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Isolation, Characterization and Optimization of Protease using *Lactobacillus* sp. and *Aspergillus flavus* isolated from the spoiled fruits

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Abstract

Protease is the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields. Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties. This paper accounts to screening and isolation of protease producing bacteria and fungi from spoiled fruits samples collected from fruit stalls at Mannargudi, Tamilnadu, India , preliminary screened for their ability to produce protease, optimization of culture condition for protease enzyme production , and assessment of the effects of incubation time, pH, temperature, carbon and nitrogen sources on protease production. The isolate *Lactobacillus sp* showed maximum activity at pH 10, incubation time 72 hrs, and at a temperature of 40^{0}C when compared to maximum activity of *Aspergillus flavus* at pH 8, incubation time of 96 hrs and temperature of 35^{o}C. Various nutrient supplements of carbon and nitrogen sources were also optimized. Highest protease production was observed with glucose as carbon source by *Lactobacillus sp* and starch by *A. flavus* . Similarly highest protease production was observed with ammonium nitrate as nitrogen source by *Lactobacillus sp*, and with sodium nitrate by *A. flavus*.

Key words: *Aspergillus flavus, Lactobacillus sp*, Optimization of culture, Protease production, Submerged fermentation.

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INTRODUCTION

Enzymes are biomolecules that catalyze chemical reaction (Grisham,1999). Almost all enzymes are proteins. In enzymatic reaction, the molecules in the substrates are converted into different molecules called the products. Enzymes function under very mild conditions of temperature, pressure and pH. Proteases are abundantly and widely distributed in biological world including plant, animal and microbes (Li *et al*., 2007). A protease, also called as peptidase or proteinase group of enzymes, that performs proteolysis is known as hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein (Hedstrom, 2002).While microorganisms are the major sources of alkaline proteases and they work better at pH range of 8 and above. Microbial proteases play a significant role in several industries such as pharmaceutical, detergent, photographic industries, waste treatment and tanning (Gupta *et al*., 2002).The protease group is vast and constitute more than 70% of commercial enzymes with diverse substrate and catalytic capabilities. The proteases have been classified based on several criteria such as targeted amino acid for hydrolysis, chemical environment and type of substrate (Cunningham *et al*., 1999). Alkaline proteases were the intact first enzymes to be produced in bulk, and current estimated value of the worldwide sales of industrial enzymes are billion and among them 75% are hydrolytic enzymes (Godfrey and West, 1996).The microbial world has been key source of various enzymes for industrial and therapeutic application since many decades. Bacterial species are on the top concern for large scale production of enzyme worldwide (Igarashi *et al*., 1979).The main advantages of the submerged processes are ease in controlling the physicochemical process, greater efficiency of nutrient absorption, and excretion of metabolites through the cells, leading to lower process times and consequently, productivity gains (Silva *et.al*., 2008). The present article deals with the isolation, characterization and optimization of protease using *Lactobacillus* spp. and *Aspergillus flavus*, isolated from the spoiled fruits.

MATERIALS AND METHODS

Sample Collection

The spoiled fruits samples such as apple, orange, banana, guava and pomegranate were collected from

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different areas of Thiruvarur district, Tamil Nadu, India. The samples were transported to the Laboratory of PG and Research Department of Microbiology , STET Women's College, Mannargudi, Tamilnadu, India for microbiological analysis. All the samples were stored at room temperature.

Isolation of Bacteria and Fungi

The bacteria were isolated from spoiled fruits by using serial dilution agar plate method. The spoiled fruits were crushed into pre sterilized mortar and pestle with distilled water to form suspension, which was serially diluted from 10^{-4} to 10^{-5} dilutions. One ml of fruit suspension from each dilution was spread over nutrient agar plates. The nutrient agar was supplemented with amphotericin B (10µg/ml) before pouring to prevent fungal growth. The inoculated plates were incubated at 37^0C for 24 hours for bacterial growth. After incubation, the morphologically different colonies of bacteria were isolated and subcultured. The bacterial isolates were maintained and stored on Man, Rogosa and Sharpe (MRS) agar slants at 4^0C for future use.

