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RESEARCH ARTICLE

FIBRINOLYTIC EFFECT OF PROTEASE ENZYME FROM *PSEUDOMONAS PUTIDA* B-18

***Pallavi K. Aradhye and Meera D. Chavan**

Department of Microbiology, Walchand College of Arts and Science, Solapur, Maharashtra, India-413006

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ABSTRACT

A fibrinolytic enzyme was produced by *Pseudomonas putida* B-18, isolated from soil. Screening of protease enzyme activity checked on milk nutrient agar, followed by fibrin agar plate for its fibrinolytic activity. Soy meal wheat powder broth used for the production of enzyme. Enzyme purification was done by ammonium salt precipitation, dialysis and DEAE cellulose. Native PAGE performed for the determination of molecular weight of the enzyme. In vitro blood clot lysis found within 19h of incubation. Effect of various environmental factors on enzyme activity was performed; in that maximum activity observed at 45^0 C temperature, pH 9.0. $FeSO_4$, $MgSO_4$, $ZnSO_4$, $CoCl_2$ and $CuSO_4$ had inhibitory action except $CaCl_2$.

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INTRODUCTION

Intravascular thrombosis, the accumulation of fibrin in blood vessels or in a chamber of heart, easily causes myocardial infarction and other cardiovascular diseases (CVD) resulted in the death of people (Yin *et al.,* 2010). Among all kinds of cardiovascular diseases, thrombosis is a frequently occurred symptom (Yuan *et al.,* 2012). The developing countries are having the high number of deaths due to thrombosis in the recent decades (Kumar Arbind *et al.,* 2011). Fibrinolytic agents are promising and highly effective in therapy for CVDs, classified in two groups as a plasminogen activator and plasmin like protein type fibrinolytic enzyme (Avhad *et al.,* 2013). A fibrinolytic or thrombolytic agent converts plasminogen to plasmin; lyses the clot by breaking down the fibrin contained in a clot. Currently several thrombolytic agents such as streptokinase, urokinase, prourokinase, reteplase (r-PA), alteplase (t-PA), reptilase, brinase and anisoylated purified streptokinase activator complex (APSAC) are available for clinical use. All those thrombolytic agents still suffer significant shortcomings including requirement of large therapeutic dose, short plasma half-life, limited fibrin specificity, reocclusion and bleeding complications (Balaraman and Prabhakaran, 2007). Now a day numbers of microbial and non microbial sources are available for obtaining the fibrinolytic enzyme. Microbial sources are bacteria, fungi, actinomycetes and algae. Non microbial sources are various fermented soy foods, earthworms, snake venom etc. In microbial sources bacteria such as *Bacillus subtilis nato (B. natto)* (Sumi *et al.,* 1987; Wang *et al.,* 2009), *Bacillus sphaericus* (Avhad *et al.,* 2013), *B. subtilis* YJ 1 (Li Jung Yin *et al.,* 2010), *B. subtilis* L-D 8547 (Jun Yuan *et al.* 2012), *Bacillis sp* strain CK-11-4 (Kim *et al.*, 1996), *Bacillus licheniformis* B4 (EF Al-Juamily *et al.,* 2012), styphylokinase from *Staphylococcus sp* (Sirinivasan *et al.,* 2013), streptokinase from *Streptococcus sp*. (Banerjee *et al.,* 2004; X Freeda Felsia *et al*., 2011; Shilpi *et al.,* 2013), urokinase from *Pseudomonas sp* (Dubey *et al.,* 2011) and protease from *E. coli* (R.H. Zhang *et al.,* 2005) can be used. Fungi such as *Aspergillus ochraceaus*, *Aspergillus fumigates*, (Kumar Arbind *et al.,* 2011), *Aspergillus* strain KH 17(Pandee *et al.,* 2013), *Aspergillus niger* and *Aspergillus flavus* (Rashmi B.S. *et al.* 2013), *Fusarium sp* (Sun Tao *et al.,* 1997), *Mucor sp*, *Penicillium sp* (Gopinath, 2011), *Rhizopus chinensis* 12 (Liu Xiao-lan *et al.,* 2005) *sp* and *Rhizomucor sp* (Usama F. Ali *et al.,* 2008) and actinomycetes such as *Streptomyces sp.* NRC411 (Abdal-Naby *et al.,* 1992), *Streptomyces megasporium* strain SDS (Chitte and Day 2000), *Streptomyces rimosus* (Victoria Geshewa, 2009), *Streptomyces sp.* CS684 (J R Simkhada *et al.,* 2010) and *Streptomyces sp.* XZNUM 00004 (Xiuym Ju *et al.,* 2012) are available. Fibrinolytic enzymes can be found in a variety of foods; such as Japanese Natto, Tofuyo, Korean Churgkook Jang, soy sauce and edible honey mushroom, fermented shrimp paste - a popular Asian seasoning. All above food sources have strong fibrinolytic

