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SCREENING AND OPTIMIZATION OF CULTURAL PARAMETERS FOR AN ALKALINE PROTEASE PRODUCTION BY ASPERGILLUS TERREUS GR. UNDER SUBMERGED FERMENTATION

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ABSTRACT

An alkaline protease-producing fungus was screened from potato grown soil fields of Bangalore and identified as *Aspergillus terreus* gr. based on morphological and microscopic features. The selected growth medium contained casein (1%) as a sole source of carbon and nitrogen. The pH of the medium was adjusted to 10.0 using sodium carbonate in order to isolate alkalophilic fungi. The best process parameters found after optimization were 5 days of incubation period, initial pH of 10.0, incubation temperature of 37 °C, 2.0% inoculum size, 1.5% casein and 2.0% soybean meal as nitrogen source supplement. All the studied process parameters significantly ($p < 0.05$) influenced the protease production by the isolate. A 4.949 fold enhancement in protease production was achieved after optimization. The increased alkaline protease production by *A. terreus* gr. using cheap nitrogen source, its thermostable nature and the alkaline pH are features which suggest its application in the detergent industry.

KEYWORDS: **Alkaline protease, casein agar, *Aspergillus terreus* gr., medium optimization**

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INTRODUCTION

Proteases (EC 3.4.21-24 and 99) are enzymes that hydrolyse proteins via the addition of water across peptide bonds. They account for about 60% of total worldwide sale of enzymes and they are thus among the largest groups of industrial enzymes. Proteases can be acidic, neutral and alkaline. The pH range of the alkaline proteases is between 7.0 and 14.0, but they are generally active between pH 9.0 and 11.0, with the exception of a few higher pH values of about 12.0 and 13.0. Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions[1]. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a vital role in the regulation of metabolism. The extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes[2]. Some of the applications of the alkaline proteases include the use in laundry detergents to allow the release of proteinaceous material from stains; in food industries such as cheese-making, baking, preparation of soya hydrolysates, and meat tenderization; in the soaking, dehairing and bating stages of preparing skins and hides; in the bioprocessing of X-ray or photographic films for silver recovery and in the management of wastes from various food processing industries and household activities[1-3].Proteases from microorganisms are in general inducible in nature, and their production is thus mostly affected by the nature of the substrate used in the fermentation. The choice of an appropriate inducing substrate is thus necessary[4]. Many of the fungi have been reported to produce extracellular alkaline proteases. Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are mostly used for alkaline proteases production because several species of these genera are generally regarded as safe[5]. *Aspergillus* species are often used for production of industrial enzymes because they can be grown on relatively inexpensive media and secrete bulk quantities of enzymes[6]. Proteases are mainly produced by submerged fermentation due to its apparent advantages in downstream in spite of the cost intensiveness for medium components[7]. Under most culture conditions, microorganisms produce extracellular proteases during the post-exponential and stationary phases and thus are generally regulated by carbon and nitrogen source. The culture conditions that promote protease production were found to be significantly different from the culture conditions promoting cell growth[8]. According to Hajji *et al.*[9], the growth medium is generally optimized with the one-variable-at-a-time method, in which all variables but one is held at a constant level, and then the optimum level of the testing variable is determined. There is no established defined medium for the optimum protease production from microorganisms; each organism has its own special conditions for maximum enzyme production[2]. The most important stage in a biological process is the optimization to improve and increase the efficiency of the process without increasing the cost[10]. Extracellular protease production in fungi is strongly influenced by medium components like carbon and nitrogen sources and by cultural parameters like incubation temperature, initial pH and incubation time[11]. Thus the optimization of media components and cultural parameters is the primary task in a biological process. The optimization of medium composition is carried out to maintain a balance between the various medium constituents thereby reducing the amount of unutilized components at the end of fermentation[12]. Although extensive work has been reported using various fungal strains, there is still a need of extensive and continuous screening for new alkaline protease-producing fungi with better properties and suitable for commercial exploitation[13]. The present study reports the screening of *Aspergillus terreus* gr. which is a potent

producer of an extracellular alkaline protease and the optimization of culture conditions for maximum enzyme production.

