Research Article

Mutational Studies on Alkaline Protease from *Aspergillus awamori*

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ABSTRACT

Extracellular proteases are naturally produced by microorganisms mainly to degrade large polypeptides in the medium into peptides and amino acids before cellular uptake. Man has commercially exploited such enzymes to assist in protein breakdown in various industrial processes. Protease enzymes constitute one of the most important groups of industrial enzymes. *Aspergillus awamori* KGSR 12 strain was used for protease production. The potential strain was kept for mutational studies. UV radiation is used as mutagenic agent. The mutant obtained i.e., Aspergillus *awamori* KGSR 12 *mu* were used for screening of protease production by plate assay and it showed 2.8 cm diameter clear zone around the colony. Screened mutated strain was employed for the production of protease through submerged fermentation and it showed 4.21 IU after 72 h of fermentation. It showed better results compared with *Aspergillus awamori* KGSR 12 parent strain.

Keywords: Mutation, UV radiation, Protease and Submerged fermentation.

INTRODUCTION

Protease is ubiquitous[1-3]. It exhibits multiple hydrolytic activities on protein to peptides and aminoacids through protein digestion, protein degradation, deproteinization or bio-degradation or bioremediation, biotransformation, etc.[2, 3-10]. As protease is omnipresent, its applications also are enormous physiologically, biotechnologically and industrially[2-4, 11, 12]. In worldwide enzyme market, while protease accounts nearly 60% of sale[2, 3, 9, 13], alkaline protease alone brings $ one billion of economy[13, 14].

Alkaline proteases are gained demand due to their high proteolytic activity with stability at alkalinity[10, 15, 16]. Researchers have strong interests in alkaline proteases because of their applications in detergent and textile industries as an auxiliary agent to release proteinaceous stains[17], degumming and softening of the silk[18-24]; in leather industry, as a substitute for lime for dehairing and release of unwanted protein from hides[10, 20, 23]; in food industry, for cheese making[20], for making soyabean hydrolysate[20, 25], sunflower protein hydrolysate[26] and for baking[20]; in medicine, for medical diagnosis[3, 23], for bioactive peptides production[27]; silver recovery from photographic films like X-ray, CT scan and MRI films[3, 20, 23, 28-31] and so on.

So along with demand comes the need for high yield of alkaline protease which is more active and stable and also safer lead to the search for fungal alkaline proteases[1, 3, 15].

The production of extracellular enzymes is impacted by inducing mutations in fungal cultures[1, 5, 32].Mutational studies were promoted due to the limited work on mutations and their great impact on high yield of enzyme[13, 32, 33].

This work was undertaken to investigate the comparative mutational studies on *Aspergillus awamori* for alkaline protease production in submerged fermentation in a search of eminent production of alkaline protease through strain improvement which is desirable[5, 34].

MATERIALS AND METHODS

Primary screening

Environmentally stressed soil samples were collected from Bangalore city ($12^0$59´ N latitude and

$77^0$35´ E longitude), Karnataka, India. They were screened for protease producers on casein agar medium. After incubated at 30^0C for 72h, the zone of casein hydrolysis was observed. *Aspergillus awamori* KGSR12 (Plate-1, 2) was shown good zone of clearance. The strain was microscopic and molecular identified. This strain was selected for mutational studies.

Screening of mutant for alkaline protease production by plate assay

The mutant fungal strains attained from above projected steps were subjected to rapid screening of alkaline protease by Plate assay (Plate-3). The organisms were grown on Casein agar medium containing (g L^{-1} distilled water) Glucose, 2; Skim milk, 0.5; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4.7H_20$, 0.52; $CuNO_3.3H_20$, trace; $ZnSO_4.7H_20$, trace; $FeSO_4.7H_20$, trace; agar, 20.0 and pH-5.0 at temperature 30^0C. The plates were inoculated with 120h culture of *Aspergillus awamori* KGSR12 mu (Plate-1) for rapid screening of alkaline protease production. The zone of clearance and the colony diameters were measured after 72h and percentage of hydrolysis for *Aspergillus awamori* KGSR12 *mu* were calculated.

