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Optimization of Protease Production from Wild and Mutant Strains of *Aspergillus* sp. Under Submerged Fermentation

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Abstract

Proteases are one of the most significant groups of commercial enzymes and contribute 60% of the world enzyme market. The choice of proteases is the microbial proteases due to their versatility, stability and distinctive properties. Today, microbial proteases are utilized in various industries *viz.*, meat processing, ripening of cheese, detergents, textile, recovery of silver from photographic image etc. Therefore, the present study was undertaken to increase the protease production from wild and mutagenic strain of *Aspergillus* sp. Wild and mutagenic strain of proteolytic soil fungus *Aspergillus* sp. was used for optimization study. Different parameters (nitrogen, carbon sources, temperature, pH and incubation time) were optimized in submerged

fermentation (SmF) for enhanced protease production. Wild strain demonstrated highest protease activity at pH 10.0 after 48 h of incubation at 37 ºC in the medium containing glucose as carbon source and yeast extract as nitrogen source whereas optimum protease activity from mutagenic strain was found at pH 10.0 after 94 h of incubation at 37 ºC in the medium containing fructose as carbon source and peptone as nitrogen source. Protease production was increased from wild and mutagenic strain of *Aspergillus* sp. after parameters optimization in SmF.
Keywords: optimization, protease activity, *Aspergillus* sp., carbon source, temperature, pH.

Introduction

Proteases are one of the imperative enzymes, contribute almost 60% of the global industrial enzyme market, which indicates their utilization in number of industries such as paper industry, detergents, pharmaceuticals, garments and food processing particularly for maturation of cheese, meat tenderizing and animal nutrition, leather and milk coagulating industry [1]. They also play an important role in the digestion of proteins in the animal digestive tract and can be obtained from plants (papain isolated from *Carica papaya*) [2], from animals (chymotrypsin, trypsin extracted from pancreatic juice) [3]. Microbes are the best source of extracellular and intracellular proteases because of the stability (under wide range of pH and temperature) and some unique properties [4].

Generally extracellular proteases catalyze hydrolysis of peptide bonds within large polypeptide chain into proteoses, peptones, small peptide fragments and free amino acids, later are absorbed into the cells whereas intracellular protease control cellular metabolism process [5]. The preference of protease source depends upon type of protease use, easy extraction procedure [6]. Generally, microbes are utilized for protease extraction because of rapid growth and multiplication of microbes, therefore able to accumulate high quantity of protease, cost effective production and extraction [7]. Among the microbes, fungi are preferred for protease production because in downstream processing, bacterial cell separation is more complex that fungal mycelium separation [8]. Besides, they also offer some advantages such as specificity to substrate, stability in wide range of pH, temperature and organic solvents [2]. The diversity of

fungal proteases is more than bacterial protease and fungi are also recognized as GRAS (generally regarded as safe), which indicate that their protease is safe for human consumption [9].

Protease producing fungi were belonging to the genera: *Rhizopus* [10], *Aspergillus* [11], *Penicillium* [12] and *Humicola* [13]. Two species of genera *Mucor* (*M. pusillus* and *M. miehei*) are well known for secretion of aspartate protease (known as mucor rennin) into the fermentation broth. Mucor rennin enzyme has lower proteolytic activity and higher milk coagulating activity, therefore utilized as a substitute of bovine rennin in cheese industry [14, 15].

Strain improvement and optimization techniques can be utilized for increased microbial enzyme production, therefore by keeping several application of protease in mind, in the current study different parameters (carbon source, nitrogen source, pH, temperature and incubation time) were optimized for enhanced protease production from wild and mutant strains of proteolytic soil fungus *Aspergillus* sp. under submerged fermentation.

Materials and methods

Microorganism

Wild and nitrous acid mutant strains of proteolytic soil fungi (*Aspergillus* sp.) were obtained from Department of Bioscience and Biotechnology, Banasthali University, Rajasthan. Culture was maintained on potato dextrose agar (PDA) slants.