MATERIALS AND METHODS

Isolation, screening and identification of alkaline protease-producing fungi

Soil samples were collected from various places of potato grown soil fields of Bangalore, mixed thoroughly in equal proportions and then screened for alkaline protease-producing fungi as follows: 1 g of smooth light brown soil sample was suspended in 10 ml of distilled water and serially diluted (up to 10^{-5}). After soil sedimentation, 0.1 ml of the supernatant of each dilution was spread on the surface of agar medium supplemented with casein as a sole carbon and nitrogen source. The modified casein agar plate contained the following constituents (g/L): casein (10), KH_2PO_4 (1), K_2HPO_4 (1), $MgSO_4$ (0.2) and agar (15)[11]. The agar was added after adjusting the pH to 10.0 with 1 M sodium carbonate before sterilization at 121 ºC for 15 min. The medium was treated with tetracycline before being poured into sterile plates. The palates were then incubated at room temperature (30 ± 2 °C) for 7 days for growth. Development of protease producing fungi was confirmed by the presence of a clear zone around the fungal colony. The colonies with highest casein hydrolyzing ability were picked up and purified by repeated streaking on the same medium and finally transferred to potato dextrose agar (PDA) slants and maintained at 4 °C and at room temperature. Isolate obtained was characterized and identified on the basis of morphological and microscopic features[14]. The isolated fungi were further identified by authentic authority (Agarkar Research Institute, Pune).

Preparation of inoculum

Anandan *et al*.[4] method was used with slight modifications. The fungal inoculum was prepared with addition of 10 ml of sterile distilled water containing 0.1% Tween-80 to the 7th day PDA slant culture of the isolate grown at room temperature (30 ± 2 ºC) and was shaken well to obtain homogeneous spore suspension. The sterile inoculation needle was used to dislodge the spores. The spore suspension to be used as the inoculum was adjusted with sterile distilled water until the optical density was 1.5-1.6 at 530 nm. One ml of inoculum was used per flask to carry out submerged fermentation.

Submerged fermentation and crude fungal alkaline protease production

One ml of inoculum was used to inoculate 100 ml of production medium contained in 250-ml conical flask. The composition of the production medium of pH 10.0 was the same as the one used for screening except that it does not contain agar. The incubation was carried at room temperature (30 ± 2 ^{o}C) for 7 days. After incubation, fungal mycelia and medium debris were separated by squeezing through twofold sterile cheese cloth and then filtering through Whatman No. 1 filter paper. The filtrate was used as a crude enzyme solution.

Determination of crude alkaline protease activity

Extracellular proteolytic activity was determined according to the modified method of Anson[15] using casein as the substrate. The enzyme solution was diluted to 1 ml with 0.2 M Tris–HCl buffer, pH 8.5. 2 ml of 0.65% (w/v) casein solution was added, shaken well, and incubated at 40 ^{o}C for 10 min. After incubation, 3 ml of 10% trichloroacetic acid (TCA) solution was added to the mixture to terminate the reaction, which was followed by additional 20 min incubation. The precipitated casein was filtered off through Whatman No.1 filter paper at 4°C and 1 ml of the filtrate was taken in a test tube. To this 5 ml of copper reagent was added, mixed well by swirling, and incubated for 10 min at room temperature. After incubation, 0.6 ml of the Folin Ciocalteu reagent (one ml diluted with 2 ml of distilled water) was added. Final readings were taken in a UV-spectrophotometer (SL 159, ELICO) at 660 nm. Blanks of the samples were prepared

by adding the TCA before the addition of substrate. The standard solution of tyrosine in the range of 0-200 mg/L was prepared in triplicate to obtain a standard curve. One proteolytic unit (PU) of alkaline protease was defined as the amount of enzyme required to liberate one µg of tyrosine per ml per min under the assay conditions.

