Int.J.Curr.Microbiol.App.Sci (2016) *5*(7): 978-989

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 7 (2016) pp. 978-989**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.507.110

Identification and Characterization of the Fibrinolytic Enzymes producing Bacterium Strain (Isolate KLG 55) from the Soil Waste

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Keywords

Fibrinolytic activity, purification, Sephadex G-50 gel permeation chromatography and DEAE Sephadex A-50 anion exchange chromatography.

Article Info

Accepted:
28 June 2016
Available Online:
10 July 2016

A B S T R A C T

The present work was conducted to characterize the fibrinolytic enzyme produced by the strains of the isolates of *Bacillus cereus sp. obtained from the soil waste.* The isolated organism was cultured in the sterile media at 37ºC for 24 hrs. The selected isolates were subjected to fibrinolytic activitiy. The fibrinolytic activity of isolates KLG 55 was higher and it was subjected for biochemical studies. Based on the study, isolate was designated as *Bacillus cereus* KLG 55. Further it was subjected to three step purification involving ammonium sulphate precipitation, Sephadex G-50 gel permeation chromatography and DEAE Sephadex A-50 anion exchange chromatography. After the purification processes, the molecular weight of fibrinolytic protease enzyme is 31kDa.

Introduction

With the increasing industrial application of fibrinolytic proteases, the search for promising strains of fibrinolytic proteases producers has became a continuous process. Proper identification and characterization of microorganisms is very important because it expands the scope for exploitation of industrially important products. Various morphological, cultural and biochemical characteristics of the isolated organisms were compared with the descriptions of the numerous bacterial species such as Bergey's Manual of Determinative Bacteriology (Buchanan *et al.*, 1974), Bergey's Manual of Systemic Bacteriology (Williams *et al.*, 1989), Bergey's Manual of Systemic Bacteriology (Sneath *et al.*, 1986), Biological and Microbiological abstracts and all other relevant journals (Fundagul *et al.*, 2009; Mousumi Banerjee *et al.*, 2011; Vyletelova *et al.*, 2002).

To isolate the potent fibrinolytic protease producing bactreria, brick red sticky soil sample was collected from various places in and around Bangalore, Karnataka, India.The soil samples were selected based on the varying characterisitics such as organic matter, particle size, colour of soil and geographical distribution. The samples were stored in sterile condition.

The isolates were initially screened for their proteolytic activities such as caseinolytic, gelatinolytic and fibrinolytic activities. Shake flask fermentation was considered for the production of fibrinolytic protease.

The promising isolate KLG 55 was subjected to taxonomic studies. The taxonomic properties were investigated for the characterization of the isolate KLG 55.

Materials and Methods

In the present work, strain that produces fibrinolytic enzyme (GD55) was isolated from the soil sample collected from different geographical locations in and around Bangalore, India.

Microorganism and Inoculum preparation

The slants were inoculated from the stock cultures and incubated at 37°C for l-3days to get maximum sporulation. Spore suspension was prepared by transferring a loopful of spores from these slants into sterile distilled water and shaking thoroughly.

Stock cultures were maintained in nutrient broth medium with 70% glycerol, cultures were preserved at -20°C (Richard *et al.*, 2009). The inoculum was prepared by transferring a loopful of the isolate to 100ml of sterile nutrient broth stock medium, then incubated it overnight at 37°C on a rotary shaker with 200rpm. A stock suspension was prepared and adjusted to 7 x $l0^3$ cells ml^{-1}.

Identification of the isolate KLG 55

The morphological characteristics, physiological properties based on the effect of the temperature, pH, growth of isolate using different concentration of sodium chloride and biochemical properties, carbon source utilization pattern, growth in the presence of various nitrogen sources and resistance or sensitivity to various antibiotics were analysed for the selected isolate.

