration. The correlation coefficient was found as rxy=0.982+ve

Fisher and Yates, (1968) [18]. By the analysis of tyrosine standard graph and comparison of own extracted enzyme, the activity of enzyme was found to be maximum in cell-free supernatant crude, and these activities decreased during purification steps. The enzyme activity of extracted enzyme was (55.51 µg/ml of FS2) and (53.49 µg/ml of S1). By the help of these activities determined the specific activity, purity, and recovery of enzyme [29, 11].

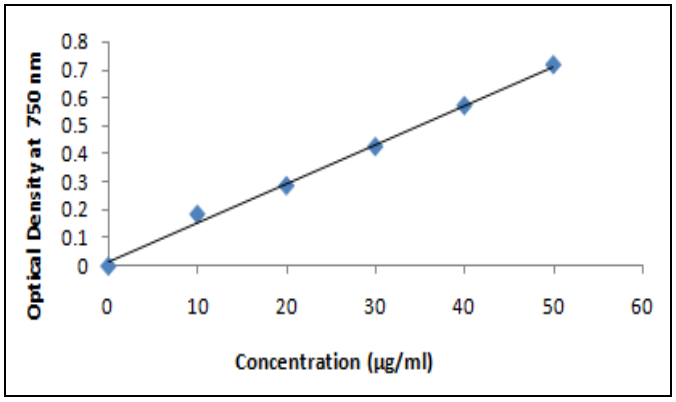

FIG. 4: ENZYME ASSAY WITH TYROSINE STANDARD

Enzyme Assay with Working Enzyme from FS_1

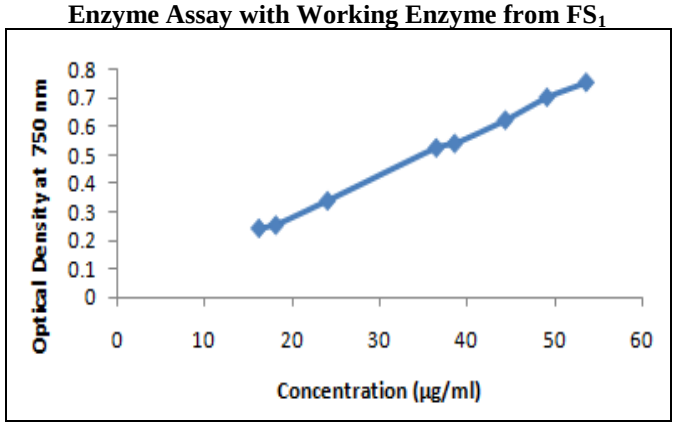

FIG. 5: ENZYME ASSAY WITH ENZYME FROM S_1

Enzyme Assay with Working Enzyme from FS_1

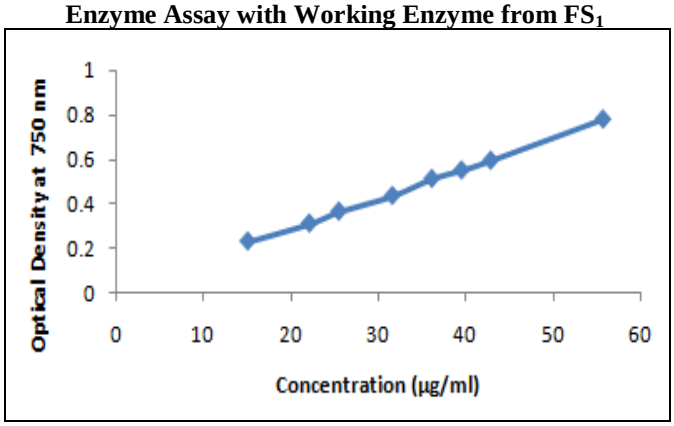

FIG. 6: ENZYME ASSAY WITH ENZYME FROM FS_2

Determination of Specific Activity: The determination of the specific activity of enzyme is dependent on the values of enzyme activity and protein concentration. The results are mentioned in **Fig. 7-11**. The maximum protein concentration found was (0.58 mg/ml of FS2), enzyme activity (0.30 µmole/min of FS2), specific activity (0.59 µmole/mg/ml in S1). The recovery of partial purified enzyme by FS2 (82.21%) and by S1 (93.10%) respectively. These characteristics of enzyme were compared with earlier reports of [4, 28, 30, 31] by the using Bacillus subtilis isolated from fermented soybean samples.

