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Study on Isolation, Optimization and Immobilization of Alkaline Protease produced by *Aspergillus Flavus*

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Research Article

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ABSTRACT

This study was conducted to isolate alkaline protease producing fungus, its identification and optimization of cultural conditions for production of alkaline protease enzyme. Preliminary screening was done on gelatin agar medium. Secondly gelatin liquification test was carried out to check the production of proteases functional at alkaline pH. The study related to process development involves optimization of different fermentation conditions towards enhancement of enzyme production for which the cultural conditions (physical and nutritional factors) during solid state fermentation for alkaline protease production by the isolated *Aspergillus flavus* were undertaken. Different types of substrates were selected for growing *Aspergillus flavus*. Alkaline protease production was found to be highest at temperature (28^{0}C), pH 8, incubation time 168 hrs, with metal ions (Mn), nitrogen source (peptone), and carbon source (sucrose) as the biggest zone of hydrolysis. The highest optical density and protein concentration was obtained at above mentioned conditions. The partial purification of alkaline protease enzyme from the culture filtrate was performed by ammonium sulfate salt precipitation which was immobilized using sodium alginate immobilization technique. Washing test resulted in complete removal of blood stain which indicates this enzyme can be proved crucial for detergent industry.

INTRODUCTION

Enzymes are proteins that catalyze biochemical reactions [1]. Protease breaks down peptide bonds to produce amino acids and other smaller peptides [2]. They are classified according to their maximal activity in a particular pH range as acidic, neutral or alkaline; or characteristic active site group as metallo, aspartic, cystein, or serine protease [3]. Although proteases producing microorganisms, plants and animals have cosmopolitan distribution in nature; microbial community is preferred over the others for the large scale production of proteases due to their fast growth and simplicity of life for the generation of new recombinant enzymes with desired properties. Alkaline protease of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures [4]. The microbial alkaline proteases find their largest use in house hold laundry with a worldwide annual production of detergents of approximately 13 billion tons [5].

The soil is a dynamic medium for microbial/ biological activities. Soil borne fungi are adopted to survive at a broad range of pH. A number of workers provided information indicating wide occurrence of fungi in soils of various habitats including rhizosphere

soils, poultry farm soils [5], garden soils, sandy soils, soil samples from butcheries etc. To produce a specific product such as protease enzyme, the mode of fermentation (solid or submerged) is very much crucial for obtaining maximum yield [6]. The cost of enzyme depends on its production strategy and downstream processing. Submerged fermentation is more extensively used for production of enzymes on commercial scale but solid state fermentation has several advantages over submerged fermentation like minimal water requirement, less complicated downstream processing etc. Among the different microbial groups attempted, filamentous fungi have scored high commercial acceptance in SSF [7].

Keeping all this in mind, the present work was designed to isolate alkaline protease producing fungi from bamboo forest soil and to optimize various culture conditions for higher enzyme production by them. An effort was made to immobilize the enzymes produced and keep them active for future use.

MATERIALS AND METHODS

Selection of sample sites

Bamboo forests soil was selected as the sample site for present investigation. Three sites were chosen *viz*. TFRI area, SFRI area and GCF area in Jabalpur District of Madhya Pradesh.

Sample collection and processing

Soil samples were collected at different sample points from the undisturbed localities employing sterile soil augers, hand trowel and polythene bags. The soil was dug out using augers up to 20 cm depth and was immediately scooped into sterile polythene bags using hand trowel. The samples were collected from 5 spots in each site and then mixed together in order to obtain a representative sample and bought to the lab for further studies. Samples were serially diluted up to 10^5 dilutions for use.

Isolation of fungi

Potato dextrose agar media and Rose Bengal agar media were prepared (pH adjusted to 10) and autoclaved. 1 ml Serially diluted samples were inoculated into pre sterilized and pre solidified PDA and RBA agar plates by pour plate method and incubated at 28^{0}C for 2-3 days.

Identification of fungal isolates

The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28^{0}C for 48 hours in order to obtain pure fungal culture. Colony morphology and microscopic examination of the fungal isolates **(Figure 1)** were used to determine reproductive and vegetative structures. Identification was carried out using a textbook of mycology [8].

