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MICROBIAL PROTEASE –SCREENING CONFIRMATION AND BIOSYNTHESIS THROUGH SUBMERGED FERMENTATION FROM *ASPERGILLUS SP*

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ABSTRACT

Proteases occur every living being in nature. They are secreted inside or outside of the cells for various physiological functions. Proteases have been successfully produced by researchers from different microbial sources. Microbes account a two-thirds share of commercial protease around the globe. Twelve *Aspergillus sp* were isolated from stressed soil samples from different areas in Bangalore. Isolated fungal strains were screened for Protease production by plate assay (casein plate). *Aspergillus sp KP* 10were selected as best protease producer (1.08 cm) and were confirmation carried out by Thin layer chromatography (Rf value is 0.71). The *Aspergillus sp KP* 10 were employed for further biosynthesis of protease studies. The different pH (6), temperature (30^0C) and inoculum size (1.25 ml) were optimized and it showed 1.9 IU of protease activity.

KEYWORDS: Protease, thin layer chromatography, Plate assay and *Aspergiluus* sp.

1. INTRODUCTION

Enzymes are found in all the kingdoms of life like archaebacteria, eubacteria, prostista, fungi, plantae and animalia. Their occurrence is all over the world. The enzymes found in every living organism have important physiological functions. In regard to enzyme production, the enzymes mainly produced by microorganisms gained the prior attention because of several reasons (Renge *et al.*, 2012).

Enzymatic applications have been enforced in a wide array of industries. Because they are specific and role of action is fast. They have a great deal of saving raw materials, energy consumption, chemicals or water compared to conventional methods of various industries. The industries of daily life products like food, paper, textile, feed, pharmaceutical, clinical and chemicals have incorporated enzymatic applications. Because they are not only influencing the above factors but also follows go-green formula cause great impact on our environment and quality of life (Jegannathan and Neilsen, 2013).

Proteases have been successfully produced by researchers from different microbial sources. Microbes account a two-thirds share of commercial protease around the globe. Since the advent of enzymology, microbial proteolytic proteases have been the most widely studied enzyme. These enzymes have gained interest not only due to their vital role in metabolic activities but also due to their immense utilization in industries (Rao et al., 1998; Sandhya et al., 2005;Younes and Rinaudo, 2015).

Proteases occur every living being in nature. They are secreted inside or outside of the cells for various physiological functions. Plant, animal, microbial, fungal and bacterial sources are employed in protease production. The present work describes on isolation and screening of protease from *Aspergillus* sp. The effect of fermentation parameters such as pH, temperature and inoculm size for the biosynthesis of protease through submerged fermentation process.

2. MATERIALS AND METHOD

Collection of samples

Soil sample was used to isolate fungal strains which produce protease. Environmentally stress soil where limitation of nutrients provides stress-tolerant fungal strains. Four different soil samples from in and around Bangalore, such as soil from leather waste, near Playing ground, Near Bangalore university campus and cultivated soil were selected for isolating Protease producing fungi and Samples were stored at 4^0C.

Media preparation

The fungi were isolated from the soil sample on Czapek Dox agar (CZA) medium. CZA medium composition is as follows. Glucose, 30g; $NaNO_3$, 2g; K_2HPO_4, 1g;

$MgSO_4$, 0.5g; KCl, 0.5g; $FeSO_4$, 0.010g; Agar, 15g and pH 6.5+_0.2 (1L Distilled water). The media was sterilized by autoclaving at 121^0C of temperature, 15lbs of pressure for 15 minutes, and then CZA plates were prepared for the inoculation of the soil sample. Fungal strains were isolated from soil samples through serial dilution method (Plate-1).