The spoiled fruits were cut into small pieces (1 cm) with a sterilized blade and surface sterilized with 0.1 % mercury chloride solution for one minute. Then pieces of fruit peels were washed with water. The sterilized pieces were inoculated into plates containing Potato Dextrose Agar (PDA) medium. Then the plates were incubated at 28°C for 5 days. After incubation, mixed fungal colonies were observed on the medium. The mycelial growth and spores were observed under microscope for identification.

Identification of Bacteria

The bacterial culture showing protease production was identified on the basis of various morphological, cultural and biochemical characteristics and its comparison was done with standard description of Bergey's Manual of Determinative Bacteriology (Garrity *et al.,* 2001).

Identification of Fungi

Based on the colony morphology and Lacto phenol Cotton Blue (LPCB) stain, the protease producing fungi were identified.(Holt *et al.,* 1994).

SCREENING FOR PROTEOLYTIC ACTIVITY

The single colonies observed on the MRS agar and PDA plates were plated again into casein agar plates (Casein 1.0g; KH_2PO_4 0.1g; $MgSO_4$ 0.2g; Agar 3.0g; pH 8.5) and were incubated at 28^0C for 3 to 5 days.

The colonies were spot inoculated at the centre of the above media plates and incubated at room temperature for 3 days. The strain that showed maximum clear zone was selected for protease production after identifying it. The pure cultures of selected isolates were maintained at 4^0C.

PREPARATION OF CULTURE INOCULUM

Bacteria

The cultures of bacteria isolated from spoiled fruit samples were used in the study was maintained on MRS agar slants at 4^0C. Inoculum was prepared by suspending a loopful of bacterial cultures into 10 ml of MRS broth and was incubated at 37^0C ± 2^0C for 24 to 48 hours.

Fungi

The potato dextrose broth containing potatoes 8g/ml; Dextrose 2.5g/ml; pH 7, were autoclaved at 121^0C for 15 minutes. It was inoculated with a single colony (which was identified *Aspergillus flavus)* and incubated at 28^0C for 3 to 5 days.

ENZYME PRODUCTION BY SUBMERGED FERMENTATION METHOD

Protease production was carried out by submerged fermentation. A 250 ml Erlenmeyer flask containing 50 ml of sterilized medium was used for production. A loopful of log phase culture was inoculated into the medium. The flask was placed in an incubating orbital shaker at 120 rpm and at 37^0C for 24 hours. Un-inoculated medium served as control. The bacterial and fungal cell mass was respectively separated by centrifugation at 10000 rpm for 10 minutes at 4^0C. The supernatant was used as crude enzyme source to determine the enzyme activity.

PLATE ASSAY BY AGAR WELL DIFFUSION METHOD

Proteolytic activity of crude enzyme was checked by agar well diffusion method on casein agar plates. Equal amount of crude enzyme (0.5µl) was dispensed in wells bored in casein agar plates. 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. Zones were measured by using zone measurement scale.

OPTIMIZATION OF CULTURE CONDITION FOR THE PRODUCTION OF PROTEASE

Optimization of pH

The optimization media were prepared and the pH was set at different pH level such as 6, 8, 10 and 12 by adding 1% N_aOH and concentrated HCl. Then the media were autoclaved. The bacterial and fungal inoculated flasks were incubated at 37^0C for 5 days and 28^0C for 7 days, respectively.

Optimization of Temperature

In order to determine the effective temperature for protease production by bacteria and fungi the optimized media were prepared and autoclaved at 121^{0}C for 20 minutes. Later it was inoculated with broth culture and was set at different temperatures *viz* ., 30^{0}C, 35^{0}C, 37^{0}C and 40^{0}C. The effect of temperature on the production of proteolytic enzyme was determined by growing the organisms at the above temperature for 5 days and 7 days for bacteria and fungi.

Optimization of Incubation period

Fermentation period is an important parameter for enzyme production. In this study, the test organisms were grown in fermentation medium. It was incubated at 37^{0}C for 24, 48, 72 and 96 hr in an orbital shaker at 150 rpm. The contents were then centrifuged at 10000 rpm at 4^{0}C for 10 minutes and protease activity was checked in the cell free extract.