Production of Fibrinolytic protease

The medium used for the production of fibrinolytic protease was composed of 0.2% fibrin, 1% fructose, 1% peptone, 0.2% KH_2PO_4, 0.04% $CaCl_2$, 0.05% NH_4NO_3 and 0.02% $MgSO_4$. The pH of the medium was adjusted to 8 using HCl/NaOH. One percent (v/v) of 24h old inoculum suspension was transferred to 100ml of growth medium in 250ml Erlenmeyer flasks. These flasks were then placed in the rotary incubator shaker rotating at 200rpm for 24h at 37°C. The broth was then centrifuged at 10,000 rpm for 10 min at 4°C and the clear supernatant (crude enzyme)was used for the estimation of fibrinolytic protease (Greenberg 1957).

Enzyme purification

Ammonium sulfate fractionation and Dialysis

Supernatant containing crude enzyme obtained after centrifugation was subjected to ammonium sulfate fractionation, at different concentrations ranging from 30-80% saturation (Olajuyigbe *et al*., 2005). The precipitates obtained were suspended in 2ml of cold saline. It was then subjected for fibrinolytic activity and total protein content. To obtain complete precipitation of the crude enzyme, the crude enzyme was subjected to ammonium sulfate precipitation at 70% saturation at 4°C by subjecting it to centrifugation at 10000rpm for 15min, It was again suspended in 100ml of cold saline solution and dialyzed (Balaraman *et al.*, 2007) in cold against 1litre of 20mM Tris-

HCL buffer at pH 8 for 24h. After dialysis, the solution was centrifuged and supernatant obtained was next subjected to gel filtration chromatography.

Gel Filtration Chromatography

The sample obtained from ammonium fractionatin was centrifuged at 8000g for 15min, and supernatant was chromatographed on a Sephadex G-50 Column (Jo *et al.*, 2011). The sample was first loaded onto a column of Sephadex G-50 (120cm X 1.0cm) and it was then followed by DEAE Sephadex A-50 column(24cm x 2.0cm), both the columns were equilibrated with 10 mM Tris-Hcl buffer at pH 8 (Peng *et al*., 2003). The column was eluted at a flow rate of lml/6min with a discontinuous gradient ranging from 0.2M to l.0M of NaCl dissolved in same buffer.

The fractions collected were determined for its total protein concentration and fibrinolytic activity. The active fractions with high fibrinolytic activity were dialyzed and used for further studies.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The active fractions showing the highest specific activity were pooled, dialyzed, lyophilized and then subjected to Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out using a 5% stacking gel and a 12% polyacrylamide resolving gel according to the method of Laemmli (Laemmli *et al*., 1970). To run the SDS gel, the electrophoresis buffer containing 25mM Tris, and 250mM glycine (Electrophoresis grade) at pH 8.8 was used. The SDS was added at a concentration of 0.1 % to the electrophoresis buffer. A molecular weight marker (Bio-Rad) was used as reference proteins, crude broth and fraction were dissolved in sample buffer containing 0.2M Tris HC1 (pH 6.8), 10% SDS, 20% glycerol, 10mM 3-mercaptoethanol and 0.05% bromophenol blue as the tracking dye and boiled for l0min to determine the protein. The treated samples were loaded in the wells of the slab gel and electrophoresis was continued till the tracking dye reached the lower end of the gel. After the complete run, the gel was soaked overnight (about 16h) in a fixative solution containing 50% (v/v) methanol and 12% acetic acid. After taking out the gel from the fixative solution, it was stained for one hour with 0.25% Coomassie Brilliant Blue R-250 in methanol/ water/ acetic acid (50: 40: 10) and the gel was finally destained in a destaining solution containing water/ acetic acid/ methanol (87.5: 7.5: 5).

Fibrinolytic Protease Assay

Fibrinolytic protease activity was assessed by the modified procedure of Greenberg (1957) using 2% bovine fibrin in 0.05 mM phosphate buffer (pH 8). 0.5ml fibrin solution with an equal volume of diluted enzyme solution was incubated for 10min at 37^0C. The reaction was stopped by the addition of 10% cold Tri Chloroacetic Acid (TCA). The mixture was centrifuged at 3,000rpm for l0 min and to the supernatant 5ml of 0.44M Na_2CO_3 was added, followed by the addition of 1ml of diluted Folin - ciocalteau reagent. The mixture was incubated for 30min at 37^0C, the sample develops color change, and the intensity of colour change will be measured at 660nm using Shimadzu UV-1700 spectrophotometer against the blank solution containing fibrin substrate. Tyrosine is used as the reference standard.Total protein concentration was determined using Lowry's method (Lowry *et al.*, 1951).