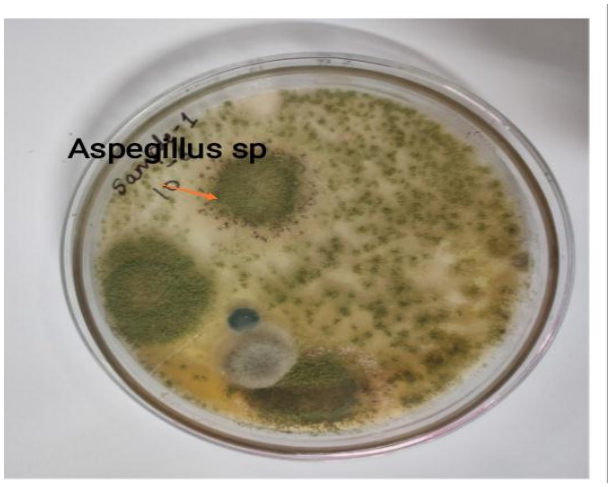

Plate-1: *Aspergillus sp.*

Screening of Alkaline Protease producers by plate assay

The composition of medium is used for screening of isolated strains for their protease activity is as observed. 2g of Glucose; 0.5g of skim milk or casein; 1.52g of KH_2PO_4; 0.52g of KCl; 0.52g of $MgSO_4.7H_2O$; trace amount of $CuNO_3.3H_2O$; traces of $ZnSO_4.7H_20$; trace levels of $FeSO_4$ and 20.0g of agar in 1L of distilled water with pH-5.0. (Karthic et al., 2014). (plate-2).

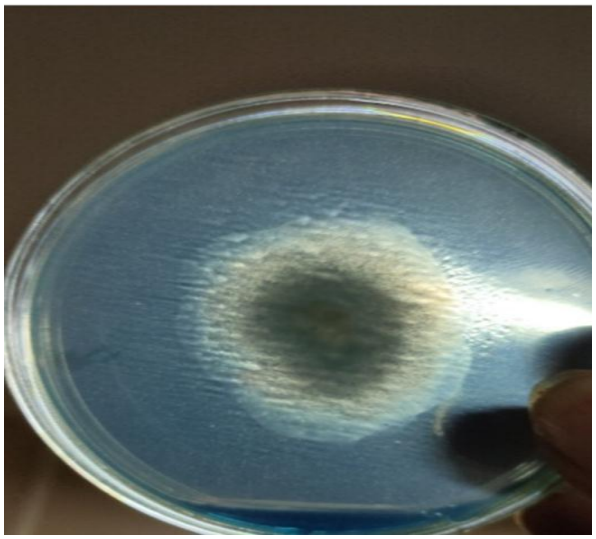

Plate-2: Plate assay.

Rapid confirmation of alkaline protease producing *Aspergillus sp*by thin layer chromatography

Rapid protease production confirmation by *Aspergillus sp* PK 10 was conducted through thin layer chromatography (TLC) of the enzyme extract (Plate 3). TLC plates were prepared and So the solvent system consists of water and phenol in a ratio of 1:1 in volume. The protease hydrolyzed product run against tyrosine amino acid to confirm the activity of protease. The enzyme activity, or an amount of tyrosine produced was roughly estimated by redness of the spot developed by ninhydrin reagent. Rf values were calculated (Radhika Pilli and K.G. Siddalingeshwara, 2016).

S 1- Standard -Tyrosine
S 2 – Tyrosine- Casein hydrolyzed by Protease
Plate-3: Thin Layer chromatography for Confirmation.

Fermentation Medium Composition

The selected *Aspergillus* sp KP 10 were cultured on production medium. The production medium consists (1L of distilled water) 30.0g of Sucrose; 2.0g of Sodium nitrate-; 1.0g of K_2HPO_4, 0.5g of $MgSO_4$. $7H_2O$; 0.5g of KCl and 0.01g of $FeSO_4$ with pH 6.8 for 96 -120h. The selected *Aspergillus* sp KP 10 were cultured on production medium. The production is carried out in Czapek Dox broth for better yield and pH 6.0, temperature 35^0C and 1.0 ml inoculum were used.

Extraction of Protease From Fermentation Medium

The samples were gathered aseptically from time to time at every 24 h. The sample extract was sieved through Whatman filter No.1. The extract was clear and was centrifuged at 2000- 3000 rpm for 15 min. The prepared supernatant was crude enzyme extract. Thus prepared crude enzyme was administered for assay of protease.