Optimization of Carbon sources

Various carbon sources such as glucose, sucrose, starch, galactose and maltose were used. The medium was prepared, autoclaved and inoculated with fermentation medium containing the culture. The flasks were incubated at 30^{0}C and at 150rpm. The samples were collected for every 3hrs up to 72hrs.The optical density of the samples was measured at 600nm.The samples were then centrifuged at 10000 rpm for 10 min and the supernatant was used as sample for protease assay to calculate the units of enzyme produced. The results were tabulated and graph was plotted.

Optimization of Nitrogen sources

Different nitrogen sources such as peptone, yeast extract, sodium nitrate, ammonium sulphate and ammonium nitrate were used. The medium was autoclaved and inoculated with fermentation medium containing the culture. The flasks were incubated at 30^{0}C and 150rpm. The samples were collected for every 3 hr upto 72hr. The optical density of the samples was measured at 600 nm. The samples were centrifuged at 10000 rpm for 10 min and supernatant was used as sample for protease assay to calculate the units of enzyme produced. The results were tabulated and graph was plotted.

RESULTS

Isolation and Identification of Bacteria

Gram positive, non-motile organism showing positive result for Methyl Red, Carbohydrate fermentation test and negative results for Indole, Voges–Proskauer (VP), Citrate, Catalase and Urease tests, were isolated. The isolates were identified as *Lactobacillus sp.* From the above results, the identification of organisms were confirmed using Bergey's Manual of Systematic Bacteriology.

Identification of Fungi

The isolated colonies were identified by lactophenol cotton blue staining technique. Under microscopic observation septate branched mycelium with yellow to green colored conidia were observed. The result was compared with standard fungal identification manual. Finally, the isolated fungal species were confirmed as *Aspergillus flavus.* These isolates were used for protease enzyme production.

Screening for Proteolytic activity

The protease producing *Lactobacillus sp* and *Aspergillus flavus* were identified with the help of the zone formation in the casein agar medium. The zone is formed due to the proteolytic activity of the organisms which cleaves protein molecules present in the casein agar medium.

Plate assay by Agar well diffusion method

The zone diameter of proteolysis by bacterial and fungal isolate in casein agar media was determined .*Lactobacillus sp* showed the highest proteolysis (20mm) in casein agar plate when compared to *Aspergillus flavus* which showed 15 mm only. This result showed that *Lactobacillus sp*s to be an effective producer of Protease

OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF PROTEASE

Optimization of pH

pH is a critical physical factor which influence the fermentation process. The effect of pH on the production of protease activity by *Lactobacillus sp* at pH 6, 8, 10 and 12 showed 70 ± 0.1 IU/ml, 65 ± 0.3 IU/ml, 85 ± 0.5 IU/ml and 30 ± 0.2 IU/ml respectively. *Aspergillus flavus* showed 60 ± 0.2 IU/ml at pH 6, 75 ± 0.4 IU/ml at pH 8, 50 ± 0.3 IU/ml at pH 10 and 15 ± 0.1 IU/ml at pH 12. *Lactobacillus sp* showed maximum protease production 85 ± 0.5 IU/ml at pH 10. *Aspergillus flavus* showed maximum protease production

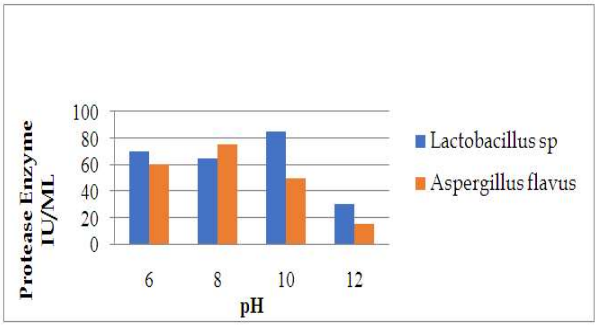

Fig.1. Optimization of pH for Protease Production

75 ± 0.4 IU/ml at pH 8 . The results were presented in Fig. 1.

