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PROCESS ECONOMIZATION OF ORGANIC AND INORGANIC NITROGEN SOURCE IN SUBMERGED MEDIA FOR ALKALINE PROTEASE PRODUCTION FROM *ASPERGILLUS AWAMORI*

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ABSTRACT

Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are (physiologically) necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Protease production were carried out by using supplementation of organic and inorganic nitrogen sources such as Beef extract, Yeast extract and peptone at concentrations ranging from 0.25% to 1.25% with increments of 0.25% and also different inorganic nitrogen sources like Ammonium chloride and ammonium sulphate at concentrations ranging from 0.025% to 0.125% with increments of 0.025%. The peptone (1%) produced 3.15 IU were best organic nitrogen source and ammonium sulphate (0.025%) appear to be good inorganic nitrogen source under submerged fermentation process and showed 2.98 IU by using *Aspergillus awamorii* KGSR12.

KEYWORDS: Protease, Organic Nitrogen, Inorganic Nitrogen, Submerged Fermentation.

INTRODUCTION

Proteases launch the largest cluster of bio-industrial enzymes with a long array of applications (Jisha et al., 2013). Microorganisms are the preeminent stop of proteases in consideration of economical, technological and ethical facets. In which, fungal alkaline proteases established increasingly interest because of production of wide variety of proteases, referred as GRAS (Generally Regarded As Safe), produce extracellular and so ease of down streaming process.

Alkaline proteases are highly stable at pH range 8 to 14 and exhibit optimum proteolytic activity. Accordingly, their applications are fashioned in detergent and textile industries, leather industry, food industry, bioactive peptide production, silk industry, silver recovery from photographic films (49). Alkaline proteases productivity from microorganisms is influenced by not only environment but also the nutritional conditions. So process economization of various nutritional and cultural sources for optimum yield of alkaline protease is easy, economical and essential in industrial production.

As a part of it, in this investigations, we report the influence of one of the major cultivate nutritional conditions, nitrogen (both organic and inorganic) ingredients on the production of alkaline protease from *Aspergillus awamorii* using submerged fermentation.

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 for the screening of protease producers on casein agar medium. After incubation at 30^0C for 3 days, the zone of casein hydrolysis was observed. *Aspergillus awamorii* KGSR12 was shown good zone of clearance and was selected for further studies. Microscopic and molecular identification of *A. awamorii* KGSR12 was done as it was referred in paper.

Optimized fermentation kinetics

Aspergillus awamorii was cultured in CzapekDox Agar medium with provided organic and inorganic source separately with various concentrations by keeping the physical parameters constant such as incubation temperature 35^0C, initial pH 6.0 and inoculum size 0.5mL as it was referred in (1t Paper1) paper.

Nitrogen sources

Organic nitrogen sources

Beef extract, Yeast extract and peptone were synthetic organic sources used to fermentation media for optimum production of alkaline protease. Various concentrations (0.25%, 0.5%, 0.75% and 1.0% of w/v) of each organic source were added to 100ml of Czapek Dox fermentation medium.

Inorganic nitrogen sources
Ammonium chloride and ammonium sulphate were used as inorganic nitrogen sources to fermentation media for optimum production of alkaline protease. Unlike the organic source concentrations, the concentrations of the inorganic were 0.025%, 0.05%, 0.075% and 0.1% of w/v of each were added 100ml of CzapekDox fermentation medium separately.

Submerged fermentation
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 (Lingappa and Vivek Babu, 2005). 1ml of spore suspension of inoculum contains a final concentration of 1 x 10^7spores/ml.

Fermentation Medium
The prepared inoculum of *Aspergillusawamori* KGSR 12 was added to the 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; $FeSO_4$-0.01 (g/L of distilled water) for 96 -120h.

Extraction of protease from production medium
The samples of volume approximately 5 mL of incubated culture broth were extracted time to time at 24 hrs in aseptic condition. The extract was collected initially by filtration through Whatman filter No.1 and then by centifugation at 2000-3000 rpm for 15 min. The supernatant was collected for enzyme preparation and for assay of protease.

Alkaline Protease assay
According to modified Keay et al., 1970, the protease activity was determined by using extracted enzyme. 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 35^0C 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 FolinCiocalteau reagent (FCR) was added, mixed thoroughly and incubated at 75^0C 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 DISCUSSION
According to Siddalingeswara and Lingappa (2010), based on casein hydrolysis on Casein/Skimmed milk agar plates, *Aspergillus awamori* KGSR12 was found to be the best protease producer of twenty five isolates isolated from eco-stressed soil samples collected from Bangalore, Karnataka, India. Thus, *A. awamori* KGSR12 was identified and selected for further studies.

