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Research Article

Screening, Identification of Alkaline Proteases Producing Fungi from Soil of Different Habitats of Amalner Tahsil [Maharashtra] and Their Applications

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Abstract

Fungal proteases had wide applications in textile, leather, food and Pharmaceutical industries. As proteases shows proteolytic activity they are helpful in proteinic stain removal hence also used in various commercial detergent industries. For fungal isolation soil samples were collected from different sites of Amalner tahsil. (Maharashtra) e.g. crop fields, near dairy areas, poultry farms etc. Those soil samples showing alkaline pH were selected for isolation of fungi on Potato Dextrose Agar plates. Then among 14 different isolates 2 were selected for their most proteolytic activity after screening on casein agar, skimmed milk agar and gelatine agar. For submerged fermentation, these selected isolates were inoculated in production media in shake flask. After 72 hrs, plate assay was performed by taking crude enzyme after filtration and centrifugation as well as by taking partially purified enzyme.(partial purification done by ammonium sulphate precipitation method). Protease activity assay was performed by agar well diffusion method, as well as blood clot lysis activity and blood stain removal ability of protease obtained from selected isolates was studied.Selected isolates were identified,among them *Aspergillus niger* gives more proteolytic activity than *Trichoderma longibrachiatum.*

Keywords: Alkaline protease; Casein agar; Skimmed milk agar; submerged fermentation; Protease assay.

Introduction

Proteases constitutes large and complex group of enzyme that plays importantrole in nutrition and various applications in medical and industrial field. A variety of organisms such as bacteria, fungi, yeast, Actinomycetes are known to produce these enzymes.(Kalpanadevi *et al*., 2008).Molds of genera *Aspergillus, Penicillium* and *Rhizopus* are especially useful for proteases (Sandhya *et al.,2005). Aspergillus clavatus* ESI has been recently identified as producer of an extracellular bleaching stable alkaline protease (Oyeleke *et al*., 2010)

The main drawback with production of bacterial protease is the requirement of cost intensive procedures for separation of enzymes from cells. On the other hand, enzyme from fungal origin offers an advantage of separation of

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mycellium by simple filtration. Fungi are an important component of the microbiota typically constituting more of the soil biomass than bacteria depending on soil depth and nutrient conditions.

Protease is the most important industrial enzyme invests an accounting for about 60% of the total enzyme market in the word (Niyonzima and More, 2013). Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, cultural conditions and ease of cell manipulation. In the last 3 decades, there has been a tremendous increase in industrial enzyme production. The growth of industrial enzyme market has expanded to nearly 85 enzymes, which are currently in commercial production. The protease enzyme constitutes two third of total enzyme used in various industries.

Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe free enzyme and having various applications in food industries, leather industries, medical and related fields and also in detergent industries etc. (Mohanasrinivasan *et al.,* 2012)

The cost of enzyme production is major obstacle in the successful applications of proteases in industries. Protease yields have been improved by screening for hyper producing strains and/or by optimization of fermentation medium. Industrial applications of proteases have posed several problems and challenges for their further improvements. (Rao *et al.,* 1998).

In the present investigation fungi were isolated from soil samples of different locations screened for proteolytic activity. The enzyme was then produced in shake flask and then plate assay for protease activity performed by using crude and partially purified enzyme.

Materials and Methods

Sample Collection

Soil samples were collected from different sites of Amalner Tahsil, Dist.- Jalgaon(Maharashtra). viz, crop fields, poultry farms, near dairy waste drainage area. These samples were taken with sterile spatula in sterile polythene bags, 2-3 cm depth of soil.

pH Determination of Soil

1gm of each soil sample was suspended in 10 ml double distilled water by shaking vigorously for 30min.Then pH determined by using pH electrode. Those soil samples showing alkaline pH were selected.

Isolation of Fungi

For fungal isolation, protocol of Sharma *et al.* (2015) was adopted. Soil samples were serially diluted up to 10^{-6} dilutions. 0.1 ml of that dilution inoculated on sterile Potato Desxtrose Agar plates by spread plate technique. Plates were incubated at room temperature for 3 days.

Screening of Fungi for Alkaline Protease Production

Fungal isolates obtained on PDA plates were screened for the proteolytic activity on casein agar(Sethi S,2015), skimmed milk agar and Gelatin agar plates by maintaining pH of media 8.5. In these medias streptomycin (1 mg/100ml) was added to restrict bacterial growth. Composition of casein agar media (gm/100ml) is- Casein, 1.0g; KH_2PO_4, 0.1g; $MgSO_4$, 0.2g; Agar, 3.0; pH 8.5.