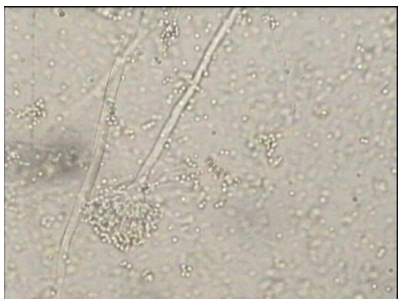

Figure 1. Microscopic image of *Aspergillus flavus.*

Preliminary estimation of alkaline protease production (clear zone of hydrolysis test)

Gelatin agar media was prepared and sterilized. Fungal culture was inoculated by point inoculation method on the prepared plates and kept at 28^{0}C for 72 hrs. After 24 hrs of incubation the plates were observed for clear zone of hydrolysis using 10% TCA solution. The fungal isolate with highest zone of hydrolysis was used for further studies **(Figure 2).**

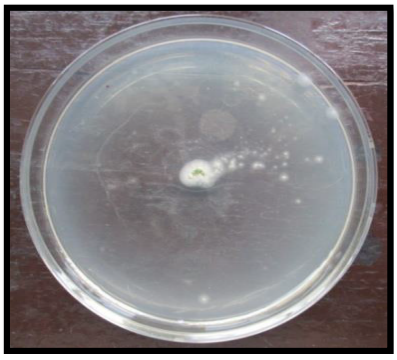

Figure 2. Clear zone of hydrolysis on gelatin agar medium.

Gelatin liquification test

Gelatin media was prepared, sterilized and stabs were prepared. Inoculation of different fungal isolates was performed in a vertical manner and incubated at 28^{0}C for 3 days. The tubes were taken out and kept at freezer for 30 min and observed for solidification.

Measurement of fungal biomass

The selected fungal culture was inoculated in presterilized PDA broth and incubated upto 5 days at 28^{0}C. The fungal mat obtained after incubation was filtered through preweighed Whatman filter paper #1, dried using oven and weighed.

Mass production

The selected fungal isolate was mass produced by solid state fermentation for which 10 g of rice broken was taken in flask and moistened with 20 ml of mineral salt solution (KH_2PO_4 -0.7 g, K_2HPO_4 – 0.7 g, $MgSO_4.7H_2O$ – 0.7 g, NH_4NO_3 – 1.0 g, NaCl – 0.005 g, $FeSO_4.7H_20$ – 0.002 g, $ZNSO_4.7H_20$ – 0.002 g, $MnSO_4.7H20$ – 0.001 g, Distilled water – 1000 ml), sterilized, cooled, inoculated and incubated at 28^{0}C for 120 hrs.

Extraction of crude enzyme

10 ml of 0.1% tween 80 solutions was added to 2 g of fermented substrate and homogenized on rotary shaker at 180 rpm for 1hr. The solids were removed by centrifugation at 8000 rpm at 4^{0}C for 15 min and the resultant clear supernatant was used for further studies.

Optimization

Optimization of culture conditions was carried out for different parameters *viz* pH, temperature, nitrogen source, carbon source, incubation period, and metal ions for protease production.

pH

3 conical flasks were taken to each of which 10 gm broken rice and 20 ml mineral salt solution was added, sterilized and cooled. The pH was adjusted at 8, 10 and 12 using 0.1 N HCl and 0.1 N NaOH. Flasks were kept in BOD incubator at 28^{0}C for 5 days.

Temperature

The same procedure was performed again except that the three flasks were incubated at different temperatures i.e. 28^{0}C, 40^{0}C and 60^{0}C for 5 days.

Nitrogen source

3 conical flasks were taken to each of which 10 g broken rice and 20 ml mineral salt solution was added which was supplemented with different (0.5%) nitrogen sources *viz* peptone, beef extract and ammonium sulphate, sterilized and cooled. The selected fungus was inoculated and incubation was carried.

Carbon source

3 conical flasks each containing 10 g of broken rice with 20 ml mineral salt solution were supplemented with different carbon sources (glucose, sucrose, starch) sterilized and cooled. All the flasks were inoculated with the selected fungi.

Metal ions

Different metal ions in the form of $MnSO_4$, $FeSO_4$, $ZnSO_4$ were added to three Erlenmeyer flask each containing 10 g of broken rice with 20 ml mineral salt solution and incubated at 28^{0}C for 5 days.

Incubation period

To study the effect of incubation time on protease production three conical flasks with similar substrate and inoculum were incubated at same temperature for different time intervals *viz* 24 hrs, 72 hrs, 168 hrs and observed after 5 days.

