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Strain improvement, production and stability of Nattokinase from UV mutant strain of *Pseudomonas aeruginosa* CMSS

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Abstract : Nattokinase (NK) is a potent fibrinolytic enzyme , which belongs to the second large family of serine proteases. NK gain significant attention in the treatment of hypertension and cardiovascular disorders (CVD's). Thus, a number of NK producers have been extensively studied, especially *Pseudomonas* sp. has extended the production level than *Bacillus* sp. with different properties. Thus the current study precedes with the strain improvement of NK producing UV mutated *Pseudomonas aeruginosa* CMSS by chemical mutagenesis. The potent mutant strain UV-EMS, treated with Ethyl Methyl Sulphonate (EMS) showed maximum NK activity. The maximum production of NK from mutant strain was determined at optimized parameters like pH 5 (1315.8 U mL^{-1}), temperature at 37°C (2413.3 U mL^{-1}), shrimp shell as nitrogen source (2355.0 U mL^{-1}) and sucrose as carbon source (3930.0 U mL^{-1}).The activity of partially purified NK was stable at pH 7, temperature at 10°C and 5mM of $MgCl_2$. The stability of NK was partially inhibited by SDS and completely inhibited by EDTA. The partially purified NK showed74% of *in vitro* clot lysis activity.
Keywords : Nattokinase, *Pseudomonas aeruginosa*, chemical mutagenesis, optimization , stability, clot lysis.

Introduction

In the present decades, the formation of blood clot in the vascular system leading to cardiovascular disorders (CVD's) and stroke. The use of thrombolytic therapy has become conventional in the treatment of major thrombotic disorders [1]. Among the thrombolytic drugs, Nattokinase (NK) proves as one of the traditional folk medicine in Japan for over 1000 years [2].Nattokinase is particularly potent treatment, and it enhances the body's natural ability to fight blood clots in several different ways; because it so closely resembles plasmin, it dissolves fibrin directly. In addition, it also enhances the body's production of both plasmin and other clot-dissolving agents, including urokinase (endogenous) [3]. A study reports that NK benefits the circulatory system through two separate mechanisms: first, through directly breaking down blood clots, and second, by converting precursors of fibrin into inactive forms. This is a significant benefit, because fibrin can be a major risk for

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developing blood clots and arterial blockages. Compounds in natto have also been identified as angiotensin-converting enzyme inhibitors, which lower blood pressure [4]. Therefore, NK has become a oral medicine as dietary supplements.The importance of NK has regained in the treatment of Alzheimer's disease and vitreoretinal disorders. Recent studies have reported the production of NK from *Bacillus* spp. and *Pseudomonas* spp.[3,5]. Therefore the enhancement of strain using simple mutation techniques and media optimization helps to fulfil the needs of large scale production for commercial use. Hence the present study focuses on the improvement of UV mutated strain *Pseudomonas aeruginosa* CMSSUV60 by chemical mutagenesis[6]. The production of NK was optimized with physical and chemical factors as well as the maximum activity of partially purified NK was analysed under stable conditions.

Material and methods

Strain and culture conditions

The UV mutant strain of*Pseudomonas aeruginosa* CMSS (Gen Bank accession number: JX112657)previously isolated from bovine milk samples was maintained in the nutrient broth and cetrimide agar slants[6].

Strain improvement

The potent mutant strain UV60 of *Pseudomonas aeruginosa* CMSS was subjected to chemical mutagenesis for the strain improvement. The chemical mutagens namely ethyl methyl sulphonate (EMS), N-methyl- N'-nitro- N-nitroso guanidine (NTG), ethidium bromide (EtBr) and sodium azide (NaN_3) were used in the concentration of 5mM. The overnight fresh culture broth of 800µL was incubated with 200 µL of the chemical mutagens separately for 30 min at 37°C. After centriguation of the culture , the pellet was washed thrice with 0.1M phosphate buffer (pH 7.5) and spread plated on nutrient agar , incubated at 37°C for 24 h. The mutants were randomly selected and maintained in the nutrient broth.

Screening of proteolytic activity-radial caseinolytic assay

The mutant strain was screened by radial caseinolytic assay. The supernatant of the mutant strains were added to the wells punched in the nutrient media was supplemented with 2% casein and incubated for 4 h at 10 ^{o}C followed by 24 h at 37^{o}C.

