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OPTIMIZATION OF FERMENTATION CULTURE CONDITIONS FOR ALKALINE PROTEASE PRODUCTION BY *Scopulariopsis* spp.

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ABSTRACT

The present study was aimed to assess alkaline protease production by a locally isolated soil fungus *Scopulariopsis* spp. The factors influencing enzyme production were optimized under submerged conditions. Maximum enzyme production occurred on 5th day of incubation on fermentation medium with initial pH 9.0, incubation temperature 37ºC, inoculum concentration 3%, casein 2% as substrate and 1.5% tryptone as nitrogen source. Under these optimized conditions, about 3-fold increase in protease activity (from 5.85 to 17.77 U ml^{-1}) was noticed. High enzyme activity at alkaline pH suggested that the fungus can be exploited for use in leather processing and detergent industries.

Key words: Alkaline protease, casein, optimization, *Scopulariopsis*, submerged fermentation

INTRODUCTION

Proteases (EC 3.4.21-24, 99), the enzymes which catalyze proteolysis, are found in plants, animals and microorganisms. The proteases derived from plants and animals are insufficient to meet the current global demands, so more focus is presently on proteases of microbial origin. Proteases of bacterial and fungal origin possess almost all the characteristics desired for biotechnological applications. Currently due to potential industrial applications there is great demand for alkaline lipases of fungal origin. They are used in food processing, laundry detergent to help in the release of proteinaceous stains, leather industry for dehairing, and tanning industry in several bioremediation processes, silver recovery, waste treatment and in the synthesis of fine chemicals (Rao *et al.*, 1998). Many fungi reportedly producing extracellular alkaline proteases belong to the genera *Aspergillus*, *Penicillium* and *Rhizopus* (Sandhya *et al.*, 2005). However, the reports on other genera are scanty especially with respect to genus *Scopulariopsis* (Sing and Vezina, 1971). The reduction in enzyme production cost by optimizing the fermentation culture conditions is a major goal of basic research of industrial applications (Li *et al.*, 2006). Recent approaches for enhancing protease yield include isolation and screening for hyper-producing strains and optimization of fermentation conditions for higher yields. Hence, the present study was undertaken to optimize fermentation conditions for alkaline protease production by *Scopulariopsis* spp. under submerged fermentation.

MATERIALS AND METHODS

Soil sample was collected from different poultry yards in Bangalore, Karnataka (India). The fungus was isolated by serial dilution plating method on skim milk agar containing skim milk powder (10%, w/v), peptone (0.5%, w/v) and agar (2%, w/v) (Sharma *et al.*, 2006) at pH 10.0 and 30°C. Formation of clear zone of casein hydrolysis served as indicator for alkaline protease production. The colonies with high casein hydrolyzing ability were characterized and identified on the basis of morphological and

microscopic features (Konemann *et al.*, 1997) and identity confirmed by National Fungal Culture Collection of India, Agarkar Research Institute, Pune (Maharastra). The fungal inoculum was prepared with addition of 10 ml sterile distilled water containing 0.1% Tween-80 to the 7th day potato dextrose agar (PDA) slant culture of the isolate grown at 30ºC. The inoculum was then adjusted with sterile distilled water until the optical density was 1.5 at 530 nm (Anandan *et al.*, 2007). One millilitre inoculum was used to inoculate 100 ml fermentation medium consisting of casein (1%), yeast extract (0.4%), KH_2PO_4 (0.05%), K_2HPO_4 (0.05%) and $CaCl_2$ (0.05%) (Pathak and Deshmukh, 2012). The incubation was carried at 30°C for 7 days. Thereafter, the broth was centrifuged in a cooling centrifuge (Remi) at 10,000 rpm for 10 min. at 4°C to remove fungal mycelia and medium debris. The clear supernatant was used as crude enzyme solution. Protease activity was assayed as per the modified method of Anson (1938) using casein as substrate. The amount of amino acids released was calculated and compared with a standard curve plotted from a known concentration of tyrosine. In brief, 0.5 ml enzyme was incubated with 0.5 ml 0.2 M Tris-HCl buffer (pH 8.5) and 2.0 ml 0.65% casein at 50°C for 10 min. After incubation, the reaction was arrested by the adding 3.0 ml 10% trichloroacetic acid. The mixture was kept undisturbed for 20 min. and the resultant precipitate removed by centrifugation at 10,000 rpm for 5 min. The supernatant (0.5 ml) was diluted to 1.0 ml with distilled water. To this, 5.0 ml alkaline cupper reagent was added, shaken well and incubated for 10 min. After incubation, 0.6 ml Folin Ciocalteu reagent (1 ml diluted with 2 ml distilled water) was added, the tubes shaken well and incubated in dark for 30 min. to allow blue colour development. The absorbance was measured at 660 nm using a UV-spectrophotometer (SL 159, Elico). One unit of alkaline protease was defined as the amount of enzyme required to liberate 1 μg tyrosine min^{-1} ml^{-1} under the test conditions.