Characterization of purified enzyme

Effect of pH on Enzyme activity

The optimal pH of the enzyme was determined between pH 3.0-12.0 was examined by incubating the enzyme solution for 30min at 37°C using the following buffer systems: 10 mM citric acid buffer, 0.05M sodium phosphate buffer, 0.05M Tris-HCl, 0.05M glycine-NaOH, 0.1M sodium phosphate buffer, 0.05M hydroxyl-chlorite buffer respectively. All experiments were done in triplicates.

Effect of Temperature on Enzyme activity

The activity of the purified enzyme was determined by incubating the reaction mixture at different temperatures (10, 20, 30, 37, 40, 50, 60 and 70°C) for 30min. Stability of fibrinolytic protease was investigated by measuring the residual activity after incubating the enzyme solution at 20-80°C for 30min in 20mM phosphate buffer at pH 8. All experiments were done in triplicates.

Hydrolysis of protein substrates

Protease activity with various protein substrates such as Bovine Serum Albumin (BSA), casein, egg albumin and gelatine was assayed by mixing 10µl of the purified enzyme and 200µl of assay buffer containing the 2mg/ml of protein substrates. After incubation at 37°C for 30min, the reaction was stopped by adding 200 µl of 10% TCA (w/v) and allowed to stand at room temperature for 10min. The undigested protein was removed by centrifugation and peptides released were assayed. The specific protease activity towards casein was taken as a control. All experiments were done in triplicates.

Determination of Fibrinolytic potential of the protease

Fibrinolytic activity was performed according to the method of Najafi *et al.*, 2005. A clean piece of pure white cotton cloth was soaked in animal blood (0.lml) for 15min and then allowed to dry at 80°C for 5min in hot air oven. Then, the cloth pieces were soaked in 2% (v/v) formaldehyde for 30min and rinsed with water to remove excess formaldehyde. The dried cloth was cut into equal sizes and incubated with crude enzyme (2ml) at 37°C for different incubation periods (10, 20, 30, 40 and 50 min). After a given incubation, the cloth was rinsed with tap water for 2min without scrubbing and then dried in open air. Visual examination of various pieces exhibited the effect of enzyme in removal of strains . The same procedure was done with the control without the enzyme exposure.

Results and Discussion

The most significant characteristics of isolate KLG 55 were analyzed (table.1) . The isolate grew well on the media. The colony surface was rough, white in colour rod shape, size in the range of 0.8 - 1.0µm. Isolate is gram positive. The isolate indicated negative result for indole test, citrate utilization test, H_2S production test, gelatine hydrolysis test. It could extensively hydrolyse starch and casein. It showed positive result for nitrate reduction, oxidation, methyl red test, voges proskauer test, arginine dihydrolase and esculin hydrolysis test. It exhibited good growth at 37°C. It could tolerate the pH levels between 5.0 and 9.0. It could not grow above 4% NaCl level. It exhibited good growth on fructose, moderate growth on dextrose, sucrose and poor growth on maltose. It could not utilize rhamnose and mannose.

Isolate KLG 55 indicated good growth with L-asparagine, moderate growth with Serine and Histidine, poor growth with Arginine and Cysteine and no growth for the sources like Valine, phenyl alamine, threonine, hydroxyl praline, methionine as it could not utilize it.

Isolate KLG 55 was sensitive to Rifampin, Chloramphenicol, Erythromycin, Ciprofloxacin, Streptomycin and Gentamicin and it is resistant towards the antibiotic Lincocin.