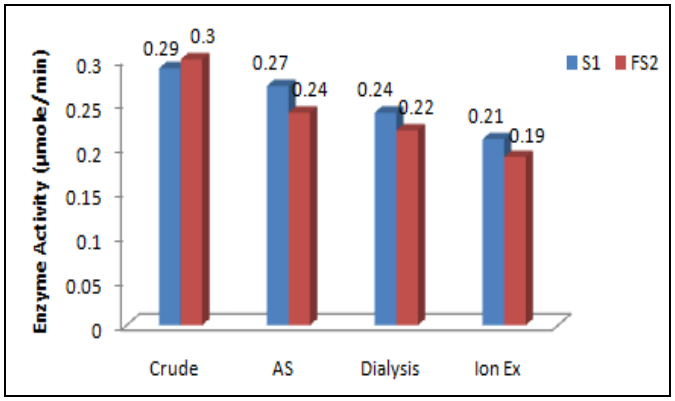

FIG. 7: ENZYME ACTIVITIES AT DIFFERENT PURIFICATION STEPS

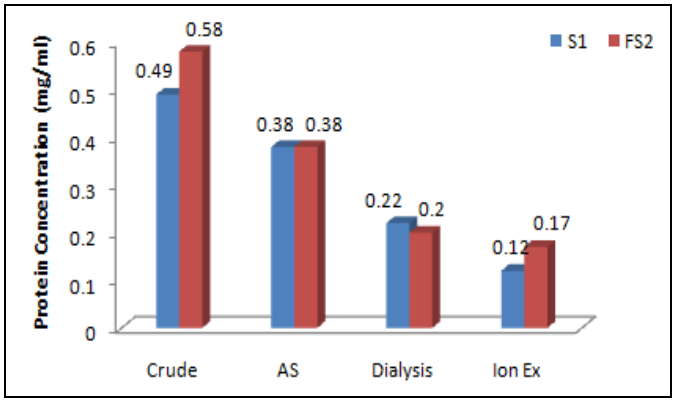

FIG. 8: PROTEIN CONCENTRATIONS AT DIFFERENT PURIFICATION STEPS

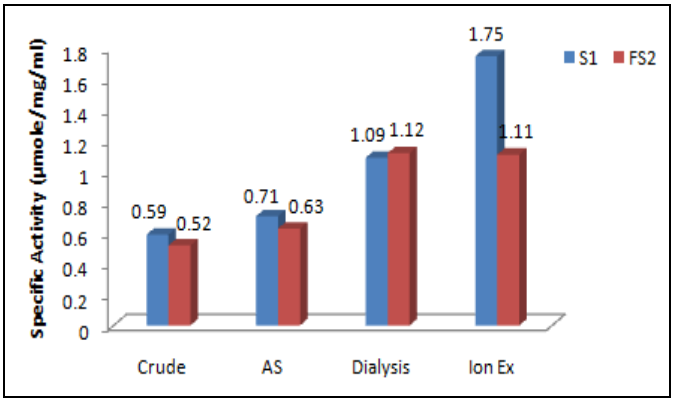

FIG. 9: SPECIFIC ACTIVITY OF ENZYME AT DIFFERENT PURIFICATION STEPS

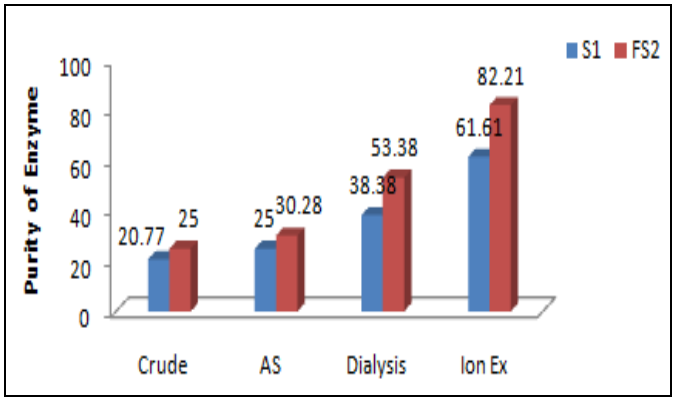

FIG. 10: PURITY OF ENZYME AT DIFFERENT PURIFICATION STEPS

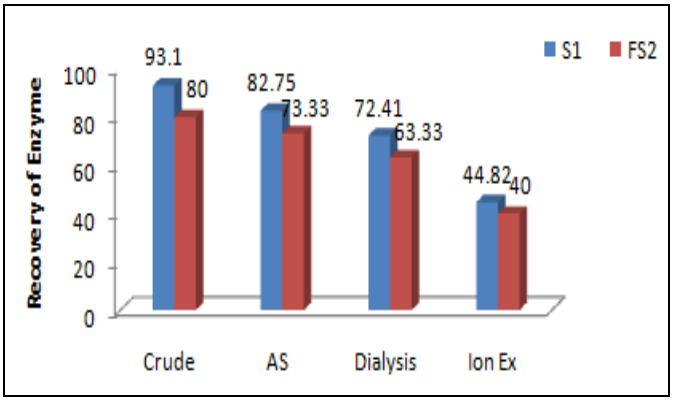

FIG. 11: RECOVERY OF ENZYME AT DIFFERENT PURIFICATION STEPS

Characterization of Purified Enzyme (Fibrin Plate Assay): Enzyme loaded into well no. 1-4 from extracted enzyme FS2 and loaded into well no. 5-8 from S1 on a fibrin agar plate and found following results in zone formation by degrading fibrin [7]. Nattokinase is a fibrinolytic enzyme and this was confirmed when loaded in well on fibrin agar plate. After incubation the extracted enzyme degraded the fibrin protein and formed the clear zone on plate **Plate 10**. The extracted enzyme from fermented soybean has good characteristics to perform fibrinolysis. These enzymes degrade fibrin protein homogenously and form zones. The present finding results were comparable with earlier reports of [5, 4, 32, 33] by all users of *Bacillus subtilis*.

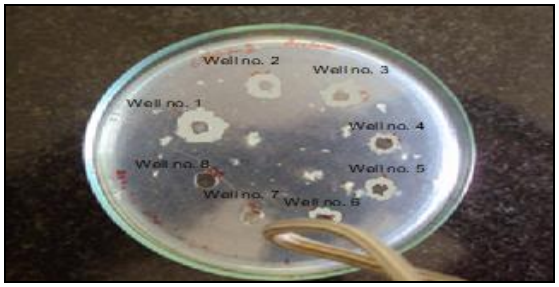

PLATE 10: DETERMINATION OF MOLECULAR WEIGHT OF NATTOKINASE USING SDS-PAGE

Determination of Molecular Weight of Nattokinase using SDS-PAGE: The enzyme sample obtained after each purification steps from the S_1 and FS_2 was subjected to SDS-PAGE to determine the molecular weight by the running them with a marker. Molecular weight was determined by observing the protein bands [20]. A mixture of egg albumin and 1% BSA was used as marker that gave protein bands, results are tabulated in following **Plate 11**. The molecular weight of purified nattokinase was calculated based on semilorgarthamic plots of the mobility of the bands on SDS-PAGE, using standard curve established with protein of known molecular weight. The molecular