****Corresponding author: Pallavi K. Aradhye,***
Department of Microbiology, Walchand College of Arts and Science, Solapur, Maharashtra, India-413006

activity (Dubey *et al*., 2011). Although many bacteria, actinomycetes, algae and fungi have been found to have fibrin-digestion abilities, the genus important GRAS strains from traditional fermented foods could produce a high yield of fibrinolytic enzymes for further commercial applications (Yin *et al.,* 2010). In present study screening and isolation of *Pseudomonas sp.*B-18 having ability to produce fibrin degrading enzyme from soil is attempted. In its further progress; production, purification characterization and in vitro applications are also analyzed.

MATERIALS AND METHODS

1.Culture selection of protease producing bacteria: Various dilutions (10^{-4}, 10^{-5}) of soil sample were spread on milk nutrient agar plates and incubated at 33^0C±2^0C for 24 to 48 h. all the colonies showing zone of hydrolysis were selected and named as B-01, B-02, B-03 and so on for further study. Microscopic, biochemical, cultural and molecular characters were studied.

2.Fibrinolytic enzyme producing bacteria: Isolate B-18 selected for fibrinolytic screening. In this step, 1% fibrin nutrient agar plates were prepared and spot inoculated the pure culture of B-18 isolate. Plates were incubated at 33^0C±2^0C for 24 h and zone of fibrin hydrolysis was observed after pouring 5% trichloro acetic acid solution.

3.Production of fibrinolytic enzyme: Isolate B-18 which showed zone of fibrin hydrolysis was further subjected for the lab scale enzyme production. Culture grown on nutrient agar slant for 24 h, loopful of culture inoculated in to 50 ml of nutrient broth and again 24 h incubation was performed. This was the inoculum preparation of B-18 isolate. Production medium selected after different trials on substrates such as casein, milk and soy meal wheat powder. In this trial soy meal wheat powder medium was found to be supportive for fibrinolytic enzyme production. Composition of the medium was as: Soy bean powder 2%; wheat powder 1.5%; Glucose 1.0% with other essential minor nutrients. 5% of inoculum, having ≈ 428 million cfu/ml, was inoculated in 500 ml of production medium. Samples were removed after each 24 h of incubation interval. These samples were centrifuged and filtered and tested for enzyme activity using casein as substrate. 5ml of 1% casein prepared in phosphate buffer of pH 7.0 + 1 ml of enzyme supernatant and incubated for 10 min at 37 ^{0}C. The reaction was terminated by adding 5 ml of 110 mM trichloro acetic acid. Again the reaction mixture was incubated at 4^0C for 30 min. after 30 min the tubes were centrifuged at 4000 rpm for 20 min. 2 ml of supernatant taken to this 5 ml of 500mM sodium carbonate and 1ml of 1:4 diluted folin cio caltaeu reagent added immediately. Incubation of 30 min at 37 ^{0}C was done. Amount of tyrosine released was measured at 660 nm with help of standard curve for tyrosine. The production was terminated after 120 h of incubation.

4.Enzyme extraction: After completion of production, the enzyme was subjected for extraction, in this method simple filtration for the removal of course media material and centrifugation at 10000 rpm for 20 minutes for the removal of cells and other fine particles was done. All the supernatant collected and labeled as crude enzyme and subjected for further characterization.