Optimization and production of NK

The production of NK from the selected mutant isolate was enhanced by classical way of optimization. The parameters such as pH, temperature, carbon and nitrogen sources plays a major role in the production of desired enzyme of interest. The optimum pH for the NK activity was studied by varying the pH levels, from 5.0 to 9.0. Similarly, the optimum temperature for the NK activity was estimated by studying the enzyme activity at different temperatures, from 30–70°C. The carbon sources such as glucose, sucrose, glycerol and maltose were selected for the NK activity . The optimization of nitrogen sources such as nitrogen shrimp shell, casein, yeast extract and tryptose shows a positive effect on the maximum NK activity. The potent mutant strain was incolutated in the optimized production medium at 37°C for 24 h, 120 *rpm*. The supernatant of the optimized medium was analysed for NK activity and protein content.

Partial Purification of NK

The ammonium sulphate precipitation method was followed for the partial purification of the enzyme. The procedure was analysed with 20%, 30%, 40%, 50%, 60%, 70% and 80% of ammonium sulphate for the culture filtrate from the potent mutant strain. Dialysis was done extensively until the protein concentrate was obtained [7].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out to determine molecular mass and the purity of the partially purified enzyme, using 4% acrylamide in the stacking gel and

12.5% acrylamide in the separating gel. Protein bands were visualized by staining with Coomassie R 250 brilliant blue with protein marker ranging from 14.3kDa – 97.2kDa [8].

Enzyme stability studies

Effect of pH and temperature

The effect of pH on NK activity was assayed at 37ºC at various pH (4.0–8.0) by incubating the enzyme solution for 30 min at 37ºC with different buffers, and then determined the residual activity by casein hydrolysis . The stability of NK was determined at different temperature range of 10ºC to 50ºC using the standard activity assay procedure at related temperature. The residual activity of NK after incubating the enzyme solution at 10ºC -50ºC for 30 min in 50 mM sodium phosphate buffer (pH 7.0) and then the enzyme activity was checked.

Effect of metal ions, surfactants and NK inhibitors on enzymatic activity

The effects of metal ions were investigated using 5mM of Mg^{2+},Ca^{2+},Zn^{2+},Mn^{2+} and Fe^{2+}. The effects of NK inhibitors were studied using phenyl methyl sulfonyl fluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA). The effects of surfactants were studied using 0.1% of the surfactants Tween-20 and 1 mM SDS. Enzyme was pre-incubated with metal ions, surfactants or inhibitors in 50mMphosphate buffer (pH 7) for 30 min at 25ºC then the residual NK activity was determined.

Casein plasminogen agarose assay

The NK activity was measured by the addition of 50µL of culture supernatant in the punched wells (1.5mm) on the casein plasminogen overlay agarose medium which was incubated at 37˚C for 12 h. The maximum activity was determined by the zone of hydrolysis around the punched wells [9].

***In vitro* clot lysis activity**

The fresh blood was purchased from Blood bank (Vellore). The clot lysis activity with 50 µL of crude enzyme , ammounium sulphate precipitate (40-60%) and dialysed enzyme along with positive and negative control was assayed in microcentrifuge tubes. Streptokinae from β.hemolytic *Streptococci* (Sigma), 10,000 KU was used as positive control and distilled water as negative control in the assay.The difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis [10].

Casein digestion assay

NK activity was determined by using casein digestion method. One unit of caseinolytic activity (U) was defined as the amount of enzyme releasing 1µM of tyrosine equivalent min^{-1}[11].

Determination of protein

The quantity of protein content was determined by Lowry's method [12].

Results and Discussion

Screening of NK producing mutant strain

The use of chemical mutagenesis on UV60 mutant strain of *P. aeruginosa* CMSS revealed the enhancement of NK activity. The growth of EMS treated strain expressed abundant growth than NTG. The growth of the strain was reduced drastically with treatment of EtBr and NaN_3. The colony morphology of the EMS mutant strain was found to be mucoid, round , smooth, convex and round which differed from the UV60 mutant strain of *P. aeruginosa* CMSS (Fig.1).