Production of protease in SmF

One ml of spore suspension (made from 6 days old slat culture of wild and mutant strains of *Aspergillus* sp.) was transferred in 100 ml of fermentation broth containing (g/100 ml): glucose, 2; yeast extract, 1; K_2HPO_4, 0.1; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.02 and pH was adjusted to 7.0. Flasks were kept at 28 $^{\circ}$C, 150 rpm for 3 days. Protease activity was measured after end of incubation time from mycelium free fermentation broth.

Protease assay

Protease activity was measured using casein as substrate according to the method of Tsuchida et al. [16].

Parameter optimization

Culture conditions were optimized for wild and mutant strains of *Aspergillus* sp. for enhancement of protease activity.

Carbon source optimization

Various carbon sources such as glucose, sucrose, starch, fructose, lactose and maltose were used at a concentration of 2% w/v to investigate their effect on protease production from wild and mutant strain of *Aspergillus* sp. Production medium containing glucose was regarded as control flask. For protease production, spore suspension was transferred in 100 ml of fermentation broth, each containing different carbon source followed by incubation for 4 days at 28 ºC, 150 rpm. Protease activity was determined from fermentation broth after separation of fungal mycelium.

Nitrogen source optimization

Previously optimized carbon sources were taken for this experiment. Organic nitrogen sources (peptone, skim milk, casein, beef extract and yeast extract) and inorganic nitrogen sources (KNO_3, $(NH_4)_2SO_4$) were utilized at a concentration of 2% w/v to study their impact on protease production from *Aspergillus* sp. Production medium with yeast extract was treated as control.

pH optimization

pH of the fermentation broth (containing previously optimized carbon and nitrogen sources) was adjusted to 4, 5, 6, 7, 8, 9 and 10 using 1 N HCl and 1 N NaOH in order to study their impact on protease activity.

Temperature optimization

Inoculated flask (containing production medium with earlier optimized carbon, nitrogen sources and pH) were placed at different temperature: 28 ºC, 37 ºC and 50 ºC to investigate their effect on protease production.

Incubation period optimization

Production medium was prepared using optimized parameters. Inoculated flasks were placed at optimized temperature for 24, 48, 72 and 96 h. Protease activity was estimated at the end of 24, 48, 72 and 96 h of incubation.

Results and discussion

Protease production in un optimized fermentation broth

Protease activity of wild and mutant strains of *Aspergillus* sp. was 19.90 ± 0.85 U/ml and 43.15 ± 0.40 U/ml, respectively at the end of 72 h of incubation at 28 ºC in the medium containing glucose (2% w/v) as carbon source and yeast extract (1% w/v) as nitrogen source.

Effect of carbon source

Figure 1 represents that glucose was found best carbon source for the wild strain (112.39 ± 11.16 U/ml) whereas optimum protease activity (118.81 ± 5.06 U/ml) was obtained from mutant strain in the presence of fructose as carbon source at 72 h of incubation. For wild strain, descending order of carbon sources for protease activity is glucose > fructose (97.21 ± 2.70 U/ml) > maltose (81.73 ± 2.55 U/ml) > lactose (77.49 ± 2.08 U/ml) > starch (55.93 ± 3.59 U/ml) > sucrose (44.99 ± 3.0 U/ml) whereas for mutagenic strain the descending order is fructose > glucose > sucrose > starch > lactose > maltose. For both the strains monosaccharide (glucose and fructose) increased protease activity. It indicates that these monosaccharide sugars were efficiently utilized by the fungus for growth and optimum production of protease. For protease production from mutagenic strain, polysaccharide sugar was found more effective than disaccharides.

Similar to our results, Rajput et al. [17] reported that protease production from *Alternaria alternate* was maximum (162 U/ml) with glucose and lowest (102 U/ml) with lactose as carbon source. Srinubabu et al. [18] reported glucose as best carbon source for proteolytic activity from *Aspergillus oryzae*. Venkat Kumar et al. [19] reported highest protease activity (790 U/ml) from *Trichospron Japonicum* with fructose (1% w/v) after 48 h of incubation.

Muthukrishnan and Mukilarasi [20] documented lactose as excellent carbon source for high level of protease activity from *A. niger* (129 U/ml), *Penicillium oxalicum* (155 U/ml), *Aspergillus ochraceous* (145 U/ml), *Fusarium solani* (169 U/ml) and *Curvularia lunata* (128 U/ml). Negi and Banerjee [1] reported highest activity of protease from *Aspergillus niger* using soluble starch as carbon source.