Quantitative estimation for production of protease

Quantitative estimation for alkaline protease production was performed as per the method of Chopra and Mathur. 2 gm of fermented substance was taken in a test tube to which 10 ml of 1% tween 80 solution was added. This was shaked on rotary shaker for 1 hour at 180 rpm. Centrifugation was carried out at 10,000 rpm for 20 min at 4^{0}C. The pellet was discarded while supernatant was used as cell free extract (enzyme solution) for further study. 1ml of 2% casein was used as protein sample to which 1ml of enzyme solution was added. It was incubated at 40^{0}C for 10 min. After this the reaction was ceased using 2ml of 10% TCA solution and kept constant for 20 min at room temperature, centrifuged at 8000 rpm for 10 min. Supernatant was collected to which 0.5 ml each of Lowery's reagent I and II was added and incubated for 20 min. after which optical density was measured at 650nm.

Partial purification of protease enzyme

Enzyme purification is one of the crucial steps during industrial enzyme production process. In present study the alkaline protease enzyme was partially purified by ammonium sulphate precipitation method. The supernatant was saturated with 20%, 40% and 80% ammonium sulphate solution.

Immobilization

Both whole cell and cell free immobilization was carried out for the fungi and protease enzyme respectively. For whole cell immobilization the fungal mat was crushed and dissolved in 2% sodium alginate solution (1:1) while for cell free immobilization the precipitates obtained upon treatment with ammonium sulphate solutions were filtered out and re-dissolved in TrisHCl. This solution was mixed with sodium alginate (2%) in 1:1 ratio. These mixtures were then dropped into the chilled solution of 0.2 M $CaCl_2$ with continuous shaking at 4^0C. Beads were washed 3-4 times with deionised water and finally with 50mM TrisHCl buffer and rechecked for its activity **(Figure 3).**

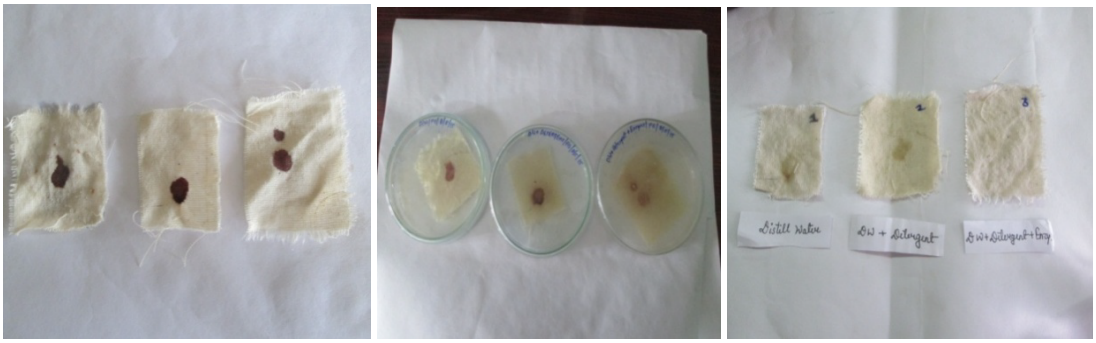

Figure 3. Washing test for protease enzyme.

(a): Blood stain on muslin cloth. (b): Incubation with (i) DW (ii) DW with detergent (iii) Detergent solution with alkaline protease enzyme. (c): Muslin cloth after washing indicates removal of blood stain.

Washing test

3 pieces of muslin cloth were taken and blood stain was placed on each of it and allowed to dry for15 min. 3 petriplates were taken, in first petriplate distilled water (DW) was added, in second distilled water and detergent was added while third petriplate contained a mixture of distilled water, detergent and alkaline protease enzyme solution. Muslin cloths with blood spot were placed one in each pertiplates and left for 15 min. The cloths were then taken out, dried and observed for removal of blood stains **(Figure 4).**

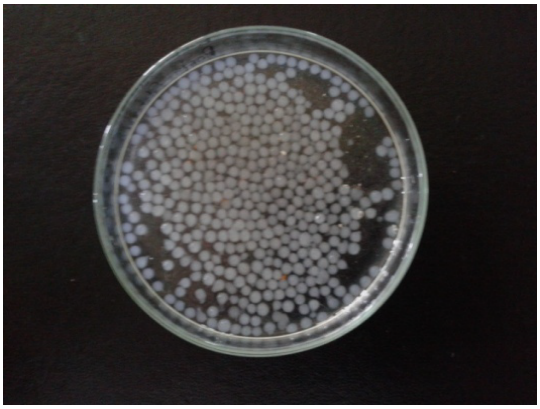

Figure 4. Immobilized enzyme beads.