5.Enzyme purification: It was carried out by ammonium salt precipitation. About 60 % of ammonium salt gives the maximum active portion of protein. After centrifugation sediment collected in 25 mM tris HCl buffer. Dialysis was performed using the same buffer for 8 to 10 hrs, with changing the buffer at 3 hrs of interval. Partially purified enzyme collected in the vials and stored at -20^0C. DEAE cellulose was used for ion exchange chromatography. In this step 2% DEAE cellulose filled in the column which was activated over night in the 25mM tris HCl buffer. Sample loaded and elution was carried out by using various concentration of NaCl. About 3 ml fractions were collected in the small test tubes. These samples analyzed at 280 nm on uv-vis- spectrometer for the presence of protein. Active portion collected together and subjected for further analysis.

6.Enzyme characterization

(i)Total protein concentration (Jayaraman, 1985) - It was determined by folins method. All the samples such as- crude enzyme, partially purified and purified enzyme fractions were tested for total protein concentration. Bovine serum albumin was used as a standard protein for the reference.

(ii)Caseinolytic activity (Tharwat, 2006) - It was performed in the terms of enzyme activity on the substrate and release of tyrosine in the reaction mixture. 0.5 ml of 1% casein prepared in 0.2M tris-HCl of pH 8.0 buffers and 0.1 ml of enzyme incubated at 37^0C for 10 min and the reaction was terminated by adding 0.5 ml of 110mM tri Chloro acetic acid. Supernatant collected after 30 min incubation at 4^0C; to this 5 ml of 500mM sodium carbonate and 1ml of 1:4 diluted folin cio caltaeu reagent immediately added and incubated at 37 ^{0}C for 30 min. The released tyrosine was measured at 660 nm and calculated with the help of standard tyrosine graph. Hence enzyme activity is defined as 'one enzyme unit is that amount of enzyme which liberates 1µmole of tyrosine in one min under the assay conditions'.

(iii)Fibrinolytic activity (Astrup and Mullertz, 1952; Kim *et al.,* 1996) - It was done on fibrin plate using plasmin as a standard reference enzyme. Fibrinolytic activity was determined by plasminogen free plate method using 0.3 U/ml of plasmin as a standard fibrinolytic protease. The fibrinogen solution [2.5 ml of 1.2% (wt/vol) human fibrinogen in 0.1M sterile sodium phosphate buffer of pH 7.4] was mixed with 0.1ml of thrombin solution (100NIH U/ml) and 7.4 ml of 1% (wt/vol) agarose solution in a petri dish. After dishes were allowed to stand for 30 min at R.T.to form fibrin clot. Six holes were made on a fibrin plate by 5mm diameter glass tubing. About 30µl of standard plasmin and test samples solution was dropped into respective well and incubated at 37^0C for 15h, after measuring the dimensions of the clear zone the number of units was determined according to standard curve by using plasmin.

(iv)In vitro blood clot lytic activity (Juan *et al.* 2012; Avhad *et al* 2013) - Blood clot lysis was checked at each stage of purification. In this method blood clots of various animals collected and subjected for lysis by adding the enzyme. Observation was made up to complete hydrolysis of blood clot.

(v)Effect of environmental factors on enzyme activity- Enzyme activity was measured at different time intervals such as 5 min, 10min, 15 min, 20min, 25min, 30min and 35 min. For effect of temperature 0^0C to 80^0C temperature were selected, reaction was carried out after pre-incubating the

substrate at respective temperature. In effect of pH 3, 4, 5, 6, 7, 8, 9, & 10 were selected; substrate casein and enzyme both were prepared in respective ionic strength and used. In effect of metals $FeSO_4$, $MgSO_4$, $ZnSO_4$, $CoCl_2$, $CuSO_4$ and $CaCl_2$ were used. 0.5 ml of various salt solutions mixed with enzyme to get final concentration of 1mM.

(vi)Determination of molecular weight (Laemmli 1970) - Native -PAGE was carried out with the tris- glycine system of polyacrylamide slab gel of 20x15x 0.1 cm (length x width x thickness) dimensions was used. Enzyme protein (mg/ml) dissolved in tris HCl buffer of pH 6.8 containing 0.5% of mercaptoethanol. Ten microliter of this sample was loaded on the gel. Gel was stained with coomassie brilliant blue stain for 1h, de-staining done for overnight. For molecular weight determination the standard protein marker was used for the reference.