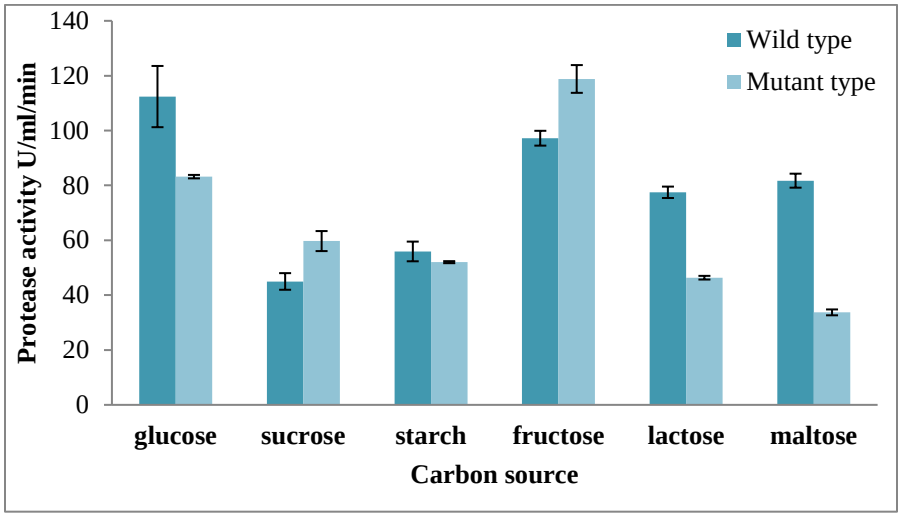

Figure 1: Effect of carbon sources on protease activity from wild and mutagenic strain of *Aspergillus* sp.

Effect of nitrogen source

Figure 2 presents that yeast extract (98.29 ± 8.67 U/ml) was found to be the best nitrogen source for wild strain whereas mutant strain demonstrated higher protease activity (99.19 ± 2.10 U/ml) with peptone. For wild strain yeast extract was excellent nitrogen source, followed by beef extract (60.98 ± 8.09 U/ml), casein (54.0 ± 1.79 U/ml), peptone (44.0 ± 2.0 U/ml), skim milk

(36.55 ± 2.42 U/ml), KNO_3 (14.48 ± 2.86 U/ml), $(NH_4)_2SO_4$ (8.63 ± 1.44 U/ml). For mutagenic strain ascending order of nitrogen sources for protease production is as follows: KNO_3 < $(NH_4)_2SO_4$ < skim milk < casein < beef extract < yeast extract < peptone. In our study organic nitrogen increased protease production whereas inorganic nitrogen decreased protease activity from both the strains. It indicates that organic nitrogen sources are efficiently utilized by fungus for growth and protease production than inorganic nitrogen sources.

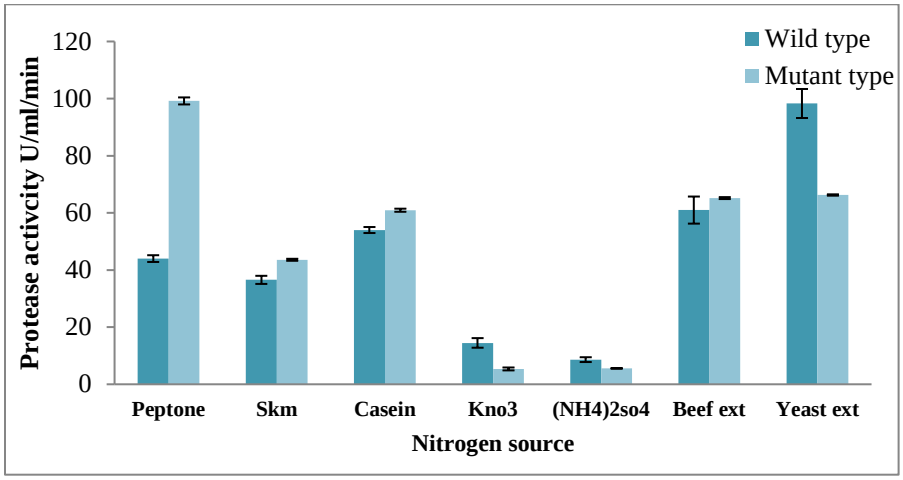

Figure 2: Effect of nitrogen source on protease activity from wild and mutant strain of *Aspergillus* sp. (Variable parameter: nitrogen sources; Constant parameter: carbon source: glucose for wild strain and fructose for mutant strain; pH 7.0; temperature 28 ºC and 72 h incubation).