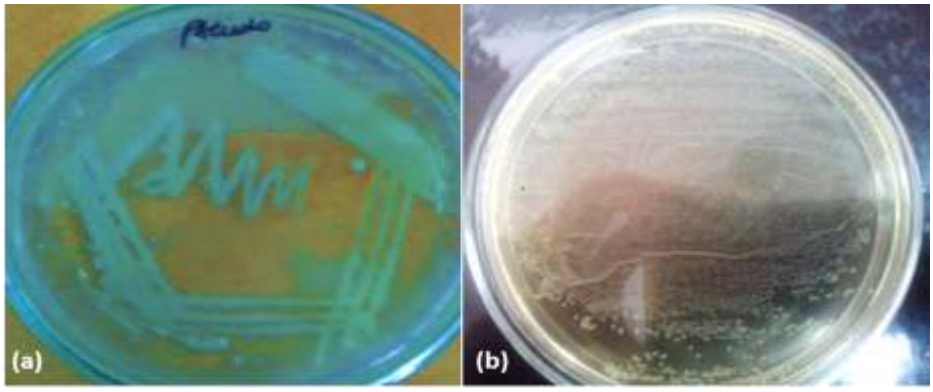

Fig.1 (a) UV60 mutant strain of *P. aeruginosa* CMSS (b) EMS treated UV60 mutant strain

The mutant strains was screened by radial caseinolytic assay. Among the chemical mutagens, EMS treated mutant strain showed maximum zone of hydrolysis when compared with the treatment of NTG. While, treatment with EtBr, expressed minimal NK activity with minimum zone of hydrolysis. The activity of NK was suppressed completely with the treatment of NaN_3.(Fig.2). Therefore, EMS treated UV 60 mutant strain of *P. aeruginosa* CMSS was used for the production of NK.

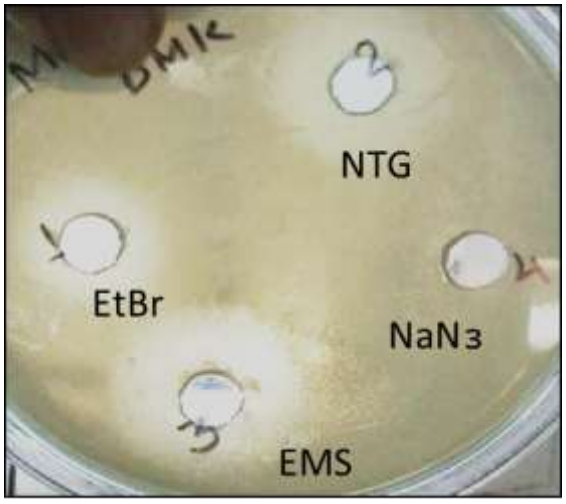

Fig.2 Radial caseinolytic assay for the chemical mutagen treated culture broth

Optimization Studies

The enhanced production of desired enzyme was followed by optimization of the process parameters . Primarily, pH and temperature affect various biological processes and play important roles in the optimization process. The optimum NK activity from the UV60-EMS mutant strain was observed at pH 5 (1315.8 U mL^{-1} and 0.177 mg mL^{-1}) and 37˚C (2413.3 U mL^{-1} and 0.106 mg mL^{-1}) (Fig.3 a & b). Based on the results, the optimum nitrogen source for the NK productivity from the UV60-EMS strain was found to be 1% shrimp shell, in the presence of which maximum NK activity of 2355 U mL^{-1} was observed. The miminum NK activity was expressed by the medium supplemented with 1% tryptose (1521.7 U mL^{-1}) (Fig.3 c). Furthermore, *P. aeruginosa* CMSS grown on 1% sucrose supplemented medium, showed maximum NK activity (3930 U mL^{-1}) than the strain grown in medium with other carbon source (Fig.3 d). The results of the present study are supported by existing reports on enhanced production of NK by UV-mutated *Bacillus* spp., which significantly demonstrated better activity compared to the wild type strain[13]. The previous studies on NK production after optimization reported that the maximum NK activity from *Bacillus* sp. was found in the medium supplemented with mannitol as carbon and soya peptone as nitrogen source [14].