Optimization of culture parameters for maximum alkaline protease production

The various factors influencing the alkaline protease production were investigated, examining one factor at a time, keeping all other variables constant except one. Once the optimization has been done with respect to a factor, it was incorporated into the experiment for the optimization of the next factor. They include incubation time, initial pH, incubation temperature, inoculum level, casein concentration and nitrogen source.

Time course study for optimal alkaline protease production

The time course for enzyme production by the fungus under submerged fermentation at room temperature (30 ± 2^{o}C) and at initial pH 10.0 was studied by inoculating the culture medium containing 1% casein with 1% (v/v) inoculum and incubating for 10 days. As the fermentation proceeds, 2.0 ml of the culture broth was withdrawn every day at the same time, filtered through Whatman No.1 filter paper at 4 °C, and then the resulted filtrate was used to determine the most suitable incubation period for maximum protease production.

Effect of initial pH of the medium on alkaline protease production

The influence of initial pH of fermentation media on protease production was investigated by adjusting the pH to 7, 8, 9, 10, 11 and 12 with a digital LI 120 pH meter (ELICO) before sterilization by adding 2 M sodium carbonate. After autoclaving at 121 ºC for 15 min, the fermentation media containing 1% (w/v) casein were inoculated with 1% (v/v) inoculum size and allowed to grow for 5 days at room temperature (30 ± 2 ºC). After fermentation period, the protease activity was evaluated as described earlier.

Effect of fermentation temperature on enzyme production

The fermentation media of pH 10.0 containing 1% (w/v) casein were inoculated with 2% (v/v) inoculum concentration. They were then incubated at different temperatures viz. room temperature (30 ± 2 °C), 37, 45 and 55 °C. At the end of the incubation period of 5 days, the culture filtrate was used for the enzyme assay.

Effect of inoculum size

The effect of inoculums size on protease production was investigated following Anandan *et al*.[4] method with slight modifications. Fermentation was carried out in 250-ml Erlenmeyer flask containing 100 ml of sterilized medium. The production medium was inoculated with different concentrations of fungal spore suspension (1 to 5%, v/v). The flasks after inoculation were incubated at 37 °C for 5 days. Later, the culture medium was filtrated through Whatman No.1 filter paper at 4°C and protease activity was determined in the filtrate as described earlier.

Effect of substrate concentration of enzyme production

To study the effect of casein concentration on alkaline enzyme production, submerged fermentation at pH 10.0 was performed by inoculating media containing different casein concentrations (0.5-3.5%, w/v) with 2% inoculum and incubating at 37 °C for 5 days. The alkaline protease activity was then determined as described earlier.

Effect of nitrogen sources

Various nitrogen sources (0.5%,w/v) viz. yeast extract, peptone, soybean meal, beef extract, ammonium chloride (NH_4Cl), potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$) and ammonium sulfate [$(NH4)_2SO_4$] were added individually to the cultivation medium of an initial pH 10.0 to study the effect of nitrogen substrate on protease production by the

fungus. The production media containing 1.5% casein were inoculated with 2% (v/v) inoculum at 37 °C. After 5 days of incubation period, the culture broth was filtered through Whatman No.1 filter paper at 4°C. The recovered filtrate was assayed for alkaline protease activity as above.The effect of concentration of the best nitrogen source in the fermentation medium for the production of protease was analyzed by varying concentration (0.5 to 3%, w/v) of the chosen nitrogen source (soybean meal) showing the highest activity. Fermentation was carried as earlier and the cell-free filtrate was used to determine the protease activity.

Statistical analysis

All the experiments were carried out independently in triplicates (n = 3) in 250-ml Erlenmeyer flasks. Data were subjected to statistical analysis and the difference between mean values was determined by the student t-test and one-way analysis of variance (ANOVA) using GraphPad Prism software. Differences were considered significant when the probability was less than 0.05.