weight of the purified nattokinase was determined to be 20-24 kDa of enzyme from FS_2 and 41-44 kDa of enzyme from S_1. These results indicated that nattokinase has monomeric structure. The molecular weight of nattokinase was generally 20- 28 kDa. Therefore, it was indicated extracted enzyme was a type of fibrinolytic enzyme and molecular weight have been reported produced from *Bacillus* sp. Strain CK 11-4, 25 k[4] *Bacillus subtilis* 44 kDa[29]; *Bacillus*

subtilis, 27.7 kDa [20]; *Bacillus megaterium* (27 kDa) and *Bacillus* sp. 24.5 kDa.

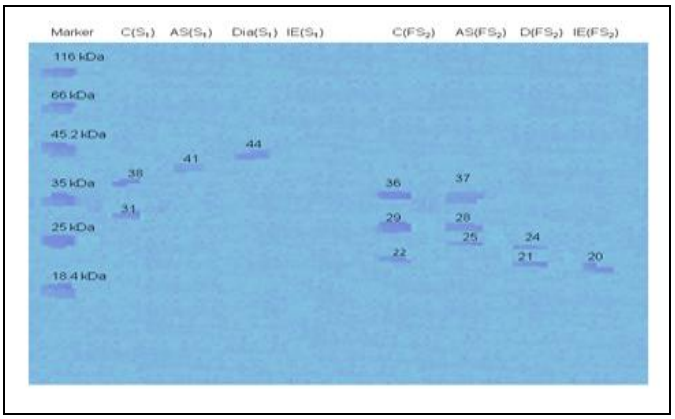

PLATE 11: SDS – PAGE

Fibrinolytic Activity: The targeted enzyme nattokinase, was believed to possess fibrinolytic properties; it functions as 'clot busters'. When applied to pre-existing clotted human blood, the enzyme successfully dissolved the thrombus. The extracted enzyme from FS2 dissolved the thrombus completely than extracted enzyme of S1 **Plate 12**. Nattokinase has property to degrade the blood clots by degrading fibrin [3]. Therefore, extracted enzyme was identified and characterized as a type of nattokinase enzyme and results were comparable with reports of [4, 22, 33].

PLATE 12: DISSOLVED THROMBUS (BLOOD CLOT)

CONCLUSION: The results showed that *Bacillus subtilis* was protease producing. The enzyme was produced under optimum production parameters and showed the fibrinolytic activity. The nattokinase enhances the body's natural ability to fight blood clots and has an advantage over blood thinners. It also helps and enhances the production of plasmin and urokinase. The nattokinase has a wide range of applications in medical research, pharmaceutical and food industries. Future prospects of the study include the characterization of the enzyme, production parameters and determination of fibrinolytic activity, specific activity and evaluation of its industrial applications.

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CONFLICTS OF INTEREST: Nil

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