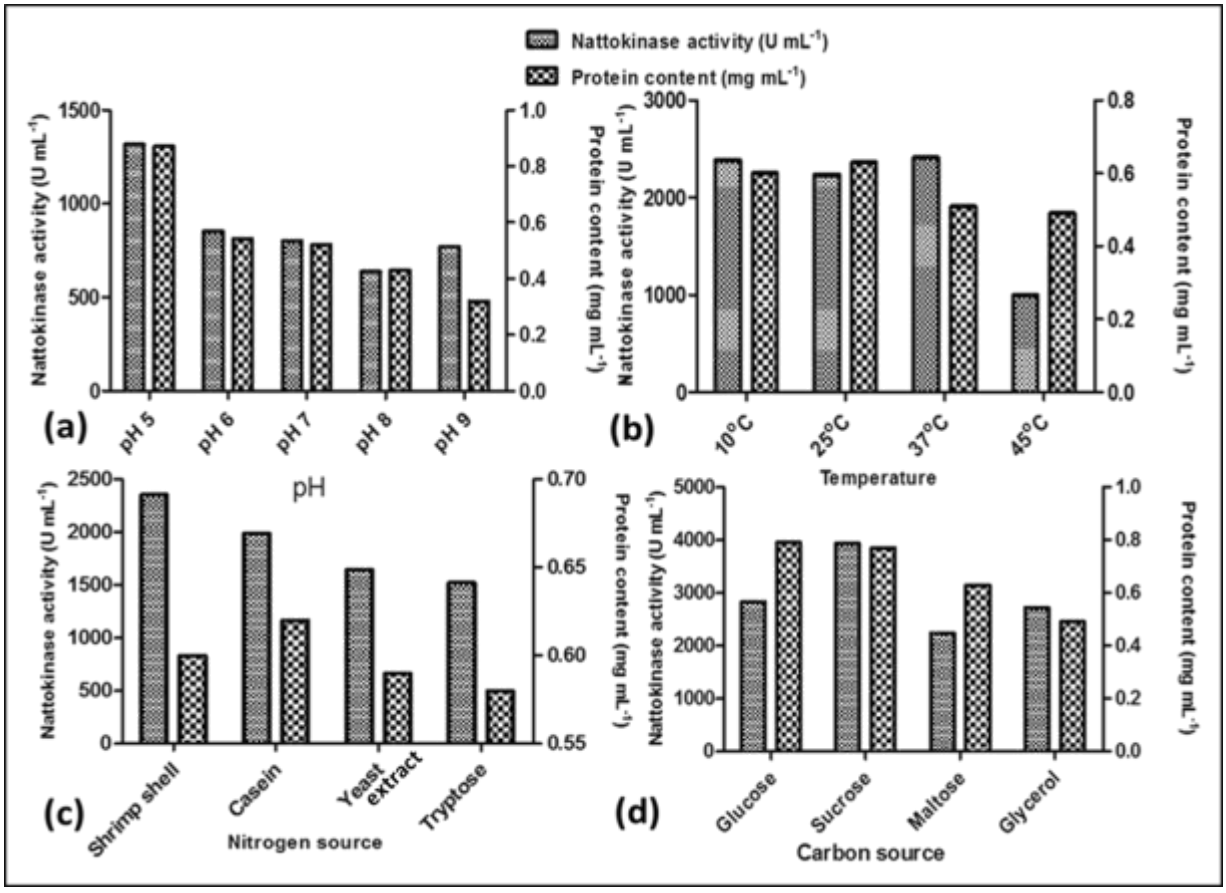

Fig.3 Media optimization of process parameters (a) pH (b) Temperature (c) Nitrogen source (d) Carbon source

Partial purification of NK

The crude culture filtrate was purified partially with ammonium sulphate precipitation method. In ammonium sulphate precipitation method, the fibrinolytic enzyme activity of UV-EMS mutant strain was 2946.6 U mL^{-1} and 3030 U mL^{-1} at at 50% and 60% saturation of ammonium sulphate. In the ammonium sulphate precipitate, the protein content was 0.62 mg mL^{-1} in case of 50% and in 60% precipitate, it was found to be 0.76 mg mL^{-1}. (Fig.4). The maximum zone of hydrolysis was determined at 60% protein precipitate in casein plasminogen overlay assay (Fig.5 a & b).