In accordance to our results, Rajput et al. [17] reported that protease activity from *Alternaria alternate* was highest (119 U/ml) with yeast extract and lowest (68 U/ml) with urea as nitrogen source. Muthukrishnan and Mukilarasi [20] have reported peptone as excellent nitrogen source for optimum protease activity from *A. niger* (187 U/ml), *P. oxalicum* (154 U/ml), *A. ochraceous* (158 U/ml), *F. solani* (195 U/ml) and *C. lunata* (134 U/ml). Srinubabu et al. (2007) reported peptone as an excellent nitrogen source for proteolytic activity from *Aspergillus oryzae*. Venkat Kumar et al. [19] reported maximum protease production (4500 U/ml) from *Trichospron Japonicum* with peptone (1% w/v) after 96 h of incubation.

Influence of pH on lipase production

Figure 3 clarifies that pH 10.0 was found to be optimum for maximum protease activity from wild strain (207.39 ± 1.73 U/ml) and mutant strain (248.33 ± 3.65 U/ml) of *Aspergillus* sp. The descending order of pH for wild strain is as follows: pH 10 (207.39 ± 1.73 U/ml) > pH 9 (158.24 ± 7.88 U/ml) > pH 7.0 (139.47 ± 3.63 U/ml) > pH 8.0 (118.67 ± 10.41 U/ml) > pH 6.0 (107.82 ± 6.02 U/ml) > pH 5.0 (100.2 ± 5.92 U/ml) > pH 4.0 (68.15 ± 3.28 U/ml) whereas the descending order for mutagenic strain was also found in the same order as in the wild strain. In our results protease activity was found higher in the alkaline pH range than in the acidic pH range. Protease activity was lowest at pH 4.0 but increases with the rise of pH and reached to maximum at pH 10.0. It indicates that fungus is capable of growing at alkaline pH (10.0), hence it is a potent producer of alkaline protease.

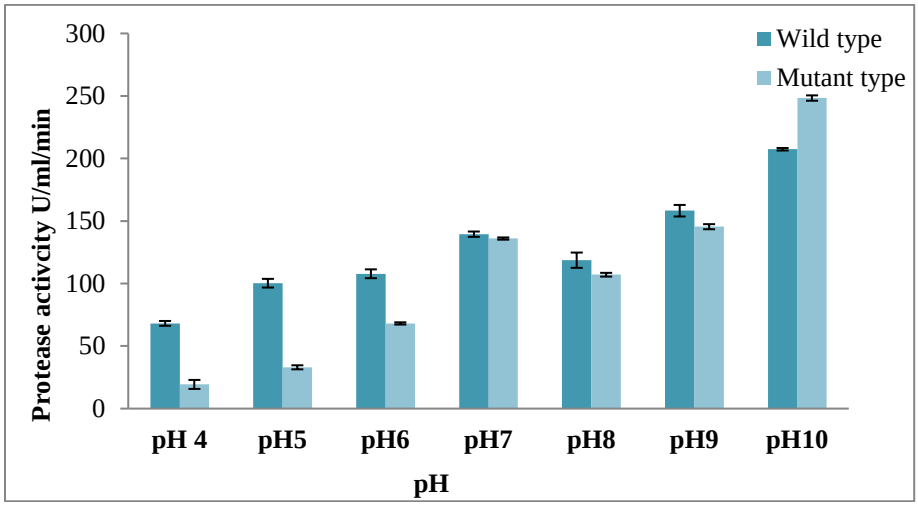

Figure 3: Effect of pH on protease activity from wild and mutant strain of *Aspergillus* sp. (Variable parameter: pH; Constant parameter: carbon source: glucose for wild strain and fructose for mutant strain; nitrogen source: yeast extract for wild strain and peptone for mutant strain; temperature 28 ºC and 72 h incubation).

