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Industrial Important Protease Screening and Optimization from Micro-Fungal Isoltaes of Ayyanar Falls Forest Samples, Rajapalalyam

S. Muthukrishnan and K. Mukilarasi

Department of Biotechnology, Ayyya Nadar Janaki Ammal College,
Sivakasi- 626124, Tamilnadu, India

Abstract: Proteases are an important class of enzymes and account for the major industrial market in the world. The optimization of protease was important process in industrial sector for strain selection. The highly Potential protease producing five microfungal species were isolated from soil, leaf litter, wood debris soil samples of Ayyanar falls forest, Rajapalayam by using Potato dextrose agar medium (PDA).The various pH, Carbone, nitrogen source conditions were optimized for protease production.Quantitative screening of extracellular fungal *protease* was done by inoculating the qualitatively Screened. Fungal species *Aspergillus niger, Aspergillus ochraceous, Penicillium oxalicum, Fusarium solani, curvularia lunata* on the submerged culture fermentation. High level *protease* production was evaluated by adding Paddy husk as a substrate, lactose as a carbon source, peptone as a nitrogen source, pH6.5 and mineral salt medium was added as a nutrient supplement. Maximum increase in *protease* production was observed with *F.solani* (219 U/ml), followed by *A.ochraceous* (211U/ml), *P.oxalicum* (198 U/ml), *A.niger* (189 U/ml), *C.lunata* (181 U/ml).

Key words: Micro-fungal · Protease · Optimization · Industrial enzyme

INTRODUCTION

Protease is one of the most important commercial enzymes and is used in food processing, detergents, dairy industry and leather making [1]. Proteases occur widely in plants and animals, but commercial proteases are produced exclusively from microorganism. Molds of the genera *Aspergillus, Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe [2]. In recent years there has been a phenomenal increase in the use of proteases as industrial catalysts. Thereare several advantages while using enzyme as biocatalyst over conventional chemicals. Most significant achievement made using these biocatalysts in last one decade is chemical free industrial process which led to environmental damage and pollution. These amazing molecules offer a high degree of substrate specificity which leads to efficient biochemical process with negligible error in product formation [3].A protease also called as peptidase or proteinase is group of enzyme that performs proteolysis known as hydrolysis of thepeptide bonds that link amino acids together in the polypeptide chain forming the protein [4]. Proteases constitute more than 70% of industrial enzyme alone and microbial sources (bacterial and fungal) are leading supplier of these enzyme. These enzymes possess catalytic activity in broad range of temperature and pH [5-6].

The groups of industrially important enzymes and modern biological techniques areessential to explore these amazing molecules forman kind currently, the largest share of the enzyme market has been held by detergent proteases which are active and stable at alkaline pH. They are also important from a physiological point of view, as they are involved in many cellular processes like protein turn over and digestion as well as fungal morphogenesis, spore formation and spore germination. Yet, there is a continued search for proteases having novel properties with known and newer applications. Filamentous fungi can effectively secrete various hydrolytic enzymes and one of the main groups of secreted enzymes in fungi is protease. Submerged as well as solid state fermentation have been employed for protease production. They usually show better results when cultured in solid-state fermentation as compared to bacteria [7]. Fungi are known to produce acid, neutral, alkaline and metallo proteases. A

Correspondig Author: S. Muthukrishnan, Department of Biotechnology, Ayyya Nadar Janaki Ammal College, Sivakasi- 626124, Tamilnadu, India.

single organism can produce more than one type of protease.The activity of protease by a strain of *A. flavus* was found stable at a wide range of pH (5-10) with optimum at pH 7.5 and temperature 50°C. The protease was found bleach stable. Isolates of *A. fumigates* produced more than 100 U/mL activity of alkaline protease. Production of protease by this species has been reported earlier by [8]. Overall, the results obtained during this investigation and those reported by other workers indicated that *Aspergillus* is one of the important genera for the production of protease.

Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under various environmental conditions of time, pH and temperature, utilizing a wide variety of substrates as nutrients [9]. Molds of the genera *Aspergillus, Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe [10]. *Aspergillus clavatus* ES1 has been recently identified as a producer of an extracellular bleaching stable alkaline protease [11]. Thereare general reports showing that different carbon sources have different influences on extracellular enzyme production by different strains [12]. The effect of different nitrogen sources like gelatin, peptone, aspartic acid, casein and acetamide has reported that nitrogen sources stimulate equal accumulation of protease in the culture medium of *Aspergillus terreus*. Although different alkaline proteases have been isolated from several bacteria and fungi, few have better properties that can be commercially exploited. Therefore, the objectives of the study were to isolate and identify protease producing fungi and optimization by different substrate, nitrogen, Carbone sources and different pH.