Components of skimmed milk agar (gm/ 100 m)l are –Yeast extract,0.5g; beef extract, 0.3g; Sodium chloride, 0.5g; Skimmed milk powder, 0.5g; KH_2PO_4, 0.1g; Dextrose, 0.3g; Agar, 3 g; pH 8.5

Fungal isolates were spot inoculated at the centre of above media plates and incubated at room temperature. for 3 days. The strain that shows maximum clear zone was selected for protease production after identifying it. The pure cultures of selected isolates were maintained at 4^{o}C.

Identification of Selected Isolates

Based on morphological characteristics by performing staining with lactophenol cotton blue and microscopic observation of spores and their arrangement, the protease producing fungi were identified and DNA sequencing of isolates and phylogeny was carried out. According to that the fungal isolate denoted by G is – *Trichoderma longibrachiatum* and isolate B is- *Aspergillus niger.*

Production of Protease Enzyme

Fermentation was carried out in Erlenmeyer flasks (250 ml) containing production media. The production media having following components in gm/100 ml – Casein 1g; Yeast extract 0.6 g; Urea 0.6g ; $NH_4H_2PO_4$ 0.6g; $(NH_4)_2SO_4$ 0.6g; NH_4Cl 0.6g; DDW 100ml; Glucose 1gm; pH 8.5.

In Production media 2ml of spore suspension of selected isolates was inoculated, flasks were kept on shaker at 120 rpm for 72hrs incubation.

Extraction of Crude Enzyme from Fermentation Media

Extraction of enzyme was done by simple filtration of media using whatman filter paper no.1 for separation of fungal mycelia(Karthik J,2014). Then filtrate was centrifuged at 10,000 rpm for 15min. Clear supernatant was taken as crude enzyme (Sind and Hamid, 2013).

Plate Assay by Agar Well Diffusion Method

Proteolytic activity of crude enzyme was checked by agar well diffusion method respectively on casein agar, gelatine agar and skimmed milk agar plates. Equal amount of crude enzyme (0.5µl) was dispensed in wells bored in casein, gelatine and skimmed milk agar plates respectively. Plates were incubated at room temperature, for 48 hrs. Zone of proteolysis was observed as clear zone of hydrolysis around agar well. For more clearance of zone casein agar plate was flooded with 5% Bromocresol green (Ponnuswamy, 2013) and Gelatin agar plate flooded with acidic $HgCl_2$. Zones were measured by using zone measurement scale.

Partial Purification of Protease

After centrifugation of filtrate at 10,000 rpm for15 min., supernatant was collected. Solid $(NH_4)_2SO_4$ was added to it by continuous mixing on ice bag (80% saturation). Then it kept for overnight period at 4°C for precipitation of protein. Again it centrifuged at 10,000 rpm for 20 min. Pellet was collected and dissolved in 15 ml phosphate buffer, and then carried out dialysis by using dialysis bag and magnetic stirrer. Again, by taking partially purified enzyme proteolytic activity was determine by agar well diffusion method.

Lab Scale Applications of Protease Enzyme

Blood clot lysis activity

Venous blood collected from healthy person was transferred in clean and dry test tubes and incubated at 37°C for 45 minutes. When clot formation takes place, serum was removed. Clot weight was measured by using formula-

Clot weight= Wt. of clot containing tube – Wt. of empty tube.

In two clots containing tubes 0.5 ml of crude enzyme obtained from both fungal isolates were added respectively. Tubes incubated at $37^0$for 90 min. Then fluid removed and tubes again weighted to observe wt. difference after clot disruption. Difference obtained in wt. before and after clot lysis was expressed as % of clot lysis. For control instead of enzyme, water is added (Chandrasekaran *et al.,* 2015)

Blood stain removal studies

Alkaline proteases also useful to remove blood stains on cloth, and can used as detergent additives. This was studied by Washing tests- 6 white cloth pieces (5×5 cm) stained with blood and dried them completely. Two sets of 3 cloths were prepared .They soaked in 100 ml distilled water (as control), 100 ml D/W+ 1% detergent and 100ml D/W +1% detergent+1ml partially purified enzyme (of both isolates separately). Kept them for 1 hour, after that results were noted.(Choudhari V.,2012).

Results and Discussions

Sample Collection

Source- Different soil samples were collected from different sites of Amalner city, Dist. Jalgaon , Maharashtra (Fig. 1). Among them, those showing alkaline pH were selected for this study. pH of soil samples used in this study is shown in Table 1.

Table 1: Determination of pH of soil samples.

Area	Soil from	pH
	Pome Granet plant	7.60
	Dadar crop	7.40
	Bajara crop	8.19
	Groundnut	8.15
Dairy	Soil near dairy waste drainage	9.00
Poultry farm	Soil from poultry farm	8.10

Isolation of Fungi from Soil Samples

14 different fungi were isolated from different soil samples on Potato Dextrose Agar plate.