Based on the results obtained, characteristics of isolate KLG 55 obtained from the soil sample designated as GUG –VI was in close comparison with the strains of *Bacillus cereus* (table. 1). Isolate KLG 55 and strains of *Bacillus cereus* have the following similarities: Colour of the colony, utilization of Dextrose, non-utilization of rhamnose and sensitivity to rifampin and chloramphenicol.

But however, some qualitative and quantitative differences could be noticed. Isolate GD55 did not grow on mannose, where as the reference culture could utilize them. Reference culture utilized gelatin and isolate KLG 55 culture could not. In view of the general close agreement and more similarities and a few differences, isolate KLG 55 can be considered to be as a new strain *of Bacillus cereus*. Hence, it is designated as *Bacillus cereus* KLG 55.

Purification of Fibrinolytic Protease from *Bacillus cereus* KLG 55

Fermentation broth containing the crude enzyme was subjected to three step procedure involving ammonium sulphate precipitation, Sephadex G-50 gel permeation chromatography and DEAE Sephadex A-50 anion exchange chromatography.

Gel Filtration Chromatography on Sephadex G-50

The crude broth obtained after fermentation was subjected to ammonium sulphate precipitation at 70%. The pellet so obtained was resuspended in cold saline (2ml) and dialysed. The dialysed enzyme was first loaded on to a column of Sephadex G-50 and then to a column containing DEAE Sephadex A-50, both the columns were equilibrated with l0mM Tris-HCL buffer, pH 8. The column was eluted at a flow rate of lml/6min. The elution profile of gel filtration chromatography is shown in the (Fig.1). The fractions collected were determined for its total protein concentration and fibrinolytic enzyme activity.

The summary of purification steps involved for fibrinolytic protease is presented in the Table.2.

Sodium Dodecyl Sulfatepolyacrylamide Gel Electrophoresis (SDS-PAGE)

The crude broth, precipitate obtained after ammonium sulphate precipitate and the purified fibrinolytic protease along with standard molecular weight markers were run on SDS-PAGE. The molecular weight of the fibrinolytic protease was determined by comparison of the migration distances of standard marker proteins (Bio-Rad). The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the R_f value (relative mobility). Depending on the relative mobility, the molecular weight of fibrinolytic protease enzyme is 31kDa.

Characterization of purified enzyme

Determination of optimum pH and pH stability

For obtaining the optimum pH level, different buffers were used such as 10mM citric acid buffer, 0.05M sodium phosphate

buffer, 0.05M Tris-HCl, 0.05M glycine-NaOH, 0.1M sodium phosphate buffer and 0.05M hydroxyl-chlorite buffer were used. The results are shown in Fig. 4. It showed optimum pH of purified enzyme is 8 and stable in the pH range of 6.5-8.0 between 2 to 20h.

Table.1 Comparison of characterization of the isolate GD55 and Bacillus cereus

Characteristics	Isolate GD55	Bacillus Cereus
Colony morphology		
Colour of isolate	White	White
Spores	Ellipsoidal	Ellipsoidal
Configuration	Circular	Circular
Margin	Irregular	Irregular
Elevation	Flat	Flat
Surface	Rough	Rough
Carbon source utilization		
Dextrose	+ve	+ve
Fructose	+ve	+ve
Sucrose	+ve	+ve
Rhamnose	-ve	-ve
Maltose	+ve	+ve
Mannose	-ve	-ve
Starch hydrolysis	+ve	+ve
Casein hydrolysis	+ve	+ve
Gelatin hydrolysis	-ve	-ve
Growth at 37^0C	+ve	+ve
Growth on 4% NaCl	+ve	+ve
Sensitivity in		
Lincocin (30µg)	-ve	-ve
Rifampin (10µg)	+ve	+ve
Chloramphenicol (20µg)	+ve	+ve
Erythromycin (30µg)	+ve	+ve
Ciprofloxacin (10µg)	+ve	+ve
Streptomycin (30µg)	+ve	+ve
Gentamycin (10µg)	+ve	+ve

Table.2 Summary of the purification of Fibrinolytic Protease from *Bacillus cereus* KLG 55