MATERIALS AND METHODS

Qualitative Screening for Protease Production: Isolated fungal isolates were screened for proteolytic activity, isolates werecultivating on the enzyme screening medium containing casein 1% incubated at 37°C for 5 days. The plates were flooded with 25% TCA (trichloroacetic acid) solution and incubated for 15 min at 115°C. On the basis of the area of clearance, five fungalpotential isolates were selected for further studies on amylase production [13].

Assay of Protease Activity: To determined protease activity 0.5 ml of diluted enzyme was added to 1.0 ml of 1% casein and 0.5 ml of glycine-NaOH buffer (25 Mm, Ph 10.0) whole mixture was incubated at 75°C for 10 min. The reaction was terminated by the addition of 3 ml of 10% TCA solution. The solution was allowed to stand for 10 min in cool and was filtered. To the clear filtrate, 5 ml 0.4 MNa2CO3 and 0.5 ml of Folin Ciocalteu reagent (FCR) was added, mixed thoroughly and incubated at 37°C for 30 min, in dark. The absorbance was measured at 660 nm.One unit of enzyme activity represents the amount of enzyme required to liberate one mg oftyrosine per min per ml under standard assay conditions, where enzyme activity is calculated by measuring mg of tyrosine released, by comparing with the standard [13].

Optimization of Enzyme Production: Enzyme production was optimized by adding different carbon source such as Maltose, Glucose, Sucrose, Fructose, Lactose, different nitrogen source such as Ammonium nitrate, Ammonium sulphate, Peptone, Yeast extract, Sodium nitrateand different substrate Wheat Bran, Rice Bran, Paddy Husk, Black gram Bran, Green gram Bran, Paddy straw, Wheat straw along with the submerged culture fermentation medium at different pH (pH 5.0, 5.5, 6.0, 6.5, 7.0).

Recovery of Enzyme: After incubation for the desired period (3rdday onwards) the fungal mycelia was removed by filtration through Whattman N0. 1 filter paper and the culture supernatant was treated as the crude enzyme for all assay

Enzyme activity =Test–(enzyme blank + substrate blank).

RESULTS

Extracellularproteaseproduction: Quantitative screening of extracellular fungal *protease* was done by inoculating *A.niger, A. ochraceous, Penicillium oxalicum, Fusarium solani, curvularia lunata* on the submerged culture fermentation medium containing 1% casein. Results in (Table -1) indicate that the highest *protease* production was observed with *Fusarium solani protease* activity reached up to (98 U/ml), followed by *A.ochraceous* (95 U/ml)*, Penicillium oxalicum* (91 U/ml), *A.niger*(87 U/ml) and *curvularia lunata*(85 U/ml).

Optimization of Protease Production

Effect of Carbon Source: Effect of different carbon sources such as lactose, maltose, sucrose, fructose, glucose, were evaluated for the production of extracellular *protease* by using quantitatively screened fungal species. Of all the carbon sources, Lactose showed considerable increase *protease* production, maximum activity was observed in *F. solani* (169 U/ml), followed by *P.oxalicum*

Table 1: Quantitative screening of protease production on submerged fermentation medium

S.No	Fungal species	Protease assay (U/ml) 3rd	5th	7th	Protein estimation(U/ml) 3rd	5th	7th
1	*Aspergillus niger*	68	87*	73	82	108*	78
2	*Penicillium oxalicum*	71	91*	72	85	112*	79
3	*Aspergillus ochraceous*	67	95*	71	89	109*	83
4	*Fusarium solani*	65	98*	75	92	111*	87
5	*Curvularia lunata*	69	85*	69	94	114*	89

* Maximum level of protease production

Table 2: Quantitative assayprotease production on 5th day of incubation

Quantitatively screened fungal species	Optimization of Protease Production(U/ml) *A.niger*	*P.oxalicum*	*A.ochraceous*	*F.solani*	*C.lunata*
Effect of Ph					
pH- 5. 0	71	69	74	73	78
pH -5.5	79	76	81	86	79
pH- 6. 0	81	79	87	95	81
pH- 6. 5	87*	91*	95*	98*	85*
pH- 7. 0	71	84	81	82	83
Effect of carbon source					
Fructose	85	98	112	115	123
Glucose	98	110	119	103	111
Lactose	129*	155*	145*	169*	128*
Maltose	112	121	103	113	119
Sucrose	99	97	102	98	99
Effect of nitrogen source					
Ammonium nitrate	113	134	145	129	123
Ammonium sulphate	98	112	123	112	109
Peptone	187*	154*	158*	195*	134*
Yeast extract	124	123	112	109	111
Sodium nitrate	119	113	106	103	101
Effect of different substrate (Agro waste)					
Wheat Bran	75	78	82	79	76
Rice Bran	55	70	96	63	56
Paddy Husk	86*	98*	99*	111*	81*
Black gram Bran	61	73	79	73	78
Horse gram Bran	73	69	79	71	69

* Maximum level of protease production

(155 U/ml), *A.ochraceous* (145 U/ml),*C. lunata* (129 U/ml), *A.niger*(128 U/ml), further obtained results were shown (Table – 2).