Optimization of Temperature

Incubation temperature plays an important role in the metabolic activities of microorganisms. Even slight changes in temperature can affect enzyme production. The effect of temperature on the production of protease activity by *Lactobacillus sp* at 30, 35, 37 and 40°C were 50 ± 0.3 IU/ml,45 ± 0.5 IU/ml, 30 ± 0.1 IU/ml and 65 ± 0.7 IU/ml , respectively. *Aspergillus flavus* were 25 ± 0.1 IU/ml at 30°C, 50 ± 0.6 IU/ml at 35°C, 45 ± 0.3 IU/ml at 37°C and 35 ± 0.5 IU/ml at 40°C. *Lactobacillus sp.* showed maximum protease production (65 ± 0.7 IU/ml) at 40°C. *Aspergillus flavus* showed maximum protease production 50 ± 0.6 IU/ml at 35°C. The results were presented in Fig. 2.

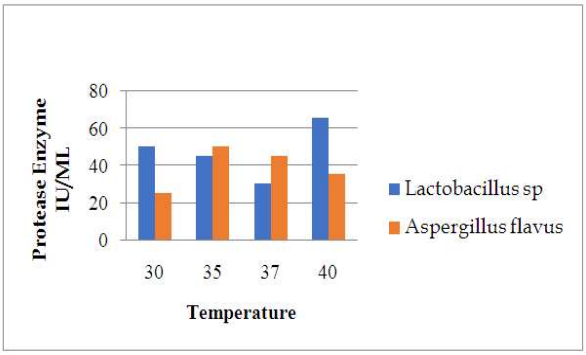

Fig.2. Optimization of Temperature for Protease Production

Optimization of Incubation period

The effect of incubation period on the production of protease activity by *Lactobacillus sp* at 24, 48, 72 and 96 hours were 15 ± 0.1 IU/ml, 12 ± 0.4 IU/ml, 25 ± 0.6 IU/ml, and 8 ± 0.5 IU/ml respectively. *Aspergillus flavus* activity were 8 ± 0.4 IU/ml at 24hrs, 10 ± 0.3 IU/ml at 48hrs, 18 ± 0.1 IU/ml at 72hrs and 20 ± 0.4 IU/ml at 96 hrs. *Lactobacillus sp* showed maximum protease production was 25 ± 0.6 IU/ml at 72hrs. *Aspergillus flavus* showed maximum protease production 24 ± 0.4 IU/ml at 96 hrs. The results were presented in Fig.3.

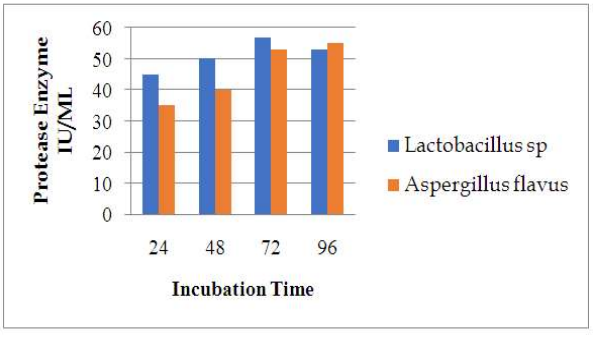

Fig.3. Optimization of Incubation Time for Protease Production

Optimization of Carbon sources

Different carbon sources have different effects on extracellular protease production based on its utilization by the particular microbes. The protease production was optimized using different carbon supplementation medium such as Glucose, Sucrose, Starch, Galactose and Maltose. In submerged fermentation, maximum protease production by *Lactobacillus sp* was recorded in the medium supplemented with glucose as carbon sources (4400 ± 45 IU/ml) and the maximum protease production of *Aspergillus flavus* was recorded in the medium supplemented with starch (3895 ± 24 IU/ml) Table1.