RESULTS

Screening, isolation and identification of alkaline protease-producing fungi

Screening for alkaline protease-producing fungi, isolated from potato field soils near Bangalore, were performed the by plate culture method. After 5 days incubation, a clear and distinct zone of protease hydrolysis was observed in the casein agar plates. The colonies with highest case in hydrolyzing ability were picked up and purified by repeated screening on the casein agar plates. One of the isolates showing higher zonesof clearance around the fungal colony was chosen for further investigations. On potato dextrose agar (PDA), colonies were typically cinnamon-buff to sand brown in color with a white yellow to deep dirty brown reverse. With lactophenol cotton blue mounting method, hyaline conidiophores and compact, columnar and biseriate conidial heads were seen. The isolate was identified as *A. terreus* gr. based on morphological and microscopic characteristics and further confirmed by National Fungal Culture collection of India, Agarkar Research Institute, Pune.

Optimization of culture parameters for maximum alkaline protease production

The various factors influencing the alkaline protease production viz. incubation time, initial pH, incubation temperature, inoculum concentration, casein concentration and nitrogen source were investigated, analyzing one factor at a time, keeping all other factors unchanged except one. Once the optimization has been done for one factor, it was incorporated into the next optimization experiment.

Time course study for optimal alkaline protease production by A. terreus gr

The protease activity was recorded every 24 h of incubation in order to determine the optimum incubation period for maximum production of enzyme. The enzyme production started after one day of inoculation and showed maximum production (5.521 PU/ml) on 5^{th} day of incubation at room temperature. The prolonged incubation period decreased the enzyme activity (Fig 1).

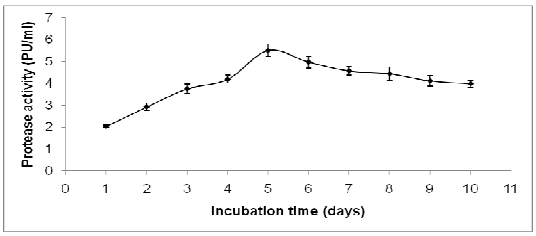

Figure 1

The Alkaline protease production on different days by Aspergillus terreus gr. was grown in the fermentation medium of pH 10.0 containing 1% (w/v) case in. 1% (v/v) inoculum was used to inoculate the media. The incubation was carried out at room temperature for 10 days. Data are mean ± SD (n = 3). The values represented differ significantly from each other at p < 0.05 using GraphPad Prism software.

Effect of initial pH of the medium on alkaline protease production

The effect of initial pH was investigated in order to determine the most suitable initial pH for the alkaline protease production by the fungus. The initial pH of the medium significantly ($p < 0.05$) influenced the production of protease by the *A. terreus* gr. The protease production increased as the initial pH of the medium increased and reached maximum at pH 10.0 (5.743 PU/ml). There was however an important decrease in protease production when the initial pH was increased from 10 to 11 and 12 (Fig 2).

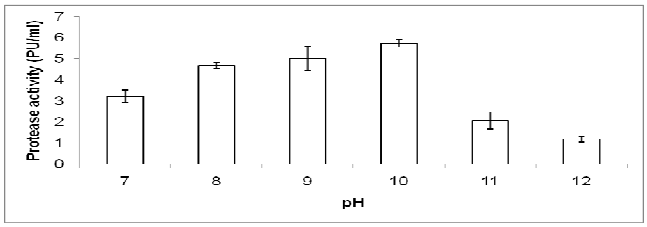

Figure 2

Effect of different initial pH on protease production by A. terreus gr. grown in the fermentation medium containing 1% (w/v) casein, inoculated with 1% (v/v) inoculum and incubated at room temperature for 5 days. The initial pH of 10.0 served as control. Data are mean ± SD (n = 3). The values represented differ significantly from each other at p < 0.05 using GraphPad Prism software.

Effect of fermentation temperature on enzyme production

The effect of incubation temperature on protease production by the fungus was studied. The analysis of variance showed that the differences among protease activities of temperatures tested were statistically significant ($p < 0.05$). *A. terreus* gr. was capable of producing protease in the range of 30 °C to 55 °C with production maximum at 37 °C (Fig 3). However, the increase in temperature beyond 37 °C led to decline in production of enzyme.