Prification Step	Total Protein (mg)	Total Ativity (U)	Specific Activity U/mg	Fold Purification	% Yield
Crude Extract	11923	14.900	1.24	1	100
70% ammonium sulfate saturation	8564	12.250	1.43	1.15	82.21
Sephadex G-50	3623	6.900	1.90	1.53	46.30
DEAE-Speakex A-50	28	1500	53.57	43.20	10.06

Fig.1 Gel filteration chromatography on Sephadex G-50

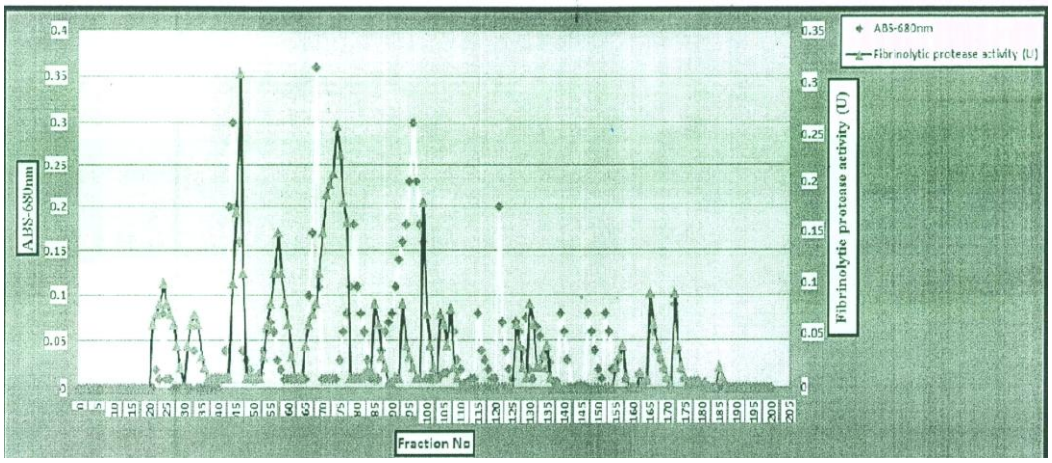


Fig.2 Anion Exchange chromatography on DEAE Sephadex A-50

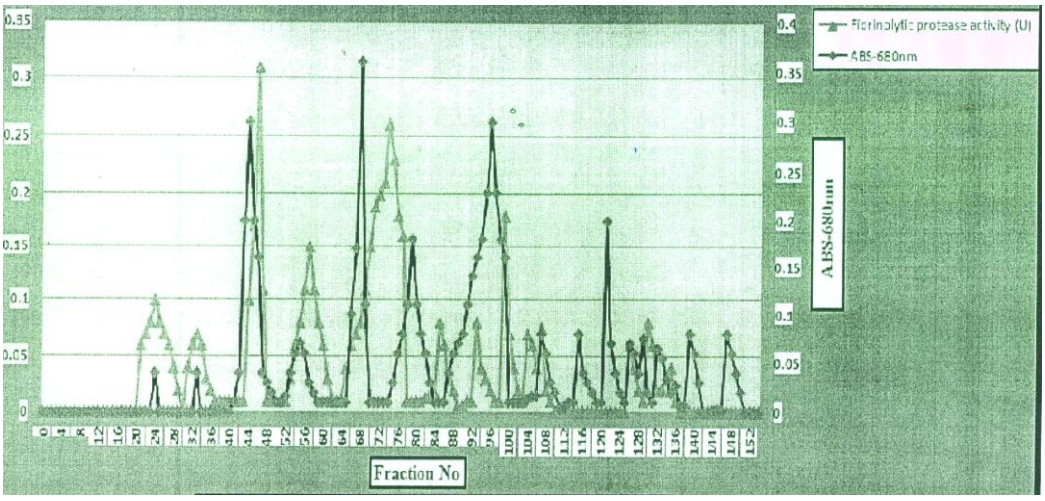
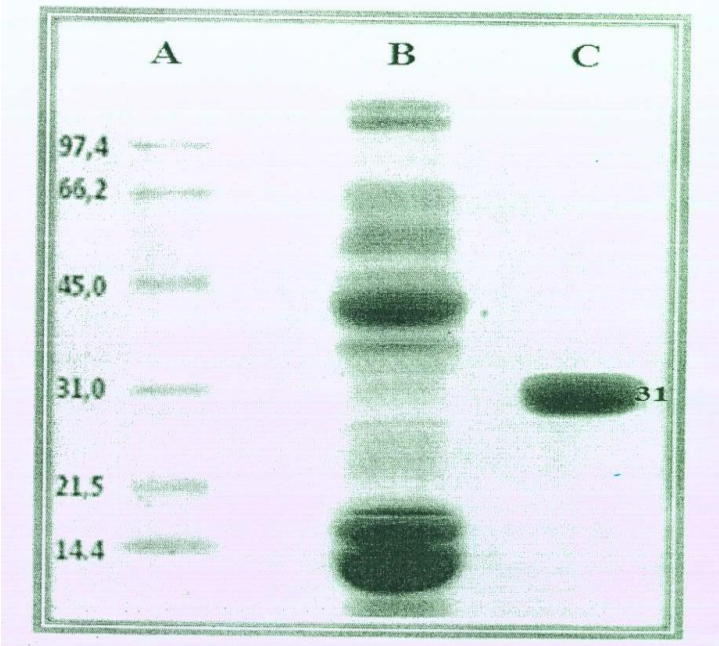