Process economization with nitrogen sources
To detect the eminent nitrogen source for alkaline protease production by *A. awamori*, the fermentation media was supplemented with three organic (beef extract, yeast extract and peptone) and two inorganic (ammonium chloride and ammonium sulphate) nitrogen sources.

El-Safey and U. M. Abdul-Raouf (2004) were showed the effect of inorganic nitrogen sources. The results of different nitrogen sources in relation to protease production by *Bacillus subtilis*. Different inorganic nitrogen source were used. The best nitrogen source for protease production was (NH_4) 2 SO_4 with enzyme level 10.96 units/m.

Organic nitrogen source as supplement
The results of alkaline protease production through process economization with organic nitrogen sources (four sets of each i.e., beef extract, yeast extract and peptone) were carried out by supplementing with 0.25%, 0.5%, 0.75% and 1.0% of w/v concentrations of production media are represented in Figure 1, 2 and 3.

It reveals that the production of alkaline protease increased with the increased conc. of beef extract and yeast extract from 0.25% (1.67 IU with beef extract, 1.34 IU with yeast extract) to 0.75% (2.54 IU with beef extract, 2.56 IU with yeast extract) and then decreased at 1.00% conc. of supplements (2.23 IU with beef extract, 2.36 IU with yeast extract) at 72nd hours of fermentation period. But in case of peptone supplement, unlike the above pattern, the alkaline protease production was increasing with the conc. of peptone and produced maximum productivity of 3.15 IU at 1.00% of peptone conc. which is the highest of all organic nitrogen supplement concentrations at 72nd hours of fermentation period.

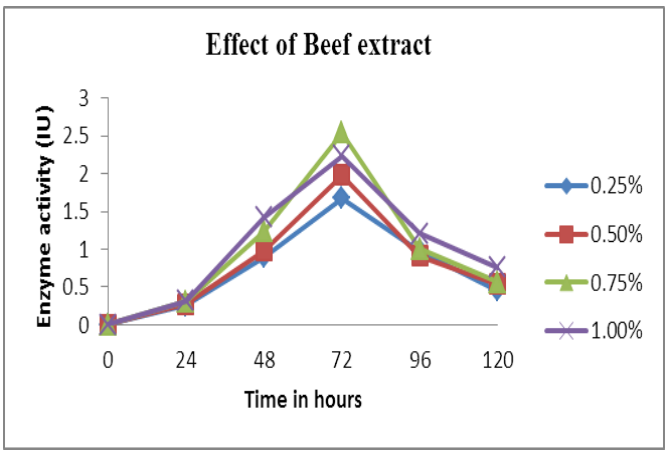

Figure. 1: Effect of Beef extract on alkaline protease production.

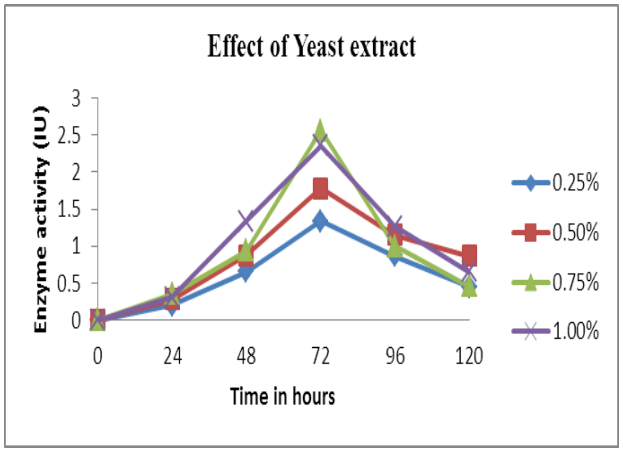

Figure. 2: Effect of Yeast extract on alkaline protease production.

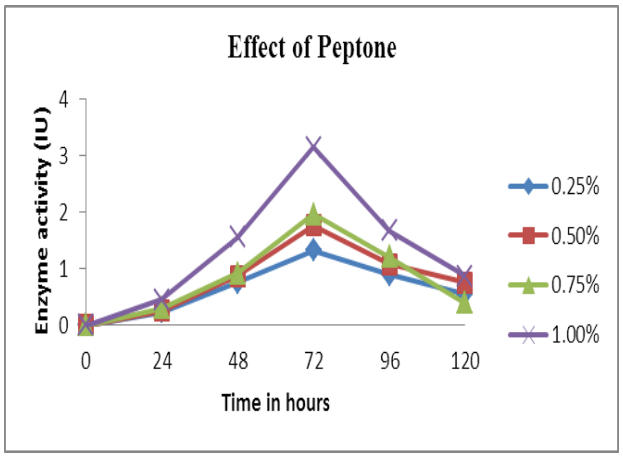

Figure. 3: Effect of Peptone on alkaline protease production.