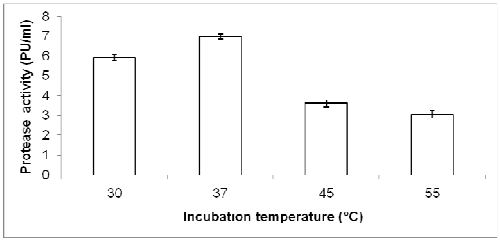

Figure 3

Effect of different incubation temperatures in protease production by A. terreus gr.The fermentation media containing 1% (w/v) casein at an initial pH of 10.0 were inoculated with 1% (v/v) inoculum and incubated for 5 days. The room temperature (30 ± 2 °C) served as control. Data are mean ± SD (n = 3). Values represented differ significantly from each other at p < 0.05 using GraphPad Prism software.

Effect of inoculum level on alkaline protease production by A. terreus gr

The effect of inoculum level on alkaline protease production was studied. One way ANOVA showed that the differences among protease activities of various inoculums used were statistically significant ($p < 0.05$). Maximum enzyme production (9.021 PU/ml) was observed with 2.0% inoculum size and further increases resulted in decreases in alkaline protease production (Fig 4).

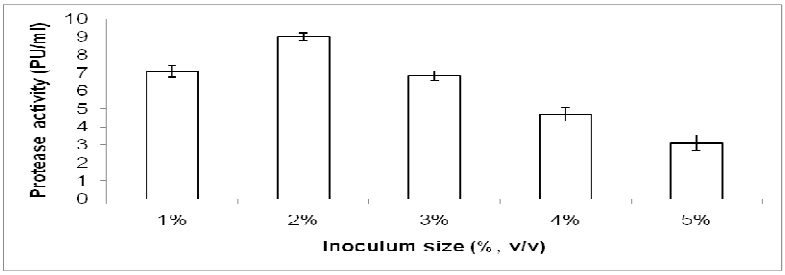

Figure 4

Effect of inoculum size on alkaline protease production under submerged fermentation by A. terreus gr. grown on the medium containing 1% (w/v) casein at an initial pH of 10, inoculated with 1% (v/v) inoculum and incubated at 37 °C for 5 days. The inoculum level of 1% served as control. Data are mean ± SD (n = 3). The values represented differ significantly from each other at p < 0.05 using GraphPad Prism software.

Effect of substrate concentration on alkaline protease production by A. terreus gr

The influence of varying concentration of casein on protease production by *A. terreus* gr. under submerged fermentation is depicted in Figure 5. One way ANOVA showed that the differences among protease activities of various casein concentrations tested were statistically significant ($p < 0.05$). The maximum alkaline protease production was recorded at 1.5% casein concentration, beyond which the activity gradually decreased.

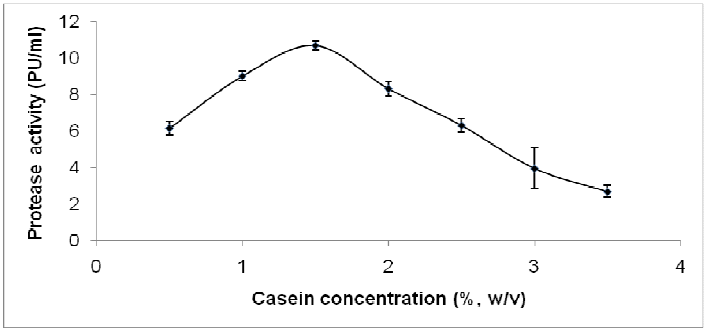

Figure 5

Effect of different concentrations of casein on alkaline protease production by A. terreus gr. The production media at an initial pH of 10 were inoculated with 2% (v/v) inoculum and incubated at 37 °C for 5 days. The case in concentration of 1% served as control. Data are mean ± SD (n = 3). Values represented differ significantly from each other at p < 0.05 using GraphPad Prism software.