The various process parameters influencing alkaline protease production were optimized individually and independent of others, and optimal conditions used in the subsequent experiments in sequential order. The fermentation medium was adjusted according to the treatment condition to be optimized and each treatment replicated thrice. The optimal incubation period at pH 10.0 and 30°C was assessed by collecting the culture supernatant at regular intervals every day for a period of 8 days. As the fermentation proceeded, 2.0 ml culture broth was withdrawn every day, centrifuged and enzyme activity determined. The effect of initial pH of medium was studied by adjusting the pH of fermentation medium in range of 7 to 12 with a digital pH meter (LI 120, Elico) using 2 M sodium carbonate. The activity was then assayed on 5th day. For optimum temperature, the fermentation medium of pH 9.0 was incubated at 30, 37, 45 and 55°C for 5 days. The inoculum levels in 1 to 5% range were studied as per Anandan *et al*. (2007). The effect of casein at various concentrations was assayed in 0.5 to 3.5%(w/v) range. Various nitrogen sources were assessed by replacing yeast extract in fermentation medium with test nitrogen source. The best nitrogen source was assayed at various concentrations varying from 0.1 to 2.5% (w/v). After each fermentation process, the culture broth was centrifuged and the supernatant used to assay protease activity as described earlier. All the experiments were carried out independently in 250 ml Erlenmeyer flasks each containing 100 ml sterilized fermentation medium with each individual treatment replicated thrice (n = 3). Means of variables and standard deviation were recorded for each experiment. The significant differences among the means for each cultural condition were investigated by one way analysis of variance and Duncan's multiple range test (DMRT) by running SPSS software at the 5% significance level.

RESULTS AND DISCUSSION

The alkaline protease-producing fungus, isolated from local poultry yard soil, was identified as *Scopulariopsis* spp. on the basis of its morphological and microscopic characteristics. Charles *et al.* (2008) isolated *Aspergillus nidulans* HA-10 from a poultry farm and feather dumping soil in Tamil Nadu (India). The enzyme production by the fungus gradually increased with incubation time and highest enzyme activity was observed on 5th day, beyond which activity showed a gradual decrease (Table 1). This decrease may be attributed to the less nutrient availability or production of toxic metabolites or protease decomposition (Anandan *et al.*, 2007). Radha *et al.* (2011) observed maximum

Table 1: Effect of incubation time, initial pH, incubation temperature, inoculum load, casein concentration, nitrogen source and tryptone concentration on alkaline protease activity (U ml^{-1}) by *Scopulariopsis* spp.

Incubation time (days)							
1	2	3	4	5	6	7	8
2.54 ± 0.40^{f}	3.26 ± 0.26^{e}	3.75 ± 0.26^{d}	$4.60\pm0.17^{b,c}$	5.85 ± 0.27^{a}	5.00 ± 0.17^{b}	4.53 ± 0.29^{c}	4.39 ± 0.15^{c}

Initial pH of the medium					
7.0	8.0	9.0	10.0	11.0	12.0
4.23 ± 0.26^{b}	5.61 ± 0.39^{a}	6.17 ± 0.38^{a}	5.98 ± 0.41^{a}	3.51 ± 0.49^{c}	2.22 ± 0.31^{d}