Inorganic nitrogen source as supplement

The results of alkaline protease production with inorganic nitrogen sources (four sets of each i.e., Ammonium chloride and Ammonium sulphate) were carried out by supplementing with 10 times less concentrations (compared to organic supplements) such as 0.025%, 0.05%, 0.075% and 0.10% of w/v concentrations of production media are represented in Figure 4 and 5.

The results reveal that alkaline protease production are maximum at low conc. (0.025%) of inorganic nitrogen supplements like 2.87 IU with NH_4Cl, 2.98 IU with $(NH_4)_2SO_4$. Then the productivity was decreased with the increase of conc. of both NH_4Cl and $(NH_4)_2SO_4$ at 72nd hours of fermentation period.

The above results reflect that organic nitrogen supplements enhance the productivity of alkaline protease with concentration whereas inorganic nitrogen supplements inhibit the productivity with concentration.

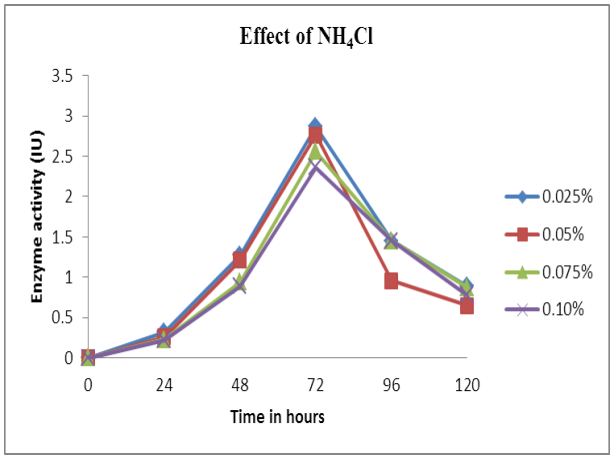

Figure. 4: Effect of Ammonium chloride on alkaline protease production.

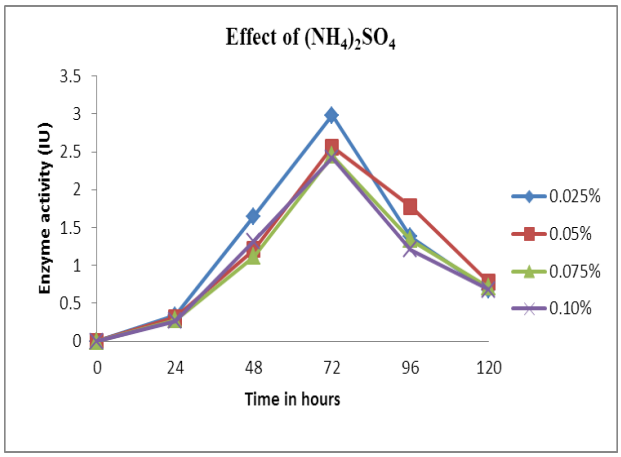

Figure. 5: Effect of Ammonium sulphate on alkaline protease production.

Mulimani and Patil (1999) were showed in organic nitrogen sources for the biosynthesis of protease. The ammonium sulphate, Ammonium nitrate, urea and potassium nitrate were used in range of 1.5%. Among the inorganic nitrogen source ammonium nitrate were showed better activity i.e 1.02 IU.

Srinubabu *et al.*, (2007) were discussed and observed that diammonium hydrogen phosphate was found to be the best nitrogen source and all other inorganic N-sources gave 80-90% protease activity compared to organic nitrogen source (malt extract). Gais *et al.*, (2009), were reported that the addition of inorganic nitrogen sources such as ammonium chloride and potassium nitrate (41.81 US/ml and 26.26 US/ml) respectively cause a decrease in protease production compared to control (77. 42 US/ml).

Sindhu *et al.*, (2009), Nitrogenous salts such as NH_4NO_3, KNO_3, NaNO3, NH_4Cl, $(NH_4)2CO_3$, $NaNO_2$, $(NH_4)2SO_4$ were incorporated at 0.5% level in the medium. *P. godlewskii* SBSS 25 showed highest activity in presence of NH_4NO_3 followed by $KNO_3$and $NaNO_3$. It showed lowest activity in the presence of $(NH_4)2CO_3$. Certain nitrogenous salts tend to decrease the pH of the culture

medium and had the adverse effect on enzyme production, although they supported the growth of the organism (Wang *et al*., 1974).

Radha *et al*., (2011), the impact of different inorganic and organic nitrogen sources on biomass and protease production were. The maximum protease activity was 2.307 U/ml1 with potassium nitrate followed sodium nitrate used in the present study than the control. As such our findings are in close agreement with Mulimani and Patil (1999).

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