RESULTS

1.Culture isolation: The milk nutrient agar plate showed various protease producing colonies. All well isolated colonies selected and subcultured on milk nutrient agar plate. Isolate B-18 selected as it showed zone of protein hydrolysis on MNA. Under microscope Gram negative rod with motile character were observed for B-18 Figure 1. Cultural characters are of typical *Pseudomonas* type with slight yellowish brown pigmentation. In biochemical characters it utilizes all type of carbon sources, ferment glucose, indol negative, starch, gelatin and nitrates reduction negative. In molecular analysis it was found to be *Pseudomonas putida* Figure 2.

Table 1. Protein concentration and caseinolytic activity of enzyme of *Pseudomonas putida* B-18

S.No.	Enzyme	Protein mg/ml	Enzyme activity EU/ml	Specific activity U/mg protein
1	Crude	3.02	5076.92	1681.10
2	Partially Purified	2.48	2403.076923	2002.56
3	Purified	0.26	1353.846154	5207.10

Table 2. Fibrin agar plate with plasmin as standard enzyme

Sr.	Lytic area (mm diameter)	Plasmin concentration (U/ml)
Std-1	7.0	0.03
Std-2	11.0	0.09
Std-3	12.0	0.15
Std-4	13.0	0.21
Std-5	13.5	0.27
Std-6	14.0	0.30
Test-7	23.0	0.38

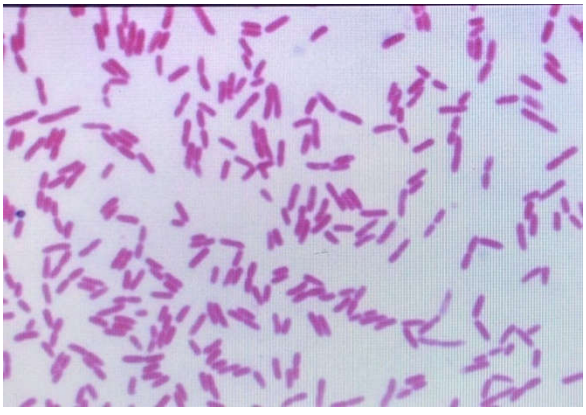

Figure 1. Microscopic observation of *Pseudomonas putida* B-18 by Gram Staining.