In accordance to our finding, Sharma et al. [21] reported highest protease activity from local fungal isolate BY-4 at pH 9.5. Srinubabu et al. [18] reported high level of proteolytic activity from *A. oryzae* at pH 10.0. Negi and Banerjee [1] reported maximum protease

production from *Aspergillus niger* when pH of the fermentation broth was adjusted to 4.0. Zaferanloo et al. [22] stated highest protease production from *Alternaria alternate* in a broad range of pH 3 to 9 but optimum pH was found to be 7.0. Similarly, Rajput et al. [17] also reported optimum protease activity (55 U/ml) from *A. alternate* at pH 7.0 while it was lowest (34 U/ml) at pH 4.0. Venkat Kumar et al. [19] also stated highest protease activity (800 U/ml) from *Trichospron Japonicum* at pH 7.0 after 24 h of incubation. Milala et al. [23] reported optimum protease activity (0.12 U/ml at pH 4.0 and 0.07 U/ml at pH 8.0) from *A. niger*.

An optimum pH of 6.5 was reported by Muthukrishnan and Mukilarasi [20] for high level of protease activity from *A. niger* (87 U/ml), *P. oxalicum* (91 U/ml), *A. ochraceous* (95 U/ml), *F. solani* (98 U/ml) and *C. lunata* (85 U/ml).

Temperature optimization

Figure 4 shows that optimum protease activity from wild strain (276.44 ± 36.82 U/ml) and mutant strain (281.30 ± 1.13 U/ml) was observed at 37 ºC. Protease activity of wild and mutant strain at 27 ºC was 211.96 ± 54.03 U/ml and 219.60 ± 1.89 U/ml, respectively. It was reached to maximum at 37 ºC and declined to 148.24 ± 23.92 U/ml for wild strain and 169.09 ± 7.41 U/ml for mutant strain at 50 ºC. Lowest activity at high temperature (50 ºC) indicates adverse effect on growth of the fungus and protease production. It might be due to that high temperature denatured the cellular protein and reduced growth of fungus and protease production.

In accordance with our results, Zaferanloo et al. [22] stated maximum protease activity from *Alternaria alternate* in the broad range of temperature (9 ºC to 50 ºC), but optimum temperature was found to be 37 ºC. Venkat Kumar et al. [19] also stated highest protease activity (1800 U/ml) from *Trichospron Japonicum* at 40 ºC after 96 h of incubation. While, Rajput et al. [17] reported that protease activity from *A. alternate* was significant within the temperature range 25 ºC to 50 ºC but it was optimum (68 U/ml) at 27 ºC. Srinubabu et al. [18] reported high level of protease activity (456 U/ml) from *A. oryzae* at 30 ºC. Optimum protease activity (0.07

U/ml) from *Aspergillus niger* was reported at 40 ºC by Milala et al. [23].

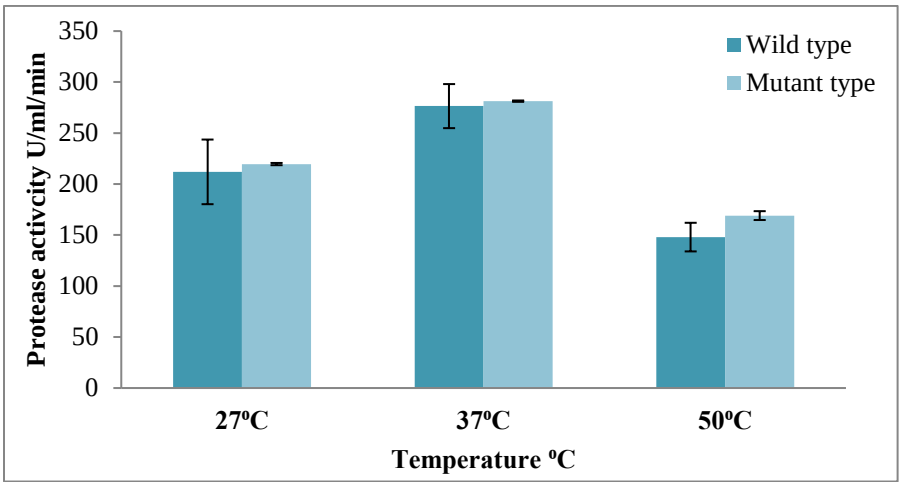

Figure 4: Effect of temperature on protease activity from wild and mutant strain of *Aspergillus* sp. (Variable parameter: incubation temperature; Constant parameter: carbon source: glucose for wild strain and fructose for mutant strain; nitrogen source: yeast extract for wild strain and peptone for mutant strain; pH: 10.0 and 72 h incubation).