Screening for Proteolytic Activity

All 14 isolates were spot inoculated in the centre of casein agar, gelatine agar and skimmed milk agar plates having pH 8.5. Among them those showing maximum zone of proteolysis were selected for this investigation, named as Isolate B and G.(Two isolates were selected.)

Identification of Selected Isolates

Based on morphological characteristics by performing staining with lactophenol cotton blue and microscopic observation of spores and their arrangement, the protease producing fungi were identified. Moreover, DNA sequencing of isolates and phylogeny was carried out. According to that the fungal isolate denoted by G is – *Trichoderma longibrachiatum* and isolate B is- *Aspergillus niger* (Fig 2).

Fig. 1: Collected soil samples from different sites.

Fig. 2. Pure culture of selected Isolates.

Plate Assay by Agar Well Diffusion Method (Sudarkodi C, 2015)

Fig. 3 shows the result obtained for zone diameter of proteolysis by fungal isolate in three different media. Aspergillus niger showed highest proteolysis in skimmed milk agar. *Trichoderma longibrachiatum* showed highest proteolysis in Gelatin agar medium. On the other hand zone diameter of proteolysis in the presence of protease enzyme, both genera showed highest activity in Gelatin agar (Fig. 4)

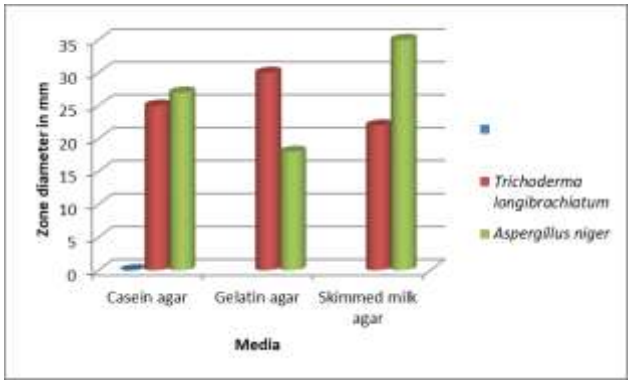

Fig. 3: Zone of proteolysis by fungal isolates.

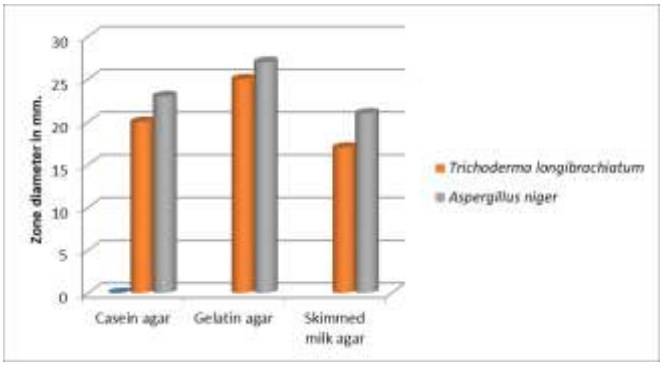

Fig. 4: Zone of proteolysis by protease enzyme.

Lab Scale Applications of Protease Enzyme

It is evident by the data shown in Table 2 that 0.1% & 0.5% clot lysis occurred by *Trichoderma longibrachiatum* & *Aspergillus niger* respectively.

Blood Stain Removal Activity

Blood stain removal activity is shown in Fig. 5.

Table 2: Blood clot lysis activity

Blood clot lysis activity of	**Wt. of empty tube**	**Wt. of blood clot containing tube**	**Wt. of blood clot**	**Protease added**	**Incubation at 37ºc for 90 min. After that fluid removed**	**Reweight blood clot tube**	**Wt. of blood clot**	**% of clot lysis**
Trichoderma longibrachiatum	4.3 g	5.0 g	0.7 g	1 ml		4.9 g	0.6 g	0.1%
Aspergillus niger	4.3 g	5.0 g	0.7 g	1 ml		4.5 g	0.2 g	0.5%