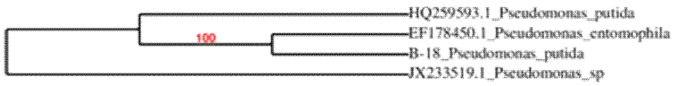

Figure 2. Phylogenetic tree for *Pseudomonas putida* B-18

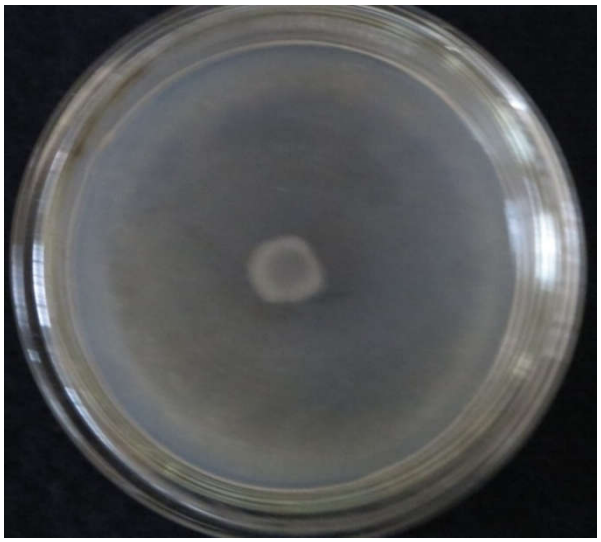

Figure 3. Fibrin hyrolyis on fibrin agar plate by B-18

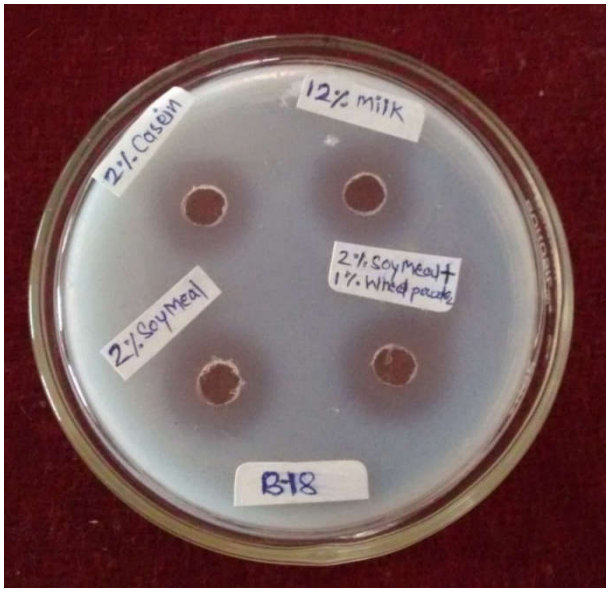

Figure 4. Soy meal + wheat powder media gives maximum zone of casein hydrolysis

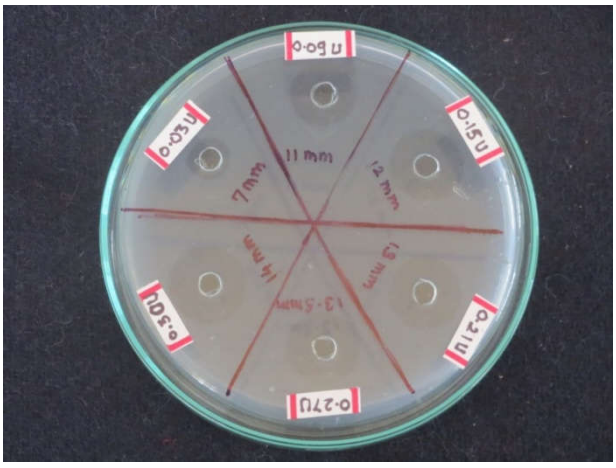

Figure 5. Fibrin agar plate with plasmin as a standard enzyme

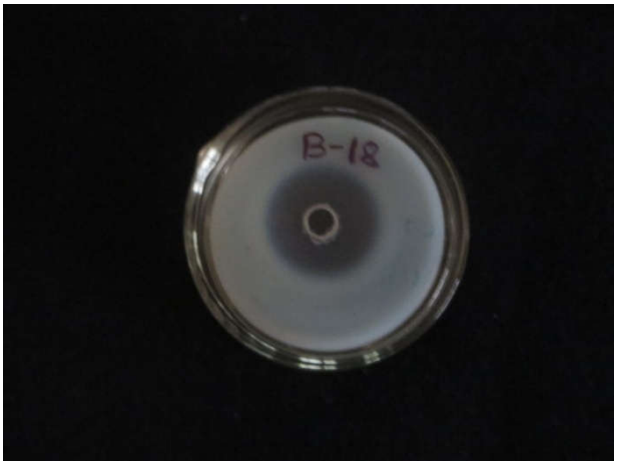

Figure 6. Fibrin agar plate with test enzyme of B-18 (0.38 U/ml form graph)