RESULTS AND DISCUSSION

Different soil samples were serially diluted up to 10-5 dilution inoculated on Potato Dextrose Agar and Rose Bengal Agar and after incubation 30 fungal isolates were obtained on the plates which belonged to 10 different genera including *Aspergillus, Penicillium, Fusarium, Rhizopus, Mucor, Alternaria, Curvularia* and three unidentified isolates. *Aspergillus sp.* was found to be dominant amongst them. Members of *Aspergillus* have been reported widely in soil since their nature allows them to have nutrients and moisture for all different studies of life cycle. The published work on global distribution of *Aspergillus* in soil has been documented by Domesch [9].The second most dominant genus in the present investigation was found to be *Fusarium* spp.

For primary screening of protease production each fungal culture was point inoculated on Reese agar medium followed by incubation at 28^0C which resulted in clear zone of hydrolysis on application of freshly prepared 10% TCA solution. Out of 10 isolates tested 6 isolates were found as alkaline protease producers. The fungal cultures showing alkaline protease production

were *Aspergillus flavus, Aspergillus niger,* species of *Fusarium, Rhizopus, Alternaria* and *Penicillium*. Out of 6 cultures tested first three had shown clear zone of hydrolysis while others gave positive results for gelatin liquefaction test. *Aspergillus flavus* gave highest zone diameter of 25 mm and hence it was used for further investigation.

Optimization

Optimization for zone diameter of *Aspergillus flavus* at different atmospheric condition like pH, temperature, incubation period and use of different metals, nitrogen source, and carbon source resulted in to presence or absence of zone with varying size. The highest diameter of zone was considered as the optimum condition for alkaline protease production **(Table 1).** Similarly highest OD and protein concentration obtained upon performing Lowry's assay for protein estimation was considered to be best for alkaline protease production by selected fungi **(Table 2).**

Table 1. Qualitative assay for protease production by clear zone of hydrolysis test.

Environmental conditions optimized		Clear zone diameter (mm)
Carbon source	Glucose	5 mm
	Sucrose	20mm
	Starch	NZ
Incubation Time	24 hours	21.6 mm
	72 hours	OG
	168 hours	OG
Metal ions	$FeSO_4$	NZ
	$ZnSO_4$	NZ
	$MnSO_4$	8.0 mm
Nitrogen source	Peptone	19.5 mm
	Beef extract	NZ
	Ammonium sulphate	NZ
pH	8	30.0 mm
	10	08.0 mm
	12	NZ
Temperature	28°C	10.0 mm
	40°C	NZ
	60°C	NZ

Table 2. Quantitative assay for protease production by Lowry's method.

Environmental conditions optimized		Optical Density	Concentration (mg/ml)
Carbon source	Glucose	1.496	13.38
	Sucrose	1.993	18.48
	Starch	1.224	10.58
Incubation Time	24 hours	0.072	1.24
	72 hours	1.820	16.70
	168 hours	2.378	22.43
Metal ions	$FeSO_4$	0.027	1.70
	$ZnSO_4$	0.740	5.62
	$MnSO_4$	1.172	10.05
Nitrogen source	Peptone	0.650	4.69
	Beef extract	0.238	0.46
	Ammonium sulphate	0.603	4.21
pH	8	1.621	14.66
	10	1.553	13.96
	12	1.354	11.92
Temperature	28°C	1.115	9.47
	40°C	0.934	7.61
	60°C	0.902	7.28

Effect of pH

Out of 3 pH ranges tested i.e. 8, 10 and 12, the diameter of zone was found to be 30 mm, 8 mm and no zone respectively. The highest diameter 30 mm at pH 8 indicates it to be best suited for this fungal isolate. Similarly highest concentration 14.66 mg/ml was obtained at the same pH. In a study conducted by Kalpana the optimum pH for alkaline protease enzyme from *Aspergillus niger* was found to 10.0. Also Derhum[10] concluded that the optimum pH for protease from *Bacillus sp.*, *Therma saquaticus* lies between 10.0 – 10.5. It shows that alkaline range suits the fungi for its own growth as well as for protease production.

Effect of temperature

Temperature is a critical factor influencing maximum enzyme activity and is necessary to optimize for industrial enzyme production. Out of 3 temperature ranges tested i.e. 28^{0}C, 40^{0}C and 60^{0}C the highest diameter of zone was found to be 10 mm at 28^{0}C while no zone were seen on other temperature ranges. Also the highest concentration 9.47 mg/ml was seen at same temperature. A similar result was obtained by Chowdhary[11] with highest alkaline protease production by *Aspergillus niger* and *Fusarium* sp. at similar temperature range. The research conducted by Nasuno and Onara showed maximum fungal biomass and protease production at 35^{0}C using *Aspergillus flavus, Aspergillus oryzae* and *Penicillium godliwskii* which supports the result obtained in current investigation.