Optimization of Nitrogen sources

Nitrogen sources play a very important role in the metabolism of the cell and enzyme production. Alkaline protease production depends heavily on the availability of nitrogen sources in the medium, which has regulatory effects on enzyme synthesis. The protease production was optimized using different nitrogen supplementation medium such as Peptone, Yeast extract, Sodium nitrate, Ammonium sulphate and Ammonium nitrate. In submerged fermentation maximum protease production by *Lactobacillus sp* was recorded from the medium supplemented with ammonium nitrate (5500 ± 46 IU/ml) as nitrogen source and the maximum protease production by *Aspergillus flavus* was recorded when sodium nitrate (4210 ± 35 IU/ml) was used as nitrogen source Table 1.

DISCUSSION

Microbial proteases have a number of commercial applications in industries like food, leather, meat processing and cheese making. A major commercial use is the addition of microbial proteases to domestic detergents for the digestion of pertinacious stains of fabrics (Sharma *et al*., 1980). It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions. So, it becomes necessary to understand the nature of proteases and their catalytic potentiality under different conditions.

Enzyme production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, $p^{H,}$ and incubation time and inoculums density. It is important to produce the enzyme in inexpensive and optimized media on a large sc ale for the process to be commercially viable (Muthulakshmi *et.al.,)*.

Protease production by microbial strains strongly depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and

Table 1. Optimization of Chemical factor for Protease Production

S. No.	CARBON			NITROGEN		
	Sources	ENZYME ACTIVITY		Sources	ENZYME ACTIVITY	
		Lactobacillus sp.	*Aspergillus flavus*		*Lactobacillus* sp.	*Aspergillus flavus*
1	Glucose	4400±45	2825±31	Peptone	3585±24	3200±20
2	Sucrose	3265±25	3500±29	Yeast Extract	4620±32	3450±25
3	Starch	3055±20	3895±27	Sodium Nitrate	4850±30	4210±35
4	Galactose	4240±35	2985±25	Ammonium Sulphate	5420±48	3585±30
5	Maltose	3350±30	3750±23	Ammonium Nitrate	5500±46	3740±32

Values were Expressed as mean ± Standard Deviation

transport of various components across the cell membranes, which in turn support the cell growth and product production (Elliah *et al.,* 2002). Aspergillus flavus showed maximal protease production at pH - 4 (Fig. 2). Identical observations were earlier recorded in *A. flavus, A. oryzae* and *A. candidus* at pH 4.0 (Nasuno and Onara, 1972; Dworschack *et al.,* 1952).

Protease enzyme activity decreased from 60!, and this could be due to the fact that at higher temperature enzymes are denatured. Gitishree *et al.* (2010) reported maximum protease production at 40!. The effect of temperature on the activity of protease enzyme produced by *A. niger* revealed that there was a sudden increase in protease production from 30-40°C with a concentration of 0.43mg/ml/sec and 0.63mg/ml/sec. However, there was sudden decrease in the activity of enzyme produced by *A. niger* when the temperature was increased above 50°C. This result agrees with the findings of Kalpana *et al.* (2008), that protease production decrease with increasing temperature from 35 to 45°C and that protease production ceases at higher temperature. Oyeleke *et al.* (2010), reported that the maximum protease yield by *A. flavus* and *A. fumigatus* was at 30°C.

Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu *et al.*,2012). Similarly, Ganesh Kumar *et al.,* (2008) reported the optimum temperature for protease production by the mesophilic fungi, synergist's species was at 35°C. Growth and protease production ceased at higher temperatures (50°C). Similar observations were also reported by Morimura *et al.,* (1994) when *Aspergillus usami* was used as test organism.

CONCLUSION

In conclusion, the result of the present study clearly indicates that the potential of *Lactobacillus sp* and *Aspergillus flavus* that could be successfully cultivated under submerged fermentation condition for the production of protease. This study suggests that it could be one of the effective approaches to reduce the cost of protease production. Apart from protease production, it also has wide application in various industries like pulp and paper industry, food industry, textile industry, poultry, cattle industry, etc. However more detailed investigation is required to characterize this microbial enzyme, which may be effectively used in the large scale production for commercial and pharmaceutical purposes in future.

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