Fig.3 Results obtained from SDS-PAGE run



Lane A : Molecular Mass Markers; Lane B: Crude Enzyme; Lane C :Purified Enzyme

Fig.4 Effect of pH on enzyme activity

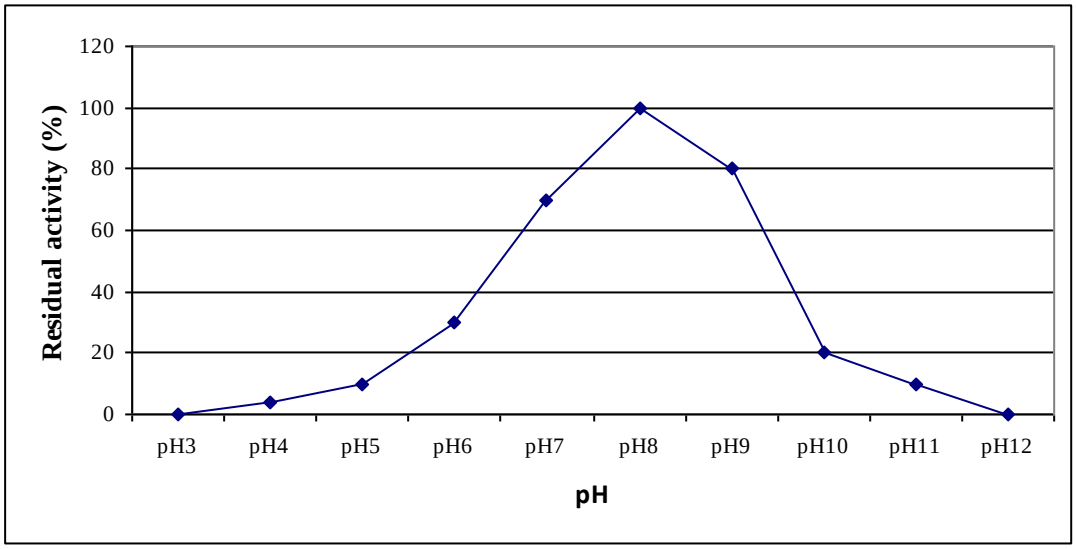