Effect of Nitrogen Source: Effect of different nitrogen sources such as, Ammonium nitrate, Ammonium sulphate, Peptone, Yeast extract, Sodium nitrate were evaluated for the production of extracellular *protease* by usingquantitatively screened fungal species. Of all the nitrogen sources, *peptone*, showed considerable increase *protease* production, maximum activity was observed in *F.solani*(195 U/ml),followed by *A.niger*(187U/ml), *A.ochraceous* (158U/ml), *P.oxalicum* (154 U/ml), *C.lunata* (134 U/ml), further obtained results were shown (Table – 2).

Effect of pH: Effect of various pH such as, pH 5.0, 5.5, 6.0, 6.5, 7.0 were evaluated for the production of extracellular **protease** by using quantitatively screened fungal species. Considerable increase in *protease* production was observed at pH 6.5 *F.solani* (98 U/ml), followed by *A.ochraceous* (95 U/ml), *P.oxalicum* (91 U/ml), *A.niger* (87 U/ml), *C.lunata* (85 U/ml) further obtained results were shown (Table -2).

Effect of Different Substrate: Effect of different substrate such as, wheat bran, Rice Bran, Paddy Husk, Black gram Bran, Horse gram bran, were evaluated for the production of extra cellular *protease* by using quantitatively screened fungal species. Of all the substrate Paddy husk showed considerable increase *protease* production in *F.solani*

Table 3: Optimum fermentation technology for Protease production

Media components	Media composition
Substrate	Paddy Husk
Carbon source	Lactose
Nitrogen source	Peptone
pH	6.5
Incubation time	5 days
Nutrient supplement	Mineral salt medium

Table 4: Protease production on optimized fermentation medium

		Protease assay (U/ml)			Protein estimation (U/ml)		
S.No	Fungal species	3rd	5th	7th	3rd	5th	7th
1	*Aspergillus niger*	99	189*	111	118	219*	178
2	*Penicillium oxalicum*	98	198*	121	123	215*	189
3	*Aspergillus ochraceous*	97	211*	132	134	245*	198
4	*Fusarium solani*	92	219*	111	145	265*	179
5	*Curvularia lunata*	93	190*	142	142	254*	181

* Maximum level of protease production

(111 U/ml), followed by *A.ochraceous* (99 U/ml), *P.oxalicum* (98 U/ml), *A.niger* (86U/ml), *C.lunata* (81 U/ml) further obtained results were shown (Table -2).

Optimized Media Composition for Protease Production: Optimum fermentation media composition for *protease* production was designed based on result obtained in the optimization of *protease* production (Table 4). High level *protease* production was evaluated by adding Paddy husk as a substrate, lactose as a carbon source, peptone as a nitrogen source, pH6.5, incubation time 5 day and mineral salt medium was added as a nutrient supplement (Table 3). Considerable increase in *protease* production was observed in *F.solani* (219 U/ml), followed by *A.ochraceous* (211 U/ml), *P.oxalicum* (198 U/ml), *A.niger* (189 U/ml), *C.lunata* (181 U/ml), further obtained results were shown (Table-4).

DISCUSSION

Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have apotential to grow under various environmental conditions of time, pH and temperature, utilizing a wide variety of substrates as nutrients [9]. There are general reports showing that different carbon sources have different influences on extracellular enzyme production by different strains [12]. The effect of different nitrogen sources like gelatin, peptone, aspartic acid, casein and acetamide has reported that nitrogen sources stimulate equal accumulation of protease in the culture medium of *Aspergillus terreus* [14]. The activity of protease by a strain of *A. flavus* was found stable at a wide range of pH (5-10) with optimum at pH 7.5 and temperature 50°C. The protease was found bleach stable. Isolates of *A. fumigates* produced more than 100 U/mL activity of alkaline protease. Production of protease by this species has been reported earlier by [8]. Overall, the results obtained during this investigation and those reported by other workers indicated that *Aspergillus* is one of the important genera for the production of protease. In this present study totally twelve fugal species were screened for the protease production, five potential species that form better halo zone formation on the casein agar medium were selected for protease production on the submerged fermentation. The highest *protease* production was observed with *F.solani* (98 U/ml), followed by *A.ochraceous* (95 U/ml), *P.oxalicum*(91 U/ml), *A.niger*(87 U/ml),and *curvularia lunata* (85 U/ml), to enhance enzyme production, the optimization of fermentation was carried out under the condition with different carbon source, nitrogen source, different pH. Optimum fermentation media composition for *protease* production was designed based on result obtained in the optimization of *protease* production. High level *protease* production was evaluated by adding Paddy husk as a substrate, lactose as a carbon source, peptone as a nitrogen source, pH6.5, incubation time 5 day, mineral salt medium was added as a nutrient supplement. Maximum increase in *protease* production was observed with *F.solani* (219 U/ml), followed by *A.ochraceous* (211 U/ml),*P.oxalicum* (198 U/ml), *A.niger* (189 U/ml), *C.lunata* (181 U/ml).