% of clot lysis = Wt. of blood clot before lysis - Wt. of blood clot after lysis

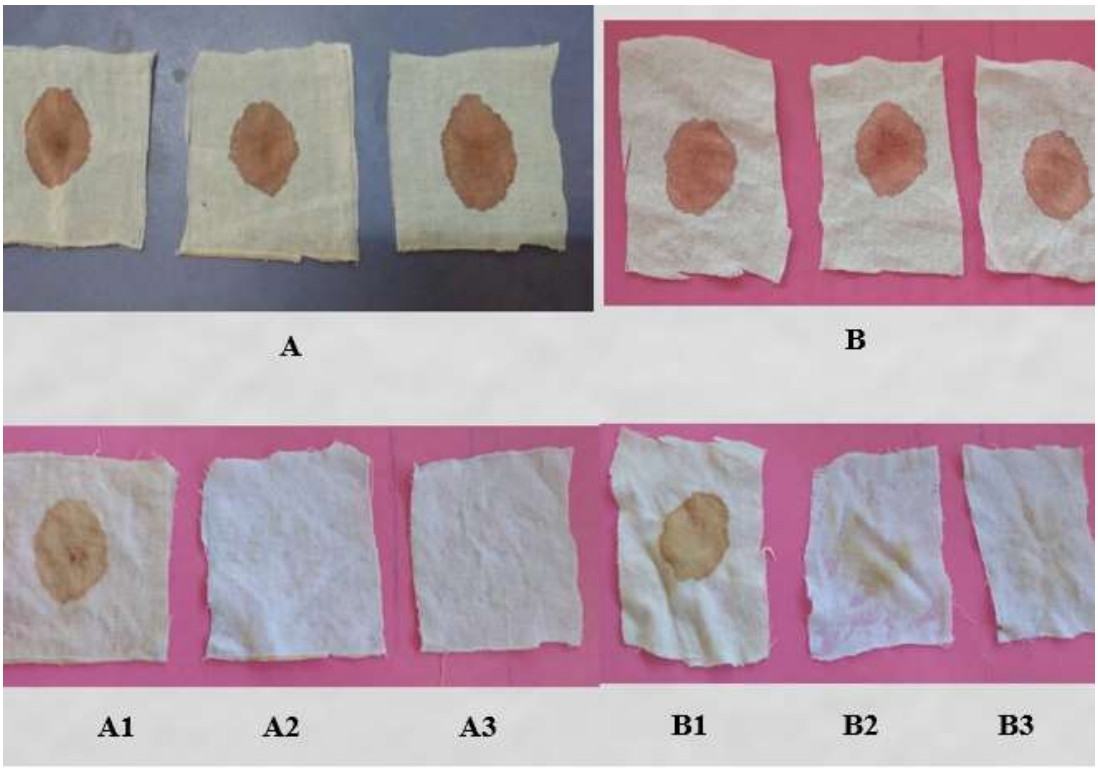

Fig. 5: Results of blood stain removal activity.

A and B- Cloth pieces stained with blood and dried, **A1, B1**- After soaked in 100 ml distilled water for 1 hour and washed.
A2, B2- After soaked in 100 ml distilled water + 1%detergent for 1 hour and then washed.
A3 – After soaked in 100 ml distilled water + 1% detergent + 1ml enzyme. (Protease from *Aspergillus niger*).For 1 hour.
B3 – After soaked in 100 ml distilled water + 1% detergent + 1ml enzyme (Protease from *T. Longibrachiatum*)

Table 3: Washing test of alkaline protease (Aftab S *et al.*,2006)

Blood stain removal	Water	Water + Detergent	Water+Detergent+Protease of *A. niger*	Water + Detergent+ Protease of *T. longibrachiatum*
	+	++	++++	+++

+ = Poor stain removal, ++ = Good stain removal, +++ = Very good stain removal, ++++ = Better stain removal.

Discussion

Two fungal isolates selected for this investigation were identified as *Trichoderma longibrachiatum* and *Aspergillus niger* according to DNA sequencing and phylogeny reports obtained from Saffron lifesciences, Navsari, Gujrat.

From resuts of study, it could be concluded that *Trichoderma longibrachiatum* and *Aspergillus niger* both are alkaline protease producers. Among them *Aspergillus niger* shows more proteolytic activity, by producing clear zones - 27mm, 18mm, 35mm on casein agar, Gelatin agar and skimmed milk agar respectively.Where as *Trichoderma* shows - 25mm, 30mm, and 22mm.zone of proteolysis. After submerged fermentation in production media having pH 8.5 and Protease extracted after 72 hours from *Aspergillus niger* gives more proteolytic activity than *Trichoderma longibrachiatum* after performing plate assay. Lab scale study to check blood clot lysis activity was done . Protease obtained from*Aspergillus niger gives 0.5%* clot lysis after 90 minutes where as Protease of*Trichoderma longibrachiatum* shows 0.1% clot lysis after 90 minutes. In washing test for blood stain removal,protease obtained from *Aspergillus niger* gives more satisfactory results than protease from *Trichoderma longibrachiatum.*

Conclusion

Protease are group of enzymes,which hydrolyze peptide bonds in proteins.And have a number of commercial applications.

Further more deatail study will provide better results and may play vital role in pharma industries as the protease is helpful for blood clot lysis.The major use of microbial protease is their addition in domestic detergents for the digestion of proteinaceous stains of fabrics.

In blood stain removal studies protease obtained from both fungal isolates effectively removes blood stains from cloth piece when added along with detergent in water. So these proteases can be effectively used a detergent additives in detergent industries.

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