Fig.5 Effect of Temperature on enzyme activity

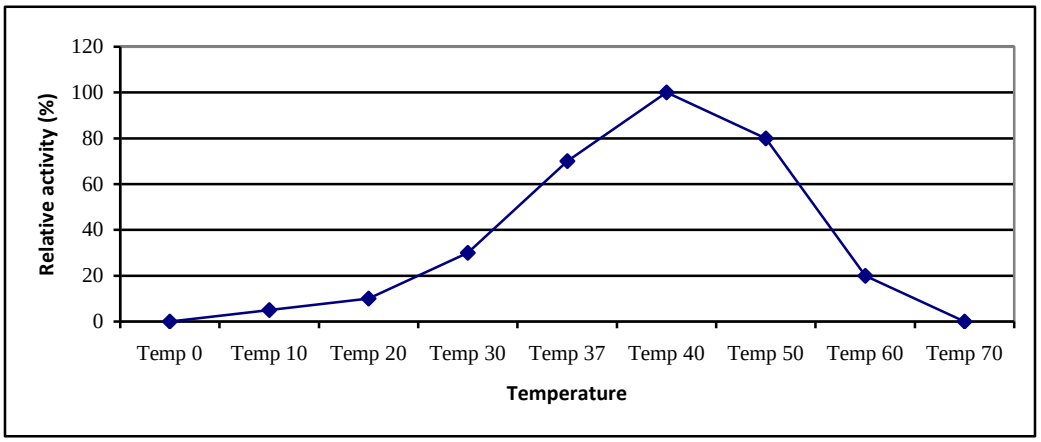


Fig.6 Fibrinolytic proteae activity against different natural substrates

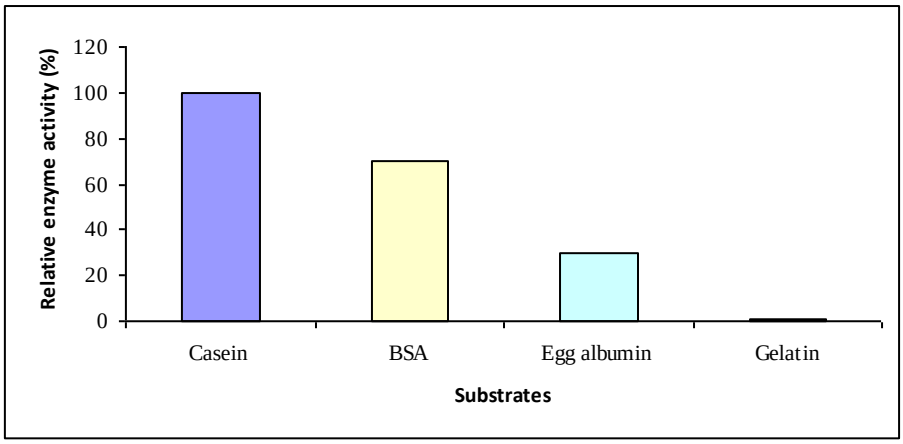
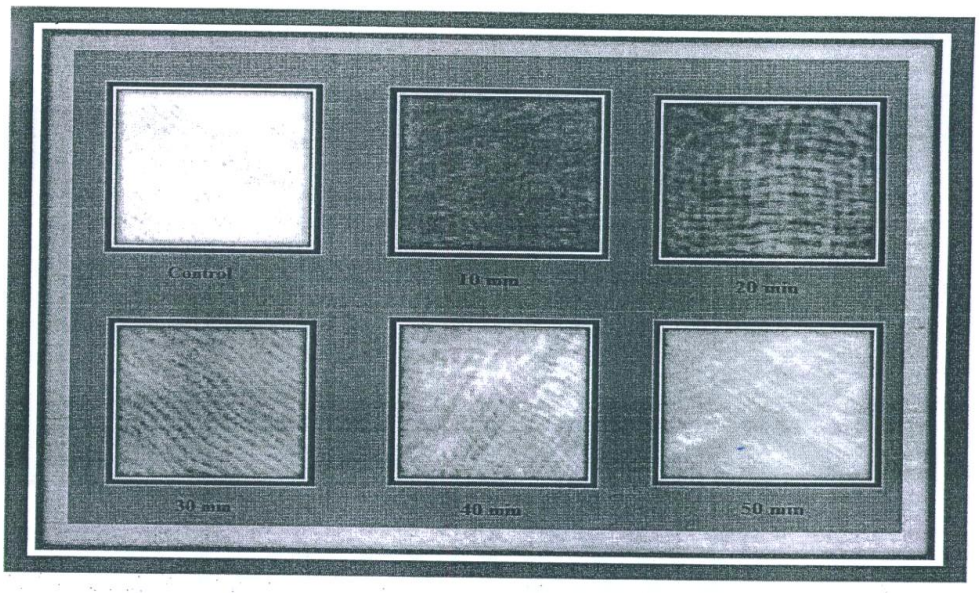