CONCLUSION

Based on the results from this study, we finally concluded that the high level protease production was evaluated by adding Paddy husk as a substrate, lactose as a carbon source, peptone as a nitrogen source, pH 6.5, in submerged fermentation of *Fusarium solani* followed by *Aspergillus ochraceus, Penicillium oxalicum*, *Aspergillus niger, Curvularia lunata*. Proteases are an important micro-fungal enzymes and the major industrial market in the world. Proteases occur widely in plants and animals, but commercial proteases are produced exclusively from micro-fungal species.The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of a microbial population. The most important among these are the medium, incubation temperature and pH. The pH of the fermentation medium is reported to have substantial effect on the production of proteases. Proteases are the leading enzymes with immensecommercial potential are widely used in industrialand therapeutic applications. Fungal protease are not limited to industrial application, there therapeutic potentialhave been classified in last few decade constitute one of the largest.

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REFERENCES

1. Negi, S. and R. Benerjee, 2006. Optimization of amylase and protease production from *Aspergillus awamor i*in single bioreactor through EVOP factorial design technique. Food Technol. Biotechnol, 44: 257-261.
2. Sandhya C, A.Sumantha, G.Szakacs and A. Pandey A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae*in submerged and solid-state fermentation. Proces. Biochem., 40: 2689-2694.
3. Xu, X., H. Yan, J. Chen and X. Zhang, 2011. Bioactive proteins from mushrooms. Biotechnol. Adv., 29(6): 667-74.
4. Hedstrom, L., 2002. Serine protease mechanism andspecificity. Chem. Rev., 102(12): 4501-24.

a5. Murakami, K., Y. Ishida, A. Masaki and H. Tatsumi, 1991. Isolation and characterization of the alkaline pro-tease gene of Aspergillus oryzae. Agricultural and Bio-logical Chemistry, 55: 2807-2711.

6. Sanatan, P.T., P.R. Lomate, A.P. Giri and V.K. Hivrale, 2013. Characterization of a chemostable serine alkaline protease from Periplaneta americana. BMC Biochem., 14: 32.
7. Pandey, A., S. Benjamin, C.R. Soccol, P. Nigam, N. Krieger, *et al*. 1999. The realm of microbial lipases in biotechnology, Biotechnol. Appl. Biochem., 29: 119-131.
8. Hossain, T., F. Das, L.W. Marzan, S. Rahman and M.N. Anwar, 2006. Some properties of protease of the fungal strain Aspergillusflavus. Int. J. Agric. Biol., 8(2): 162-164.
9. Ikram -Ul-Haq, Hamid Mukhtar and Hina Umber, 2006. Production of protease by pencilliumchrysogenum through optimization of environmental conditions. Journal of Agricultural and Social Sciences, 1813-2235/02-1- 23- 25.
10. Sandhya, C., A. Sumantha, G. Szakacs and A. Pandey, 2005. Comparative evaluation of neutral protease production by Aspergillusoryzae in submerged and solid-state fermentation. Process Biochemistry, 40: 2689-2694.
11. Hajji, M., A. Rebai, N. Gharsallah and M. Nasri, 2008. Optimization of alkaline protease production by Aspergillus clavatus ES1 in Mirabilis jalapa tuber powder using statistical experimental design. App.Microbiol. Biotechnol., 79: 915-923.
12. Nehra, K.S., S. Dhillon, K. Chaudhary and R. Singh, 2002. Production of alkaline protease by Aspergillus species under submerged and solid state fermentation. Ind. J. Microbiol., 42: 43-47.
13. Abirami, A., K. Meenakshi, R. Kanthymathy, R. Bharathidasan, Mahalingam and A. Panneerselvam, 2011. Partial purification and characterization of an extracellular Protease from Penicillium janthinellum and Neurospora crassa. European Journal of Experimental Biology, 1(3): 114-123.
14. Ashour, S.A., H.M. EL Shore, M. Metwally and S.A. Habib, 1996. Fungal fermentation of Whey incorporated with certain supplements for the production of protease. Microbios., 86: 59-69.