Effect of carbon source

The effect of addition of different carbon sources in the form of monosaccharide and polysaccharide could influence the production of enzyme. For this purpose out of 3 carbon sources tested i.e. glucose, sucrose and starch, the use of sucrose gave best result with the zone size of 20 mm, optical density 1.993 and protein concentration 18.48 mg/ml. The production of protease was comparatively lesser upon using glucose as well as starch as the carbon source. Malathi and Chakraborty[12] reported sucrose to be an efficient carbon source for *Aspergillus flavus* while glucose was observed to be effective for *Conidiobolous coronatus* as reported by Lakshman[13].

Effect of incubation period

An experiment was designed to investigate the effect of different incubation period on alkaline protease production by *Aspergillus flavus*. The time periods taken were 24 hours, 72 hours and 168 hours. The highest zone of hydrolysis was obtained upon incubating the Reese agar plates for 24 hours with diameter of 21.6 mm while on performing Lowry's assay for protein estimation, highest optical density 2.378 and protein concentration 22.43 mg/ml was found at an incubation of 168 hours.

Effect of metal ions

The production of alkaline protease seems to vary with use of different metal ions in the media. Out of 3 metals used $FeSO_4$, $MnSO_4$ and $ZnSO_4$used, the highest zone of diameter 8 mm, optical density 1.172 and concentration 10.05 mg/ml was obtained with MnSO4. In a research conducted by Yuan-Chi-Su [14] it was reported that Mn^{2+} and Ca^{2+} activated the enzyme activity.

Effect of nitrogen source

Different organic and inorganic nitrogen source were tested to study the effect of nitrogen on alkaline protease production. Out of which best nitrogen source was found to be peptone with the zone diameter of 19.5 mm, optical density 0.650 and concentration 4.69 mg/ml. Also Chinnaswamy reported protease activity with peptone and beef extract which enhance the production of protease enzyme.

Soild state fermentation

The obtained alkaline protease producing *Aspergillus flavus* was mass produced using solid state fermentation. Solid state fermentation offers many advantages including use of expensive substrates, simple downstream processing and lower energy requirements with submerged fermentation. For this purpose broken rice used as protein substrate and it was proved as good solid substrates for growing fungi as enough growth was obtained after 7 days of incubation. This finding coincide with that of Sharma[15] who reported that solid state fermentation offer numerous advantages including high volumetric productivity, relatively higher concentration of the product and requirement for simple fermentation equipment. The fermented product can also be used directly as a source of enzyme.

Partial purification and immobilization

Enzyme purification is one of the crucial steps during industrial enzyme production process. In present study the alkaline protease enzyme was partially purified by ammonium sulphate precipitation method. The supernatant was saturated with 20%, 40% and 80% ammonium sulfate solution. The best precipitation was obtained with 80% saturation which was properly dissolved in Tris – HCl and gave best enzyme activity after immobilization.

The partially purified protease enzyme was immobilized in sodium alginate beads. The beads were weighed to be 2.385 gm. The beads were found to be functional as upon testing them in casein broth followed by Lowry's method, the optical density was increased. The recorded optical density for cell free immobilized beads was 1.980 while for that of whole cell immobilization was 1.432. This indicates that cell free immobilization was more beneficial and economical in regard to protease production by *Aspergillus flavus*.

Washing test

Apart from several commercial applications of proteases there use in detergent industry for de-staining is the most important potential of them. In this investigation the partially purified alkaline protease enzymes were used to remove blood stain from muslin cloth. The distilled water hardly removed any stain. A detergent solution resulted in little removal while a combination of

detergent solution with purified alkaline protease enzyme completely removed the blood stain from muslin cloth. This indicates that the protease enzyme obtained from the isolated strain Aspergillus flavus can be of great use and significance in detergent and textile industries.

CONCLUSION

Protease enzymes are of great industrial importance. This study indicates that Aspergillus flavus is a potent protease producing fungi. The alkaline protease production with Aspergillu sflavus was found to be highest at temperature (28^0C), pH 8, incubation time 168 hrs , metal ions (Mn), nitrogen source (peptone), carbon source (sucrose) with the biggest zone of hydrolysis, highest optical density and largest protein concentration at above mentioned conditions. Washing test resulted in complete removal of blood stain which indicates that along with many other industries, this enzyme can be specially proved very crucial for detergent industry.

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