Quantitative Assay of Protease Activity

Protease activity was resoluted through the modified process of Keay *et al.*, (1970). A mixture of 0.5 mL suitably diluted enzyme extract, 1.0 mL 1% casein and 0.5 mL phosphate buffer (10 mM, pH 6.8) is incubated at

35^0C for 10 min. The reaction was ceased with 3 mL of 10% TCA solution. The solution was let to stand for 10 min in cool and then was filtered. Further, 5 mL of 0.4 M Na_2CO_3 as well as 0.5 mL of Folin Ciocalteau reagent (FCR) was added to the clear filtrate. Mixed vigorously and incubated at 37^0C for 30 min in dark and measured the absorbance at 660 nm.

International unit (IU) is One protease unit which was defined as the amount of enzyme that released 1 µg of tyrosine per mL per minute under the assay conditions mentioned above.

Optimization of Fermentation Kinetics For Biosynthesis of Protease

Effect of Initial pH on Protease

250 mL Erlenmeyer flasks contained 100 mL of fermentation media were prepared and initial pH of the media were adjusted. The adjusted initial pH of fermentation media were ranging from 3-7 with increments of 1.0. Consequently prepared flasks were cotton plugged and sterilized by autoclave at 15 lbs, 121^0C for 15 min. The flasks were aseptically inoculated with freshly prepared spore suspension and incubated.

Effect of Temperature on Protease

100mL of the fermentation media were collected separately in 250 ml Erlenmeyer flasks and prepared for submerged fermentation. Thus prepared flasks were incubated at different temperatures like 25^0C, 30^0C, 35^0C and 40^0C.

Effect of inoculum Size on Protease

The inoculum was prepared by 168h freshly prepared culture of *Aspergillus sp* KP 10 at different levels i.e., 0.25, 0.50, 0.75, 1.0 and 1.25 mL and then inoculated and fermentation studies were carried out. As it was mentioned before, the media was extracted during every condition of pH or incubation temperature or inoculum size to prepare crude enzyme for each and was used to assay the protease activity.

RESULTS AND DISCUSSION

In the present study, twelve potent fungal strains were designated, out of Twelve (12) isolates were isolated from soil samples collected at varying environmental stress conditions, they were isolated from in and around Bengaluru Karnataka, India.

The results of plate assay were employed for screening of Protease producers. Plate assay method was used to select potential strains by observing clear zone in the plate around the colony. The protease producers i.e *Aspergillus sp* were segregated as poor protease producers have less than 6mm of zone of diameter belong to medium protease producers and fungi have 6 to 9 mm of zone of diameter, which were good Protease producers and fungi have above 9mm of zone of diameter which were said to be potential producers. According to above segregation, *Aspergillus sp* KP10 emerged as the greater zone of diameter 1.08 cm and considered as potential strain for protease production among the strains isolated from the soil. So the fungal strain KP10 was selected for the further studies. Karthic *et al.*, (2014) studies on screening of protease production by *Aspergillus oryzae* through casein hydrolysis and detected 1.15 cm clear protein hydrolytic zone around the colony. Our results are good agreements with Karthic *et al.*, (2014).

The results on the confirmation of alkaline protease production from fungal strain using Thin Layer Chromatography were presented in Plate 3 and Table 1. The catalyzed casein samples were subjected to TLC for rapid confirmation. The crude enzyme extracts ran against standard tyrosine sample which exhibited similar Rf value (0.88) was selected. In the present study, *Aspergillus sp KP* 10 exhibited Rf value of 0.71 which is almost the same Rf value to standard (0.73). These results clearly indicated that the metabolite produced by *Aspergillus sp KP* 10was confirmed as protease. Radhika Pilli and Siddalingeshwara K G (2016) were reported on confirmation of protease by TLC and showed Rf value of 0.84. and our results were concides with Radhika Pilli and Siddalingeshwara K G (2016).

Table 1: Confirmation of Protease from *Aspergillussp KP* 10 by Thin Layer Chromatography.

