Research Article

Enhancement of protease production from *Aspergillus* sp. by nitrous acid treatment

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Abstract

Background: Proteases are crucial category of enzymes, account for the main global industrial market and are utilized in dairy industry, detergent industry, pharmaceutical, food processing and leather manufacturing. **Objectives:** The purpose of the present study was to identify the proteolytic soil fungal isolate by macroscopic and microscopic observations and to enhance the extracellular protease production by random mutagenesis of *Aspergillus* sp. using HNO_2 as chemical mutagen. **Materials and methods:** Point inoculation of fungus was done in potato dextrose agar (PDA) plates for its macroscopic examination whereas lactophenol cotton blue staining of purified fungal colony was prepared for its microscopic observations. For chemical mutagenesis, spores of *Aspergillus* sp. were incubated with nitrous acid for different time durations thereafter protease activity was determined for screening of potent mutant strain. **Results:** Potent proteolytic soil fungal isolate was recognized as *Aspergillus* sp. based on its morphological characters observed directly from PDA plate culture and microscopic characters observed under compound microscope. Among the exposure time, mutagenic strain of *Aspergillus* sp. obtained after 90 min exposure with nitrous acid exhibited high level of protease activity (43.15 ± 0.40 U/ml) in submerged fermentation (SmF) which was 116% higher than wild strain (19.90 ± 0.85 U/ml). **Conclusion:** Fungal isolate was recognized as *Aspergillus* sp. by classical method of identification. Thereafter, protease activity was successfully increased up to 116% by chemical mutagenesis of *Aspergillus* sp. using nitrous acid as mutagen.

Keywords: Proteases, chemical mutagenesis, nitrous acid, *Aspergillus* sp., microscopic

Introduction

Proteases are protein cleaving enzymes are found in animals, microorganisms and plants and play important role in physiological processes such as digestion of protein in stomach and intestine, blood clotting, cell growth and tissue arrangement (Rao et al., 1998). Microbial proteases have attracted a vast deal of consideration in the past few years due to their applications in different industrial processes such as food, leather, detergent, dairy, textile and pharmaceutical preparations (Saran et al., 2007). Microbial proteases are among the principal hydrolytic enzymes and have been investigated broadly. This group is largest groups of industrial enzymes and contributes for nearly 60% of the global enzyme sales (Zambare et al., 2011). Among microbial proteases, fungal proteases have attracted attention because fungi can be cultivated on low cost substrate, capable of producing huge quantity of enzyme into production media, separation of enzyme is easier and properties of enzymes are superior (Anitha and Palanivelu, 2013). Generally microbial proteases are extracellular in origin therefore directly secreted into fermentation broth hence, simplifies separation of enzyme (Savitha et al., 2011).

Strain improvement is the improvement in the productivity of desired metabolite by genetic alteration of the newly isolated microbial strain (Sidney and Nathan, 1975). Microbes generally excrete industrially significant metabolites in very little concentrations by their intrinsic regulatory system. Although the amount of desired metabolite might be enhanced by optimizing the cultural situations, eventually the productivity is regulated by the organism genome (Stanbury et al., 1995). In the past few years, the rapid boost in the application of proteases in

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different areas has placed stress and demand in both qualitative improvement and quantitative enrichment via strain improvement. Such improved strains offer several advantages such as reduce cost of production, produce metabolite in increased quantity etc. Today isolation of true mutant strains carries beneficial genetic modification for improved productivity is the big task (Haq et al., 2005; Sivaramakrishan et al., 2006).

This genetic manipulation in the microbial strain may be site specific and random. In random mutagenesis microbial strains are treated with physical and chemical mutagens for screening of high yielding strains. Among mutagens, chemical mutagens have been reported as potent mutagenic agents and insert stable modifications in DNA sequence. They induce transitions from G: C→A: T (Miller, 1972) and have privileged influence on DNA replication (Joh et al., 2004; Paul et al., 2005; Wu et al., 2006).

Nitrous acid (HNO_2) causes oxidative deamination of the amino group (NH_2) of adenine and cytosine into an ether group, therefore changing their base pairing. Because hydrogen bonding potential of nitrogenous bases are strongly affected by change in the amino group of the base into keto group (Azin et al., 2001; Rubinder et al., 2002; Szafraniec et al., 2003).

In view of this background, the purpose of the present investigation was to identify the proteolytic soil fungal isolate and to enhance the protease production in submerged fermentation by treatment of fungal spores with chemical mutagen (nitrous acid).