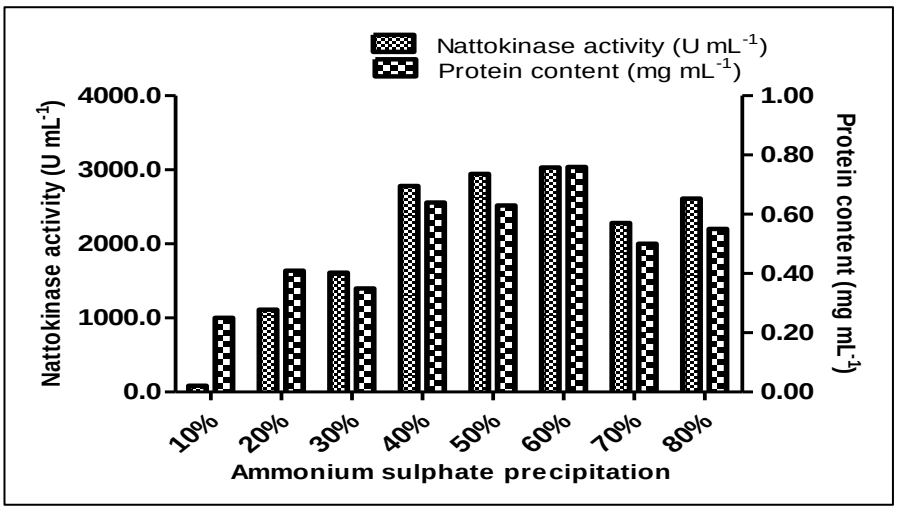

Fig.4NK activity and protein content of the ammonium sulphate precipitates

Fig.5 Caseinolytic agar plate activity. Figure (a) and (b) showing the zone of clearance at different ammonium sulphate salt concentration. Figure (b) shows that the maximum NK activity at protein precipitate from 60% saturation.

Effect of pH and temperature

The effect of pH on enzyme activity was determined over a pH range of 4.0 to 9.0. The NK was highly active in the pH range of 6.0 – 8.0 with an optimum at pH 7.0. The enzyme activity was retained and 80.75% (2446.7 U mL $^{-1}$) and 86.25% (2613.3 U mL $^{-1}$) of its original activity at pH 6.0and 7.0,respectively (Fig 6a). The stability profile of NK showed that the enzyme is stable at 10°C. The enzyme retained more than 86.3% of its initial activity after 60 min incubation at 10°C, respectively. However, the enzyme retained only 64% activity after 60 min incubation at 20 °C (Fig.6 b).

Effect of metal ions, surfanctants and inhibitors

The characterization of partially purified nattokinase from UV-EMS mutant strain of*P.aeruginosa* CMSS, examined the effects of some known enzyme inhibitors and divalent metals on their activities. The nattokinase activity increased significantly at the presence of 5 mM , Mg^{2+} (2196.7 U mL $^{-1}$), but the NK activity decreased in Zn^{2+} (Fig 6 c). The enzyme activity was highly influenced by Tween 20 as surfactant (1196.7 U mL $^{-1}$) .The significant activity was decreased by SDS (Fig 6 d). The nattokinase activities were not affected by most metal ions but the NK was completely inactivated at the presence of 5 mM PMSF (280.0U mL $^{-1}$) . EDTA, a chelator of divalent cations, had no significant effect on the activity of the purified nattokinase (113.3U mL $^{-1}$) (Fig 6 d). However, inhibitor of serine protease (PMSF) had significant effect on the activity of the purified nattokinase, indicating that nattokinase were serine protease.