S.No.	SAMPLE	Rf value
1	Standard Tyrosine	0.73
2	Tyrosine hydrolyzed product from Casein	0.71

The results obtained in the present study on the effect of initial pH in submerged fermentation of alkaline Protease production by *Aspergillus sp KP 10* is represented in Table-3. It reveals that the Protease production rose with the increasing of pH of the medium from pH 3.0, up to pH 6.0 and then further increase in initial pH caused the declining of Protease yield. These increasing peaks were observed up to 72 hours of fermentation period and thereafter the decreased yield as fermentation period increased. The maximum Protease activity 1.2 IU was obtained at pH 6.0 for 72 hours of fermentation period. The least Protease activity was obtained at pH 3.0 with *Aspergillus sp* KP 10 strain and it showed 0.2 IU at 96 hours of fermentation period.

Some microorganisms exhibited growth and optimum productivity at initial pH of high alkalinity (pH 10.0) for *Aspergillus oryzae* and *B. licheniformis* TISTR 1010;

furthermore, *B. circulans* accounted optimum initial pH 10.5 and *Bacillus sp.* 2-5 reported optimum initial pH 10.7 (Srinubabu *et al.*, 2007). Karthic *et al.*, (2014) were highlighted effect of pH on protease production and represented pH 6.0 was found optimum and our results are good agreement with Karthic *et al.*, (2014).

Table 2: Effect of different pH on Protease production.

pH	Protease Enzyme activity (IU)
3	0.2
4	0.35
5	0.60
6	1.2
7	0.9
8	0.72

The results obtained in the present study on the effect of temperature in submerged fermentation of Protease production by *Aspergillus sp* KP 10 is represented in Table 3. It reveals that the Protease production was increased along with the increase of temperature of the medium from 25^0C, up to temperature 30^0C with optimized constant pH of 6.0. These increasing peaks were observed up to 72 h of fermentation period and thereafter the decreased yield as temperature levels and fermentation period increased. The maximum Protease activity 1.8 IU was obtained at temperature 30^0C for 72 h of fermentation period. The least Protease activity was obtained at temperature 40^0C with *Aspergillus sp* KP 10 strain and it showed 0.3 IU at 72 h of fermentation period.

During alkaline protease fermentation, optimum incubation temperature of 30^0C for some microorganisms announced were *Pencilliumchrysogenum* IHH5, *Aspergillus oryzae* 637, *A. tamarii*, *Rhizopus sp.*, *Rhizopus oryzae*, *Pseudomonas aeruginosa* MCM B-327, P. aeruginosa PseA, *Bacillus sp*. like *B. cereus*, *B. coagulans* (Sharma et al., 2017). Karthic *et al.*, (2014) were described 35^0C were showed optimum temperature for maximum production and our results were coincides with results of Karthic *et al.*, (2014).

Table 3: Effect of temperature on Protease production.

Temperature in ^{0}C	Protease Enzyme activity (IU)
25	0.7
30	1.8
35	1.3
40	0.3

Table 4: Effect of Inoculum size on Protease production.

Inoculum Size (in ml)	Protease Enzyme activity (IU)
0.25	0.47
0.50	0.98
0.75	1.2
1.00	1.4
1.25	1.9.

The inoculum size on protease synthesis results were obtained in submerged fermentation of Protease production by *Aspergillus sp* KP 10 is represented in Table-4. Out of five inoculum size tested (0.25, 0.50, 0.75, 1.0 and 1.25 mL), 1.25 mL inoculum was found to be the most suitable for high production ofProtease by *Aspergillus sp KP 10* in submerged fermentation at 72 h of fermentation and it showed 1.9 IU and it showed lowest activity at 0.25 ml of inoculum size i. e. 0.47IU at 72 hr of fermentation period. It is clear that the Protease production steadily increased with the increasing in the size of the inoculum until it reaches to the magnitude when enzyme productivity became maximum, thereafter no appreciable change in production of Protease with high inoculum size could be observed.

Vishwanatha *et al.*, (2010) discussed the use of 1mL for optimization studies for the production of protease produced a yield with specific activity of $4.8x\ 10^5$ U/g by *Aspergillus oryzae* MTCC 5341 at 120 h of fermentation period. Radha *et al.*, (2011) marked protease production by *Aspergillus sp.* yielded maximum of 3.167 U/mL with 1% inoculum size. Karthic *et al.*, (2014), discussed that 1.7 IU/ mL was the highest enzyme yield in 1.0% of initial inoculum size conditions. Our results are good agreement with Karthic *et al.*, (2014).

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