Fig.7 Fibrinolytic potential of the protease



Determination of optimum temperature of purified enzyme

The activity of the purified enzyme was determined at different temperatures ranging from 10°C - 70°C for 30min. The optimum temperature recorded was at 37°C and enzyme activity was gradually declined at temperature beyond 40°C. The results are shown in Fig.5.

Hydrolysis of protein substrates

When assayed with native proteins as substrates, the protease showed a high level of hydrolytic activity against casein and poor to moderate hydrolysis of BSA and egg albumin, although the hydrolysis was hardly observed with gelatine. The results are presented in the Fig.6.

Fibrinolytic potential of the protease

The blood strain was removed from a white cotton cloth by incubating the cloth in purified fibrinolytic protease for different time intervals. It was seen that protease produced by *Bacillus cereus* KLG 55 had high capability of removing the blood strain (50min), which indicates its potential in detergent industries. Anwar *et al.*, (1997) reported the effectiveness of protease on blood strain removal. In the present study the removal of blood strain by *Bacillus cereus* KLG 55 producing protease enzyme is a promising additive for detergent industry. Vijayalakshmi *et al.*, (2011) reported the removal of blood strain by *Bacillus* RV.B2.90.

In conclusion, over the last 10 years, several effective thrombolytic agents have been identified and characterized from microorganisms, earthworms, plants, snake venoms, insects and leeches. The thrombolytic agents are of interest as useful tools for understanding fibrinolytic mechanism and as potential therapeutic drugs. Extracellular fibrinolytic proteases produced by *Bacillus cereus* species are of main interest from a biotechnological perspective, and are not only in scientific fields of protein chemistry and protein engineering but also in applied fields such as foods and pharmaceutical industries. The genus *Bacillus* contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains *of Bacillus cereus.*

Microbial enzymes are preferred because they are generally cheaper to produce; their enzyme content is more predictable, controllable, limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties.

The most significant characteristics of *Bacillus cereus* KLG 55 are rod shaped, gram-positive microorganism it grew well on the media. The colony surface was rough, white in colour.

However, studies on the production of fibrinolytic proteases from *Bacillus cereus* remain unexploited.

Different natural substrates collected from various places of Bangalore were screened for the isolation of potent fibrinolytic protease producing *Bacillus cereus sp.* The isolates formed from the culture samples were tested for their proteolytic activity on milk casein agar medium.. The taxonomic studies were earned out for the selected promising isolate (KLG 55). A close scrunity of the literature indicated that the isolate (KLG 55) was related to *Bacillus cereus* but different in some biochemical characteristics. In view of some significant

differences from the reference culture, the isolate was considered to be the novel strains of *Bacillus cereus* and designated as *Bacillus cereus* KLG 55.

The isolate (KLG 55) was used for the production of fibrinolytic protease was subjected to purification. The crude enzyme was first purified in a three step procedure involving ammonium sulphate precipitation, Sephadex G-50 gel permeation chromatography and DEAE Sephadex A-50 anion exchange chromatography. The protease was purified 43 fold. The SDS-PAGE of the enzyme showed that it has a molecular weight of 31kDa.

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How to cite this article:

Gopinath, S.M., and Lingappa, K. 2016. Identification and Characterization of the Fibrinolytic Enzymes producing Bacterium Strain (Isolate KLG 55) from the Soil Waste. *Int.J.Curr.Microbiol.App.Sci.* 5(7): 978-989. doi: http://dx.doi.org/10.20546/ijcmas.2016.507.110