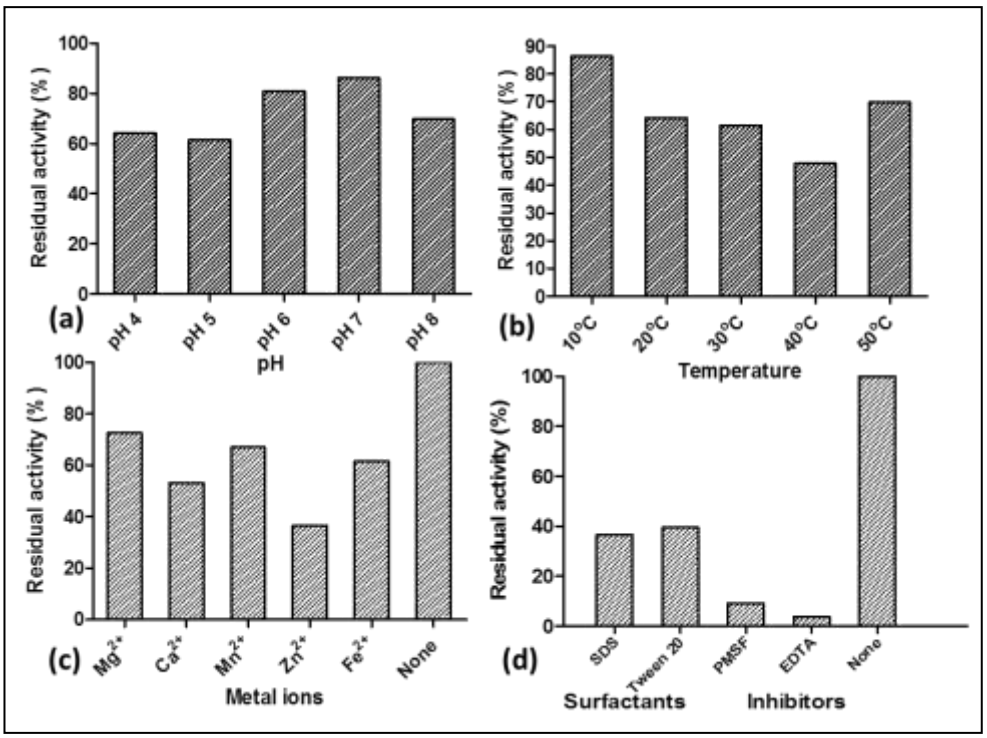

Fig.6 Effect of stable conditions on NK activity (a) pH (b) Temperature (c) Metal ions (d) Surfactants & Inhibitors

***In vitro* clot lysis activity of NK**

The percentage of clot lysis of crude (30.89%) and partially purified nattokinase (74.5%) was significant when compared with positive control (87.14%) (Streptokinase). Maximum blood clot lysis was observed with partially purified NK (74.5%) with in 30 min (Fig 7a& b). Thereby, it can be concluded from the results of exposure of UV mutant strain to chemical mutation was the most effective in producing favorable mutation that enhanced NK production with a higher activity.

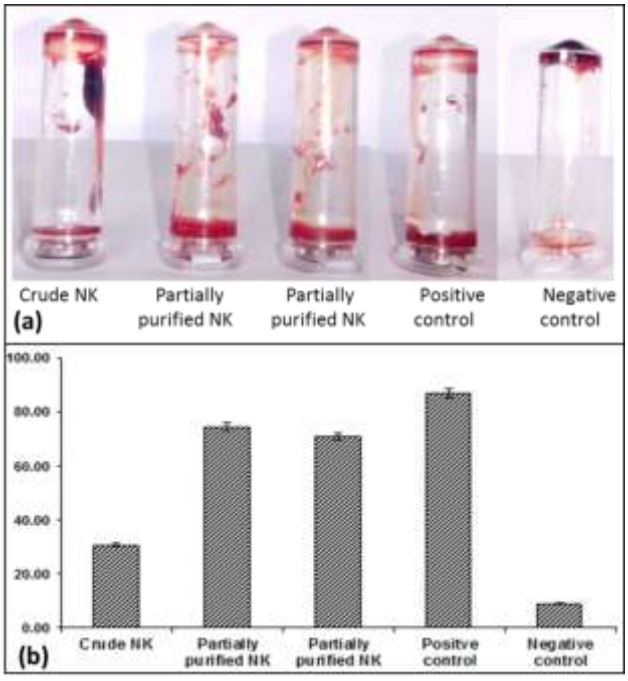

Fig.7 NK activity- (a) *In vitro* blood clot lysis assay (b) Percentage of clot lysis

SDS-PAGE

The molecular weight of the partially purified NK from UV-EMS of *P.aeruginosa* was found to be 27 KDa, which was comparable by the banding patterns along with the protein marker (Fig.8). This confirms the NK produced by UV-EMS of *P.aeruginosa*CMSS. Many reports supports the current study with microbial NK that had similar molecular weight as *P. aeruginosa* CMSS NK, included the fibrinolytic alkaline protease of *Fusarium sp*. BLB (27 kDa), the metalloprotease of *C. militaris*, the serine protease of *Bacillus amyloliquefaciens* DC-4 (28 kDa) [15,16].