Figure 7. In-vitro blood clot lysis with human blood clot

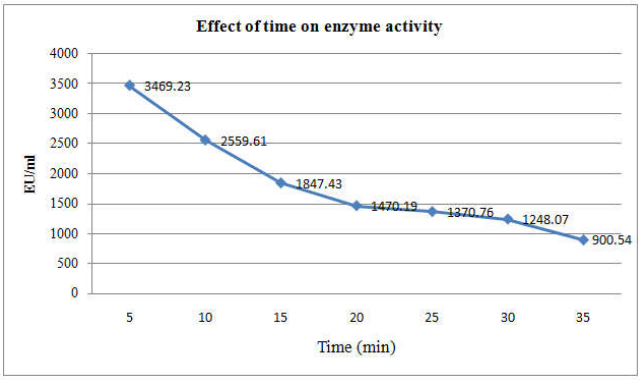

Figure 8. Effect of time on enzyme activity

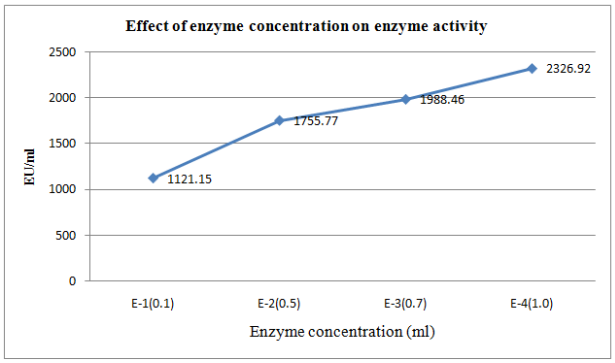

Figure 9. Effect of enzyme concentration on enzyme activity

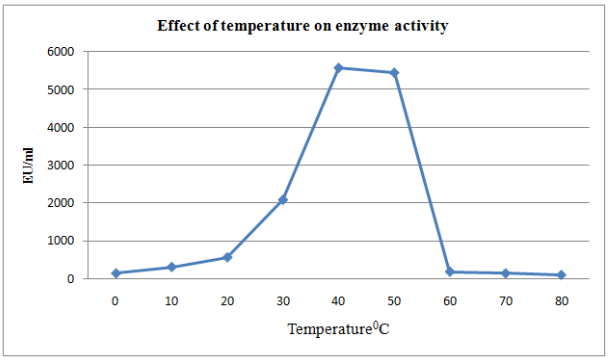

Figure 10. Effect of temperature on enzyme activity

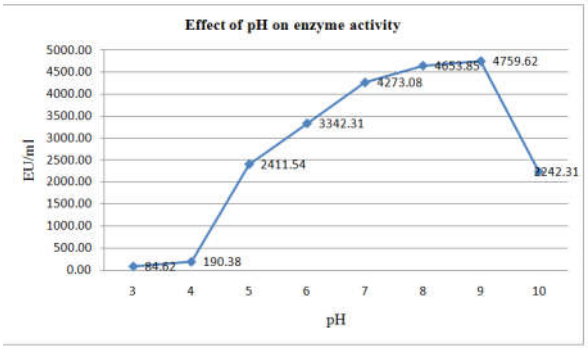

Figure 11. Effect of pH on enzyme activity

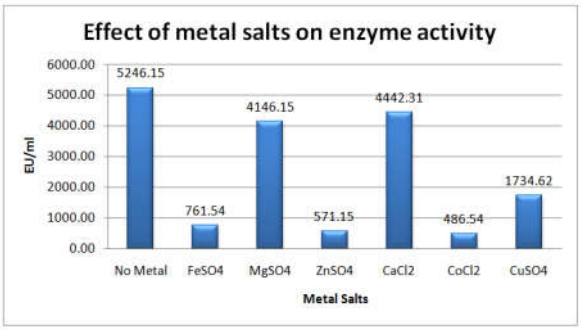

Figure 12. Effect of metal salts on enzyme activity

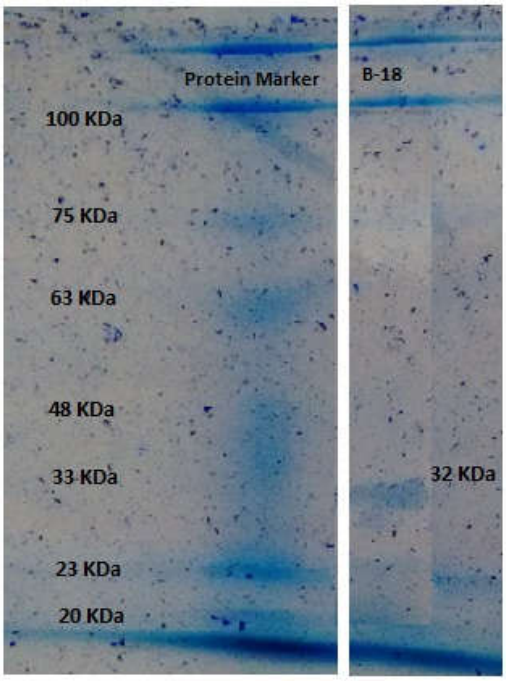

Figure 13. Native -PAGE of purified enzyme. Lane -1 Protein marker, Lane-2 B-18 enzyme

2.Fibrinolytic enzyme producing bacteria: On fibrin agar plate B-18 showed about 20 ±1mm of zone of hydrolysis after 24 h of incubation Figure 3.