Effect of nitrogen sources on alkaline protease production

Alkaline protease production by *A. terreus* gr. was compared among media containing different types of nitrogen sources. The fungus was able to use all the tested nitrogen sources with maximum production in the presence of soybean meal followed by beef extract whereas ammonium sulfate exhibited the least protease production by the isolate (Fig 6). Alkaline protease production was doubled by the addition of 0.5% (w/v) soybean meal to the cultivation medium (from 10.693 PU/ml to 21.554 PU/ml).

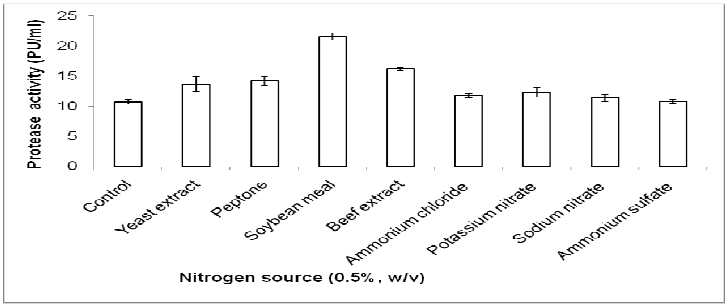

Figure 6

Effect of the different nitrogen sources (0.5%, w/v) on protease production by A. terreus gr. The fermentation media at an initial pH of 10.0 were inoculated with 2% (v/v) inoculum and incubated at 37 °C for 5 days. There was no addition of nitrogen source in the control medium. Data are mean ± SD (n = 3). The values represented differ significantly from each other at p < 0.05 using GraphPad Prism software.

Effect of different concentrations of soybean meal on protease production by A. terreus gr

The fermentation medium was further optimized with various soybean meal concentrations in the range of 0.1 to 3.0%. Maximum protease production was achieved at 2% although protease activity was present at all concentrations investigated (Fig 7).

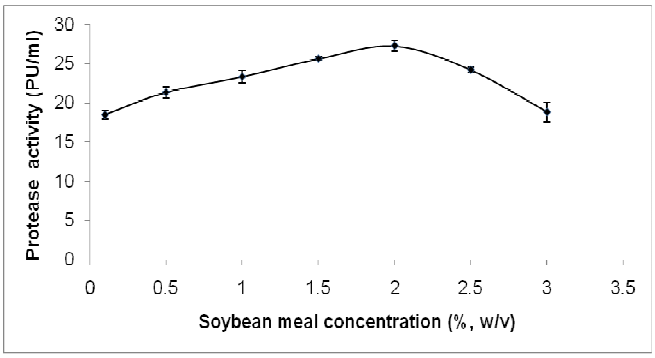

Figure 7

Effect of different concentrations of soybean meal on protease production by A. terreus gr. The production media at an initial pH of 10 were inoculated with 2% (v/v) inoculum and incubated at 37 °C for 5 days. The soybean meal of 0.5% concentration served as control. Data are mean ± SD (n = 3). The values represented differ significantly from each other at p < 0.05 using GraphPad Prism software.

DISCUSSION

Isolation, screening and identification of alkaline protease-producing fungi

Screening and isolation for alkaline protease-producing fungi were carried out. A clear and distinct zone of protease hydrolysis was observed in the casein agar plates after 5 days. Sodium carbonate was used to adjust the growth medium to pH 10.0 in order to isolate alkalophilic fungi. Grant *et al.*[16] reported that sodium carbonate is generally the major source of alkalinity in natural environments and its addition to the isolation media enhances the growth of alkalophilic microorganisms. Casein was used to screen for alkaline protease producing fungi and served as inducers. Kumar and Takagi[2] suggested that in formulating a medium for screening alkaline proteases, the medium must contain inducer for enzyme and should not contain any constituent that prevents enzyme production.The isolate was identified as *A. terreus* gr. Sandhya *et al.*[5] reported that proteases have been mainly produced by *Aspergillus*, *Penicillium* and *Rhizopus* as several species of these genera