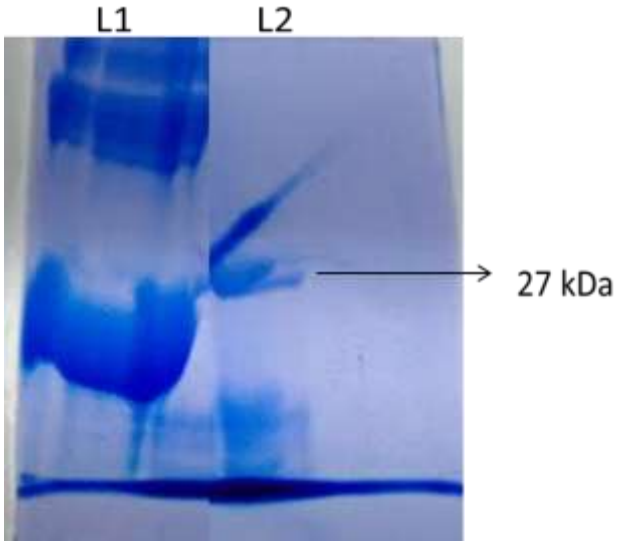

Fig.8 SDS-PAGE : L1 –protein marker L2 – partially purified NK

Conclusion

In the present scenario, enzymes play a very important role as active ingredients in various applications in the fields of food, detergent, medicines, etc. Nattokinase is one of the fibrinolytic enzymes which possess applications in pharmaceutical and food industry.The results from the present study paves way for the improved activity of the fibrinolytic enzyme NK by chemical mutagenesis and media optimization. The future studies focused on the isolation of NK gene the and to study the molecular structure such as amino acid sequence as well as secondary and tertiary structures.

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References

1. Devi CS, Sinha D, Sharma V, Mohanasrinivasan V. Screening for staphylokinase producing *Staphylococcus* spp. from different environmental samples. Asian J Pharm Clin Res., 2012,5(4):125–28.
2. Debajit, Yadav RNS, Ankush Lubana S, Kumar A. Production, purification and characterization of nattokinase from *Bacillus* sp. Asian J Pharm Clin Res., 2012, 5(3):124-25.
3. Sumi H, Hamada H, Nakanishi K, Hiratani H. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. Acta Haematol., 1990,84(3):139–43.
4. Kim JY, Gum SN, Paik JK, Lim HH, Kim KC, Ogasawara K,Inoue K, Park S, Jang Y, Lee JH. Effects of nattokinase on blood pressure: a randomized, controlled trial. Hypertens Res., 2008, 31(8):1583-88.
5. Wang SL, Chen HJ, Liang TW, Lin YD. A novel nattokinase produced by *Pseudomonas* sp. TKU015 using shrimp shells as substrate. Process Biochem., 2009, 44(1):70-76.

6. Subathra Devi C, Mohanasrinivasan V, Ravi Shanker S, Swathi T, Vaishnavi B, Jemimah Naine S, Suyash P.Exploring the *in vitro* thrombolytic activity of nattokinase from a new strain *Pseudomonas aeruginosa* CMSS. Jundishapur J Microbiol., 2015, 8(10):235-67.
7. Sambrook J, Frisch EF, Maniatis T. Molecular Cloning. Cold Spring Harbour Laboratories, New York: 1989.
8. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature,1970,227(5259):680-85.
9. Sahni G, Kumar R, Roy C, Rajagopal K, Nihalani D. Clot specific SK proteins processing altered plasminogen activation characteristics and a process for their preparation. 2007. US Patent No. 7,163,817, B2.
10. Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF. Development of an in vitro model to study clot lysis activity of thrombolytic drugs. Thromb J., 2006,4:14.
11. Dubey R, Kumar J, Agrawala D, Char T, Pusp P. Isolation, production, purification, assay and characterization of fibrinolytic enzymes (nattokinase, streptokinase and urokinase) from bacterial sources. Afr J Biotechnol., 2011, 10:1408–20.
12. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem., 1951, 193:265-75.
13. Mohanasrinivasan V, Subathra Devi C, Biswas R, Paul F, Mitra M, Selvarajan E. Enhanced production of nattokinase from UV mutated *Bacillus* sp. Bangladesh J Pharmacol., 2013, 8(2):110–15.
14. Al-Juamily EF, Al-Zaidy BH. Optimization conditions of production fibrinolytic enzyme from *Bacillus lichniformis* B4 local isolate. Br J Pharmacol Toxicol., 2012, 3:289-95.
15. Cui L, Dong MS, Chen XH, Jiang M, Lv X, Yan G. A novel fibrinolytic enzyme from *Cordyceps militaris*, a Chinese traditional medicinal mushroom. World J Microbiol Biotechnol., 2007, 24(4):483-99.
16. Peng Y, Huang Q, Zhang RH, Zhang YZ. Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi, a traditional Chinese soybean food. Comp Biochem Physiol B Biochem Mol Biol., 2003, 134(1):45–52.

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