Materials and methods

Fungus and its identification

Proteolytic fungus previously isolated from soil sample was utilized for identification and strain improvement. For macroscopic examination, fungus was point inoculated on to the centre of the PDA plate followed by incubation in appropriate conditions. For microscopic examination, a drop of lactophenol cotton blue stain was transferred on to the centre of a microscopic glass slide. A small group of the fungus, usually with spores and spore bearing structures were transferred from the PDA Petri plate grown culture, into the stain drop, using a flamed, cooled needle. The material was lightly teased with needle for proper mixing of stain with fungus structures. A cover slip was placed over the preparation taking care to avoid trapping of any air bubbles in the stain (Aneja, 1996). Preparation was observed for the presence of conidial head, conidiophores, conidia and mycelium at 10X and 40X objective lenses of a compound microscope and images were captured by the attached camera.

Mutagenesis using HNO_2

For this purpose, 0.1 M solution of sodium nitrite was prepared in 50 mM sodium acetate buffer, pH 5.0. Spore suspension of *Aspergillus* sp. was mixed and incubated with HNO_2 reagent for 15, 30, 45, 60, 75 and 90 minutes at 28 ºC in a shaker incubator. Treated spores were withdrawal after corresponding time duration and centrifuged at 10000 rpm for 5 min for recovery of pellet containing treated spores. Pellet was wished 2-3 times with 1 ml of sterile distilled water for removal of any traces of HNO_2. Finally pellet was mixed with 0.5 ml of sterile distilled water and used for inoculation on to the PDA plates (Sharma et al., 2016).

Screening of mutagenic strains for protease activity

Screening of mutant strains was done by transferring 1 ml of spore suspension from each of the mutant culture (15 min to 90 min) in 100 ml of fermentation broth. The composition of fermentation broth (g/L) is as follows: glucose, 20.0; yeast extract 10.0; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.2 and pH was adjusted to 7.0. Flasks were kept in an incubator at 28 °C, 150 rpm for 3 days followed by estimation of protease activity form culture supernatant using casein as substrate by the method as explained earlier by Tsuchida et al. (1986).

Results and discussion

Identification of proteolytic fungal isolate

Figure 1 presents that previously isolated lipolytic fungus was identified as belonging to *Aspergillus* genera based on direct observation of its morphological characteristics on PDA Petri plate culture and microscopic features of lactophenol cotton blue stained slide at 40 X magnification. Macroscopic characters were as follows: Surface colour of colony was black (figure 1a) with powdery texture and heavy sporulation whereas reverse colour was whitish yellow with radially furrowed zonation (figure 1b). Microscopic characters were as follows: Septate and hyaline hyphae were observed (figure 1c). Conidiophore stipes were large, transparent, smooth walled, with length ranging from 500 to 3000 µm long and were becoming dark at the top and terminating in a circular vesicle with size of 25 to 72 µm in diameter (figure 1d and 1e). Metulae and phialides were entirely covering the surface of vesicle. Biseriate, black, larger and globular conidial head was observed. Conidia (5 µm diameter) were circular and in the form of linear chain (figure 1f) they were attached to phialides.

Choudhary and Jain (2012) reported that soil mycoflora were identified by Petri plate culture and lactophenol cotton blue staining as belonging to the genera *Aspergillus*,

Chrysosporium, *Curvularia*, *Fusarium*, *Mucor*, *Paecilomyces*, *Rhizopus* and *Penicillium*. *Aspergillus* and *Fusarium* sp. were maximum in number. Gaddeyya et al. (2012) reported identification of fungi isolated from soil of agricultural field. Fungi were identified as *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium* and Trichoderma based on their morphological characteristics on Petri plate culture and microscopic properties in stained slide. Rohilla and Salar (2012) reported identification of 33 soil fungi belonging to 10 genera: 7 genera from *Ascomycota*, 2 from *Deuteromycota* and 1 from *Zygomycota*. Similarly these classical approaches for soil fungi identification were also used by Gandipilli et al. (2013).