are generally regarded as safe. However, the genus of choice for large scale production of industrial enzymes is *Aspergillus* since most of its species can grow on relatively inexpensive media and can secrete bulk quantities of enzymes[6]. Some of the protease producing-*Aspergillus* species isolated from soil include *Aspergillus niger* from soil sample of Coimbatore (Tamil Nadu)[17], *Aspergillus terreus* from butcheries soil sample at Zagazig, Sharkia (Egypt)[18], *Aspergillus* sp. from soil contaminated with abattoir waste collected from rural areas of Tirupati (India)[19] and *Aspergillus flavus* from soil of a sugarcane field of Coimbatore (India)[20].

Optimization of culture parameters for maximum alkaline protease production

The various factors influencing the alkaline protease production were studied. All the investigated culture parameters significantly ($p < 0.05$) influenced alkaline protease production by *A. terreus* gr. It is vital that the alkaline protease-producing fungi be provided with optimal growth conditions to increase enzyme production, making enzyme production cost effective and economically feasible. Moon and Parulekar[8] found that the culture conditions that promote protease production are significantly different from the culture conditions promoting cell growth. There is no established defined medium for the optimum protease production from microorganisms; each organism has its own special conditions for maximum enzyme production[2].

Time course study for optimal alkaline protease production by A. terreus gr

The enzyme production showed maximum production on 5th day of incubation at room temperature. The differences among protease activities on different days were statistically significant ($p < 0.05$). However, when the activity of day 5 was compared to the one of day 6 with student t-test, the difference was not statistically significant ($p < 0.05$). The fungal culture can thus be harvested on day 5 or 6. A broad incubation period ranging from 4 to 9 days for maximum alkaline protease production by *Aspergillus* sp. has been previously reported[12,18,20,21]. The main advantage of enzyme production by *A. terreus* gr. is a shorter incubation period which will reduces operational cost and the decomposition of the enzyme created by protease itself during the submerged fermentation process. The production of enzymes increases with the incubation time to a certain extent. After that, the enzyme production by the microorganism decreases. It was also noticed that prolonged incubation period decreased the enzyme activity and this might be due to due to insufficient availability of some nutrients in the growth medium or decomposition of protease. Romero *et al.*[22] suggested that this decrease can be attributed to the reduced availability of nutrients and the production of toxic metabolites, whereas Anandan *et al*.[4] proposed that the decrease in the protease production is due to the autolysis of the protease. Oyeleke *et al*.[21] found that the production of proteases by *A. flavus* and by *A. fumigates* increased with increase in time. *A. flavus* recorded a higher protease yield than *A. fumigates* and this difference in protease yield was attributed to the differences in their genetic makeup.

Effect of initial pH of the medium on alkaline protease production

It is well known that the initial pH of the medium is an important factor affecting the production of protease. It can affect the growth of the microorganisms either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces[20]. The protease production increased as the initial pH of the medium increased and reached maximum at pH 10.0. This shows the alkalophilic nature of the fungus. The present experimental results indicate that the isolate is sensitive to change in pH because the change in initial growth medium pH affects enzyme production. It has been noted that the most important characteristic of alkalophilic microorganisms is their strong dependence on extracellular pH for cell growth and enzyme production[2]. Maximum

alkaline protease production by different *Aspergillus* sp. has been reported at various optimum initial pH of the medium[17,19,21,23]. The pH requirements vary from species to species and even in different stains of the same species isolated from different habitats. This might be attributed to the differences in their genetic makeup.

Effect of fermentation temperature on enzyme production

Incubation temperature is an important environmental factor for the production of proteases by microorganisms because it affects growth rates of microorganisms, regulates the synthesis of the enzyme and also the enzyme production by changing the properties of the cell wall[24]. *A. terreus* gr. was capable of producing protease in the range of 30 °C to 55 °C with production maximum at 37 °C. However, the increase in temperature beyond 37 °C led to decline in production of enzyme proving that temperature plays a major role in protease production. The incubation time of 37 °C for enzyme production was also reported for *A. funiculosus* G. Smith[25]. The present experimental results showed a decrease in enzyme activity at elevated temperatures and this may have been due to the decomposition of the proteolytic enzyme by autolysis. Likewise, protease production ceased at higher temperature for *A. flavus*[20]. Conn *et al.*[26] explained that the enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure.