Incubation time optimization

Figure 5 indicates that wild strain showed high level of protease activity (332.23 ± 1.25 U/ml) at 48 h of incubation whereas mutant strain demonstrated 619.25 ± 3.04 U/ml after 96 h of incubation. Protease activity for mutant strain at 24 h of incubation was 527.49 ± 1.96 U/ml. It increased with the rise of incubation time (48 h, 72 h) and reached to maximum at 96 h of incubation. Our findings suggest that wild strain can be used to accumulate large quantity of protease within very short period of time.

In agreement with our results, optimum protease activity (0.07 U/ml) from *A. niger* was reported after 24 h of incubation by Milala et al. [23]. Negi and Banerjee [1] reported highest activity of protease from *Aspergillus niger* after 96 h of incubation.

An optimum fermentation period of 5 days was reported by Muthukrishnan and Mukilarasi [20] for protease production from *A. niger* (87 U/ml), *P. oxalicum* (91 U/ml), *A. ochraceous* (95 U/ml), *F. solani* (98 U/ml) and *C. lunata* (85 U/ml).

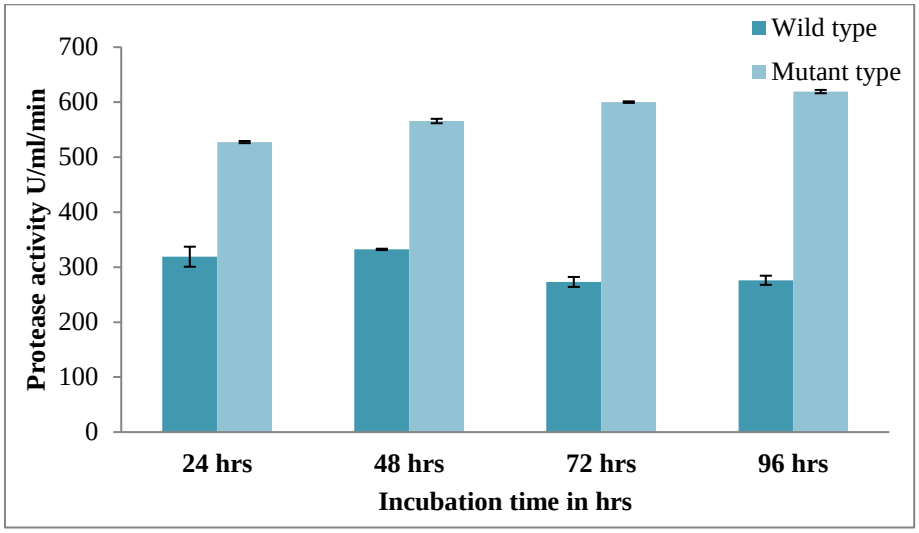

Figure 5: Effect of incubation time on protease activity from wild and mutant strain of *Aspergillus* sp. (Variable parameter: incubation period; Constant parameter: carbon source: glucose for wild strain and fructose for mutant strain; nitrogen source: yeast extract for wild strain and peptone for mutant strain; pH: 10.0; temperature 37 ºC and 72 h incubation).

Conclusion

The purpose of present investigation was to increase protease production from wild and mutagenic strains of *Aspergillus* sp. by process parameters optimization. Protease activity from both the strains was found higher in optimized medium if compared with unoptimized medium. Fermentation broth containing glucose and yeast extract was found suitable for maximum protease activity from wild strain at 48 h of incubation at 37 ºC, pH 10.0 whereas Production medium with fructose and peptone was found suitable for optimum proteolytic activity of mutant strain after 96 h at 37 ºC, pH 10.0.

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