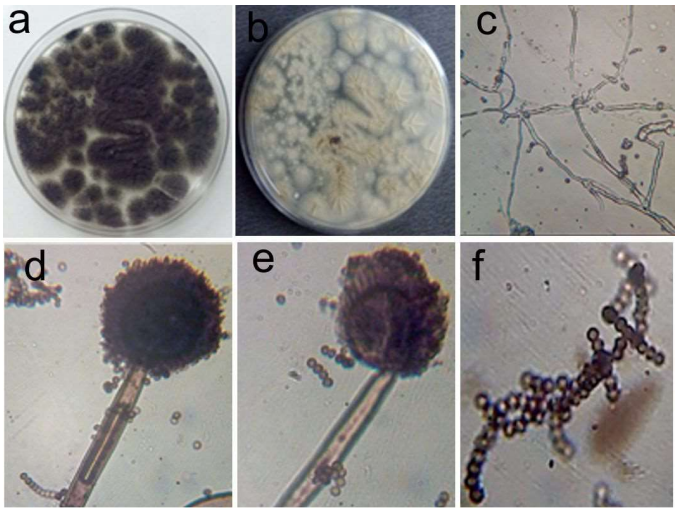

Figure 1: PDA Petri plate and microscopic pictures of *Aspergillus* sp. **(a):** surface view of 4 days old culture; **(b):** reverse view of 4 days old culture; **(c):** fungal mycelium; **(d):** black conidial head and spherical vesicle; **(e):** conidiophores stipe linked to conidial head at apex and **(f):** chain of globose conidia.

Mutagenesis using HNO_2

Results showed in table 1 represents that all the mutagenic cultures exhibited increased protease activity and specific activity as compared to wild strain but protease activity (43.15 ± 0.40 U/ml) and specific activity (11.63 ± 0.54 U/mg) of mutant strain (AS_{90}) was highest as compared to protease activity (19.90 ± 0.85 U/ml) and specific activity (5.27 ± 0.38 U/mg) of wild strain (AS). It was 116% increase in protease activity for mutant strain (AS_{90}) as compared to wild strain. Mutagenic strain AS_{90} was obtained after 90 minutes exposure of *Aspergillus* spores with HNO_2. Exposure of spores with nitrous acid for 15, 30, 45, 60, 75 and 90 minutes increased protease activity up to 39%, 66%, 35%, 56%, 96% and 116%, respectively. The present results suggest that HNO_2 increased proteolytic efficiency of *Aspergillus* sp. in all the exposure time (15 to 90 min). Therefore, nitrous acid was found to be strong chemical mutagen for enhanced protease activity of *Aspergillus* sp.

Table 1. Protease activity and specific activity of nitrous acid mutagenized strains ($AS_{15}$to AS_{90}) of *Aspergillus* sp. after 72 h of incubation.

Time of exposure with HNO_2 (min)	Mutagenic strains	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Percent increase in activity
0	*Aspergillus* sp.	19.90±0.85	3.9	5.27±0.38	100
15	AS_{15}	27.63±0.52	2.7	10.13±0.46	138.84
30	AS_{30}	33.01±1.44	3.3	10.01±0.95	165.87
45	AS_{45}	26.93±0.48	3.7	7.28±0.78	135.32
60	AS_{60}	31.14±0.80	3.3	9.42±0.60	156.48
75	AS_{75}	39.14±0.78	3.5	11.14±0.90	196.68
90	**AS_{90}**	**43.15±0.40**	**3.7**	**11.63±0.54**	**216.83**

AS: *Aspergillus* species.

Zambare (2010) observed 1% survival rate when spores of *Trichoderma reesei* were exposed for 20 min in UV light. A total of 20 UV mutants were screened for qualitative and quantitative protease production in SmF and solid state fermentation (SSF). Out of 20 UV mutants, UV-8 demonstrated 9 mm diameter of zone of hydrolysis in skim milk agar plate and protease activity of 199.6 ± 6.5 U/ml and 552.6 ± 3.5 U/ml in SmF and SSF, respectively. UV radiations were also utilized for increased production of proteases from *Bacillus* sp. (Wang et al., 2007; Javed et al., 2013; Basavaraju et al., 2014; Immaculate and Patterson, 2014). UV and EMS (ethyl methane sulfonate) were also used as mutagens for increased protease production from *Aspergillus niger* (Radha et al., 2012) and *Aspergellus oryzae* (Yousaf et al., 2010). EMS is an alkylating agent that inserts point mutations by changing the A-T base pairing to G-C base pairing (French et al., 2006). Yadav et al. (2013) developed mutant strain of *Aspergillus flavus* by chemical mutagenesis using 4-nitroquinoline oxide, secreted 38% higher protease than wild strain. Mehtani et al. (2017) reported sequential mutagenesis for increased production of proteases from halotolerant actinomycetes using acridine orange, ethidium bromide and UV rays.

Conclusion

Soil fungal isolate based on our previous experiments was used for recognition by macroscopic and microscopic analysis and identified as belonging to genera *Aspergillus* with the help of its characters we have studied and authentic manual. Then *Aspergillus* sp. was subjected to chemical mutagenesis using nitrous acid in an attempt to increase protease activity in SmF. Highest protease activity and

specific activity was observed after 90 min incubation of spores with nitrous acid. In the present study nitrous acid was noted as potent mutagenic agent who increased protease activity of *Aspergillus* sp. up to 116%.

Declaration of interest

We report no declarations of interest.

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