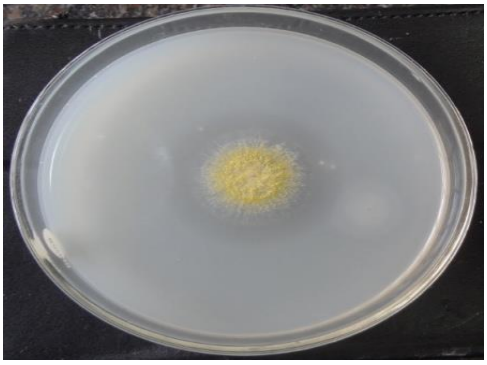

Plate 1: Plate assay of *Aspergillus awamori* KGSR12 *mu*

Mutational studies

The strain *Aspergillus awamori* KGSR12 (Plate-2) was induced through a physical mutation by subjected to UV radiation for enhancing the production of Alkaline protease. *Aspergillus awamori KGSR12* (Plate-2) spore suspensions were prepared and were irradiated using a 15W Philips UV lamp at various distances (5, 20, 40, 60 cm) for 15 min[32]. The irradiated spore suspensions were inoculated on Czapek Dox agar plates. The present study deals with the parent strain and the best mutant strain which was isolated. The strain was labeled as *Aspergillus awamori* KGSR12 *mu* (Plate-3) and used for screening and production of alkaline protease.

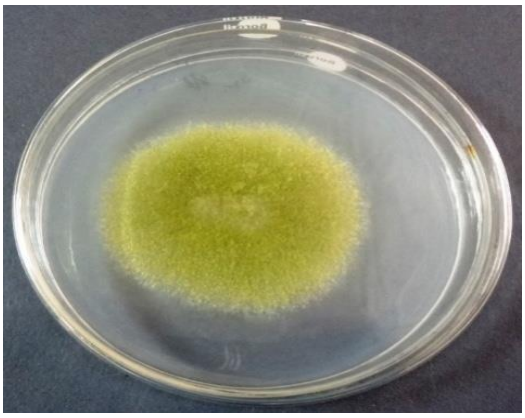

Plate 2: *Aspergillus awamori* KGSR12

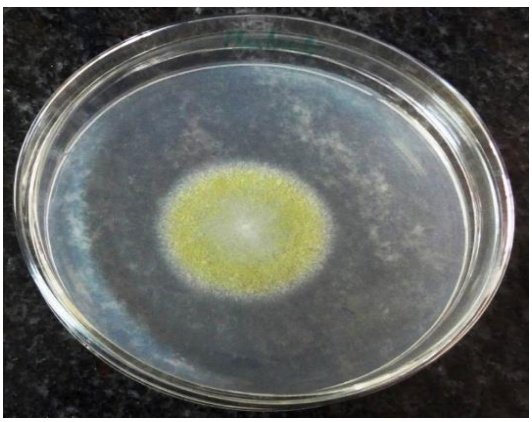

Plate 3: *Aspergillus awamori* KGSR12 *mu*

Comparative fermentation studies on Parent and Mutant strains of *Aspergillus awamori* KGSR12

Inoculum

The homogenous spore suspension was prepared by adding 10mL of 0.01% Tween 80 solution to 168h fresh culture slant and was suspended the spores well with the sterile loop which was used as a inoculum[35]. 1mL of spore suspension of inoculum contains a final concentration of 1 x 10^7spores/mL.

Fermentation Medium

The fermentation medium composition is Sucrose-30.0; Sodium nitrate-2.0; K_2HPO_4-1.0, $MgSO_4$. $7H_2O$-0.5; KCl-0.5; $FeSO_4.7H_20$ -0.01 (g L^{-1} distilled water); pH 6.8 for 96 - 120h.

Submerged fermentation

250mL Erlenmeyer flasks were used for submerged fermentation. 100mL of fermentation medium was prepared in each flask and autoclaved at 121^{0}C, 15lbs for 20 min and cooled to room temperature. Then 1 mL of freshly prepared inoculum suspension was inoculated aseptically in each one and the broth was thoroughly mixed and incubated for 72 to 120h at 35^{0}C.