Incubation temperature (°C)			
30	37	45	55
8.15 ± 0.22^{b}	8.90 ± 0.32^{a}	6.50 ± 0.44^{c}	5.70 ± 0.38^{d}

Inoculum concentration (%)				
1.0	2.0	3.0	4.0	5.0
8.95 ± 0.34^{b}	9.90 ± 0.34^{a}	10.38 ± 0.22^{a}	6.45 ± 0.51^{c}	4.18 ± 0.11^{d}

Casein concentration in medium (%)						
0.5	1.0	1.5	2.0	2.5	3.0	3.5
5.31 ± 0.71^{f}	10.29 ± 0.39^{c}	11.29 ± 0.39^{b}	12.53 ± 0.18^{a}	8.68 ± 0.27^{d}	6.59 ± 0.60^{e}	4.23 ± 0.30^{g}

Nitrogen source replacing equivalent casein (%)							
Yeast extract	Peptone	Tryptone	Beef extract	Ammonium chloride	Potassium nitrate	Sodium nitrate	Ammonium sulfate
12.63 ± 0.36^{b}	11.27 ± 0.42^{c}	15.59 ± 0.37^{a}	$10.63\pm0.33^{c,d}$	7.56 ± 0.50^{e}	10.41 ± 0.42^{d}	8.20 ± 0.38^{e}	6.70 ± 0.62^{f}

Tryptone (%, w/v)					
0.1	0.5	1.0	1.5	2.0	2.5
12.53 ± 0.28^{d}	15.47 ± 0.68^{c}	16.62 ± 0.28^{b}	17.77 ± 0.38^{a}	11.85 ± 0.28^{e}	7.80 ± 0.34^{f}

Data presented are mean±standard deviation for each treatment. In rows the values with different superscripted alphabets significantly differ from each other at $P_{0.05}$.

protease production by *Aspergillus* species on 5th day of incubation. Maximum protease production in present study was achieved in medium with initial pH 9.0 although statistically at par with pH 10 (Table 1), thereby dipicting alkalophilic nature of this fungus. Anandan *et al.* (2007) observed maximum protease production by *Aspergillus tamari* when initial pH of the medium was maintained at 9.0. In present study maximum enzyme production was observed at 37°C and minimum at 55°C (Table 1). The enzyme production gradually decreased when incubation temperature was increased beyond 37°C. This may be ascribed to the denaturation of protein at higher temperature (Conn *et al*., 1987).

Maximum protease production was obtained when fermentation medium was inoculated with inoculum @ 3% (Table 1). Use of higher inoculum levels lead to the decrease in enzyme production, probably due to the shortage of nutrients. The higher protease activity was in medium having 2% casein. At lower casein concentration, the enzyme yield was low, whereas higher casein concentrations decreased enzyme production. It appears that the amino acids and/or peptides in casein act as specific inducers in enhancing alkaline protease production. Samarntarn *et al.* (1999) showed increased alkaline protease yield by *A. oryzae* U1521 when casein was used as a source of peptides and amino acids. In present study a decrease in protease activity was also noted with higher casein concentration. This decease in enzyme production at higher casein level may be attributed to substrate repression (Ogrydziak, 1993). Maximum protease activity was recorded when tryptone was used as nitrogen source in medium (Table 1). Radha *et al*. (2011) also found tryptone as best organic nitrogen source for maximal protease production by *Aspergillus* species. The concentration of 1.5% tryptone in medium as nitrogen source gave highest protease yield. It was noticed that protease production increased with increase in tryptone concentration upto 1.5% and declined thereafter. Complex organic

nitrogen sources like tryptone being rich in amino acids and short peptides displayed enzyme repression when concentration used was high (Ogrydziak 1993). It may be concluded that alkaline protease-producing *Scopulariopsis* spp., isolated from local soil, is thermotolerant and alkolophilic in nature. The remarkably high enzyme activity at alkaline pH suggests that the fungus is promising alkaline protease producer and may find application in leather processing and detergent industries.

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