3.Production and extraction: Various substrates such as casein, milk, and soy meal + wheat powder showed support for the enzyme production. Out of that soy meal wheat powder combination gives the better result as 15 ±1 mm zone on fibrin agar plate Figure 4. Intermittent samples removed at each 24 h of production showed the enzyme activity in increasing order for first three days EU/ml (0h- 84.61, 24h- 4738.46, 48h- 6007.69) and found steady later (72 h- 5034.61, 96h- 5076.92, 120h- 5076.92) and hence that was the end point of enzyme production. The extracted enzyme having pale yellow brown color and clear from all cell debris was labeled as crude enzyme.

4.Purification and Enzyme characterization: At each purification step enzyme activity determined it was tabulated in terms of enzyme activity U/ml and its specific activity. In enzyme characterization total protein concentration, caseinolytic activity and fibrinolytic activity was calculated for three forms of enzyme i.e. crude, partially purified, purified enzyme Table 1. Fibrinolytic activity on fibrin agar plate with plasmin as standard enzyme was also successfully determined Figure 5 & 6. Fibrinolytic units calculated for the B-18 fibrinolytic enzyme was 0.38 U/ml Table 2. In-vitro blood clot lysis experiment was the key point of this study. Blood clot (5%) gets dissolved by enzyme (5ml) within 19 h. of incubation at 37^0C, for comparison control with normal saline was used Figure 7. In testing of effect of environmental factors on enzyme activity, various parameters were studied, in that the maximum activity for given enzyme was found to be at 5 min time, E-4(1.0ml) enzyme concentration, 45^0C temperature, 9.0 pH and $FeSO_4$, $MgSO_4$, $ZnSO_4$, $CoCl_2$ and $CuSO_4$ had inhibitory action except $CaCl_2$. All the data have shown in Figure 8, 9, 10, 11& 12. Molecular weight was determined by native PAGE it was found 32 KDa Figure 13.

DISCUSSION

Microorganisms have the ability to produce various enzymes. In that proteolytic enzyme is one the important kind of enzyme having various application. To the point of current study, the protease enzyme is used for the degradation of fibrin i.e. blood clot. The bacterial isolate was the *Pseudomonas putida* B- 18. It is a Gram negative bacterium, having ability to produce protease enzyme. In earlier studies nattokinase enzyme from *Pseudomonas* sp. was isolated and studied (Wang *et al*., 2009). Gene for urokinase was expressed in *E.coli* using suitable vector (Dubey *et al.,* 2011). Use of soy meal wheat powder was also a part of reducing the coast of production medium. Other substrates such as fibrin (Borah *et al*., 2012, Kumaran *et al.,* 2011 Nagwa Tharwat 2006), milk casein (Kim *et al.,* 1996), rise chaff/husk (Gopinah *et al.,* 2012, Yin *et al.,* 2010), sunflower oil cake (Mona Rashad *et al.,* 2012), oat meal (J.R. Simkhada *et al.,* 2010) glycerine (Victoria Gesheva, 2009) etc. can be used and found good substrate for fibrinolytic activity. Use of fibrin, serum, casein increases the cost of production medium while rise husk, sunflower oil and soy meal wheat powder like substrates are cost effective. Hence it has been always preferred the use of natural substrates than the synthetic one. Chinese (Douchi), Korean (Chung kook-Jang soy sauce, edible honey mashroom), Japanese (Natto, Tofuyo), are the fermented soy products and are found to be good source of fibrinolytic enzyme. Purified enzyme can be given in the form of tablets, liquids or granules; orally or through injection (Jun Yuan *et al.* 2012). Their role may be either like t-PA (activates the plasminogen to plasmin) or direct action on the fibrin clot as plasmin like enzyme. But all these studies require the extensive enzyme study and the live animal trials.

CONCLUSION

From the data present in the literature and the current research outputs we can conclude that the microbial enzyme and its fibrinolytic use will be the future need of the world, to reduce the risks and deaths due to number of complications of blood clotting disorders.

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