Extraction of crude alkaline protease from production medium

The culture media samples were extracted of volume approximately 5 mL at 24 h of successive intervals aseptically. The extract was filtered through Whatman filter No.1. The filtrate was centrifuged at 2000-3000 rpm for 15 min, supernatant were used for enzyme preparation. Thus prepared crude enzyme was used for assay of protease.

Alkaline Protease assay

The protease activity was determined by the modified method proposed by Keay *et al*.[36]. 0.5 mL of suitably diluted enzyme is 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 35^{0}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 M Na_2CO_3 and 0.5 mL of Folin Ciocalteau reagent (FCR) was added, mixed thoroughly and incubated at 75^{0}C for 30 min, in dark. The absorbance was measured at 660 nm.

International units (IU)

One protease unit was defined as the amount of enzyme that released 1µg of tyrosine per mL per minute under the above assay conditions.

RESULTS AND DISCUSSIONS

In present study, twenty five strains were isolated to screen the production of alkaline protease by plate assay. *Aspergillus awamori* KGSR12 was selected as it was the potent alkaline protease producer. It exhibited 1.2cm

diameter of zone of clearance and 46.15% of hydrolysis around the colony. This potent strain was used for mutational studies. Because the need of selecting a suitable strain is immensely demanded that is capable of producing commercially acceptable yield for bioprocessing.

The mutant strain *Aspergillus awamori* KGSR12 *mu* was also kept for screening of alkaline protease synthesis by plate assay (Plate-1) and the strain which was kept at a distance of 60cm from UV lamp showed better protease activity. It was also formed comparatively higher zone of diameter of 2.8cm and 64.1% of hydrolysis around the mutant colony. Furthermore, both the parent and the mutant strains were used to produce alkaline protease in fermentation medium. The mutant *Aspergillus awamori* KGSR12 *mu* was showed the highest production of alkaline protease of 4.21IU at 72h fermentation period in submerged fermentation (Figure 1). While comparing with mutant, the parent strain *Aspergillus awamori* KGSR12 could yield only 1.88IU at 72h fermentation period. The mutant *Aspergillus awamori* KGSR12 *mu* produced 2.24 times higher yield than the parent *Aspergillus awamori* KGSR12.

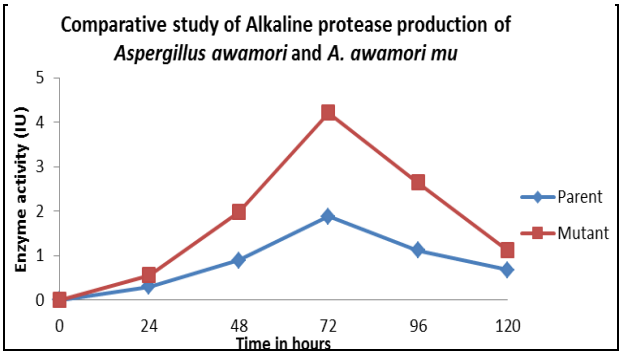

Figure 1: Compression of Parent and Mutant *Aspergillus awamori* KGSR12 and *Aspergillus awamori* KGSR12 *mu*

Review of literature revealed that mutagenic irradiations, usually X-ray, gamma rays and UV rays are used to target fungi. Because the wavelength and penetrating power was high for X-ray and gamma ray, lead to greater unclear damages which lead to lethal effects in target organisms[32, 33]. They are powerful mutagens cause damage of nucleus, so they are avoided. Therefore, mild mutagens like UV rays with shorter wave length than visible light are considered to be ideal for mutational irradiations[32, 33]. Kang *et al.*[37] revealed that mutant *Aspergillus niger* yielded maximum production of various enzymes through UV irradiation. Suntornsuk and Hang[38] also referred that UV irradiation lead to strain improvement of *Rhizopus orygae* in production of glucoamylase. Mutational studies on *Aspergillus niger* by UV rays enhanced the hemi cellulolytic and cellulolytic activity[39].

Basavaraju *et al.*[5] reported that UV mutant *Bacillus* strains expressed 28% improvement in alkaline protease production compared to wild strain. The enzyme activity was found to be 3.45U for wild and 4.78U for the mutant. The report of Djamel *et al.*[40] showed that UV mutant of Pencillium strain S08M4 gave an over-yield of 1400U/ml. Our results are good agreements with the results of Basavaraju *et al.*[5].

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