Effect of inoculum level on alkaline protease production by A. terreus gr

The level of inoculation is one of the key factors for fungal growth and alkaline protease production for submerged fermentation. Maximum enzyme production was observed with 2.0% inoculum size and further increases resulted in decreases in alkaline protease production. With higher inoculum concentrations the nutrient might have been consumed rapidly and overall resulted in less alkaline protease production. Sarao *et al*.[27] also noticed a rapid increase in alkaline protease production with an increase in inoculum level due to fast degradation of the substrate. In general, at lower inoculums levels, the yield is very low, whereas large inoculum levels are inhibitory in nature[28]. Hesseltine[29] suggested that the decrease seen with large inoculum size could be attributed to the shortage of the nutrients available for the large biomass and faster growth of the culture. Studies by Muthulakshmi *et al.*[20] showed that higher inoculum levels decreased protease production owing to clumping of cells which could have reduced sugar and oxygen uptake rate. Various inoculum levels in the range of 1 to 10% were found to maximally produce alkaline proteases by different *Aspergillus* sp.[4,19,20]. The inoculum concentration has thus profound effects on protease production depending upon the characteristics of the strains.

Effect of substrate concentration on alkaline protease production by A. terreus gr

The maximum alkaline protease production was recorded at 1.5% casein concentration, beyond which the activity gradually decreased. Casein was used as carbon and nitrogen source. Under most culture conditions, microorganisms produce extracellular proteases during post-exponential and stationary phases and thus are generally regulated by carbon and nitrogen source. Proteases from microorganisms are in general constitutive or partially inducible in nature, and their production is mostly affected by the nature of the substrate used in the fermentation. The choice of an appropriate inducing substrate is thus necessary[4]. A study by Kamath *et al.*[11] on the optimization of cultural conditions for protease production from *A. niger*, using casein as substrate, has shown the highest proteolytic activity at 1.5% casein concentration on day 5. Casein induced protease production at low concentrations.

However, it acted as substrate repressor at high concentration[30].

Effect of nitrogen sources on alkaline protease production

Inorganic and organic forms of nitrogen are metabolized in most microorganisms to produce amino acids, nucleic acids, proteins, and cell wall components. The fungus was able to use all the tested nitrogen sources with maximum production in the presence of soybean meal. Similarly, soybean meal was best nitrogen source for protease production by *Aspergillus* sp.[12,19]. Fermentation media containing organic nitrogen sources gave higher protease productions when compared to inorganic nitrogen sources. Several reports have demonstrated the use of organic nitrogen sources leading to higher enzyme production than the inorganic nitrogen sources in *Aspergillus* species[19].

Effect of different concentrations of soybean meal on protease production by A. terreus gr

Maximum protease production was achieved at 2%. A trend on the inhibitory effect of soybean meal was observed in alkaline protease production at an increasing concentration and this might be to nitrogen metabolite repression. The nitrogen metabolite repression was also reported as a regulatory on the synthesis of protease in many types of yeast[30].

CONCLUSION

A potent alkaline protease-producing fungus was screened from soil sample and identified as *Aspergillus terreus* gr. It was found to be a better thermotolerant and a better alkolophilic fungal strain which can therefore be utilized in detergent industry and other biotechnological applications. The optimization of culture conditions played a central role in improving the enzyme production through the submerged fermentation process. A 4.949 fold enhancement in protease production (from 5.521 PU/ml to 27.325 PU/ml) was achieved after optimization. This increase was especially due to the supplementation of a cheap and readily available soybean meal. This strongly indicates that the isolate can be economically used for commercial alkaline protease production. Purification and characterization of an alkaline protease from *A. terreus* gr. are under progress.

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