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RESEARCH ARTICLE**EFFECT OF FERMENTATION KINETICS FOR THE BIOSYNTHESIS OF PROTEASE FROM *ASPERGILLUS AWAMORI***Radhika Pilli¹, *Siddalingeshwara K G²¹Research & Development Centre, Bharathiar University, Coimbatore, India²Scientific & Industrial Research Centre, Bangalore, India

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ABSTRACT

Among the various groups of microorganisms filamentous fungi are most widely exploited because of their ability to grow on complex medium and production of wide range of extracellular enzymes. This study highlights the production of extracellular protease biosynthesis were carried out by using *Aspergillus awamori* was evaluated under different fermentation kinetics by employing submerged fermentation method. The protease producers detected by the clear zone (casein hydrolysis) around the colony by simple plate assay method. *Aspergillus awamori* KGSR 12 was the potential strain among the fungal isolates. The Protease biosynthesis was increased their yield after the optimization of fermentation parameters. The optimum pH 6.0 (1.96 IU), temperature 35°C (2.12 IU) and inoculum size 0.5 ml showed 2.23 IU.

Key words: Fermentation Kinetics, *Aspergillus awamori*, Protease, Plate assay.

INTRODUCTION

Microorganisms have been endowed with vast potentials of new biocatalysts (enzymes). They produce an array of enzymes, which have been exploited commercially over the years. In recent years, the potentials of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Jayani *et al.*, 2005; Alva *et al.*, 2007).

Proteases (EC 3.4.21-24) are enzymes that hydrolyze proteins and form a bulky cluster of enzymes which are ubiquitous in nature and most central category of enzymes from an industrial point of view. An extensive range of microorganisms has great potential to produce alkaline proteases under suitable growth conditions (Arulmani *et al.*, 2007).

The industrial demand for highly active preparations of proteolytic enzymes with appropriate specificity and stability to extreme pH and temperature continues to stimulate the search for new enzyme sources. Although the majority commercial proteases originated from bacteria belonging to the genus *Bacillus*, fungi exhibit a wider variety of proteases than bacteria. In addition, fungi are normally GRAS (generally regarded as safe) strains and they produce extracellular enzymes, which can be recovered easily from the fermentation broth. Certain genera such as *Aspergillus*, *Penicillium* and *Fusarium* have been reported to produce neutral proteases (Sandhya *et al.*, 2005).

Among the various groups of microorganisms filamentous fungi are most widely exploited because of their ability to grow on complex solid substrates and production of wide range of extracellular enzymes.

The aim of the present study was to evaluate *Aspergillus awamori* as a producer of protease under submerged fermentation by using designed medium with different fermentation kinetic parameter.

MATERIALS AND METHODS**Microorganism and maintenance**

The fungi used in this study were collected from stressed environmental soils. The soils were taken from different regions of Bangalore (12°59' N latitude and 77°35' E longitude) of Karnataka. Fungal strains were isolated from soil samples through serial dilution method (Waksman, 1927). The stock cultures of the isolates were maintained by sub-culturing them on CzapekDox Agar slants and 120h to 168 h incubated cultures were stored at 4°C.

Protease screening-Plate assay

The isolated strains were screened for their protease production by plate assay where protease producers were detected by the clear zone (casein hydrolysis) around the colony and were used for further studies. Totally thirty five fungal strains were isolated and the best protease producer fungal strain was identified as *Aspergillus awamori* KGSR 12. Tentatively identified in the laboratory and was confirmed by molecular level. The screening medium is as follows. Glucose, 2; Skim

milk,0.5; KH₂PO₄,1.52; KCL,0.52; MgSO₄.7H₂O,0.52; CuNO₃.3H₂O, trace; ZnSO₄.7H₂O, trace FeSO₄, trace; agar, 20.0 and pH-5.0 (g/L distilled water) (Plate-1).

Inoculum preparation

The fungal inoculum was prepared by adding 10ml of 0.01% Tween 80 solution to 168 h culture slant and was suspended well with the sterile loop to obtain homogeneous spore suspension (Lingappa and VivekBabu, 2005). Each mL of spore suspension of inoculum contains a final concentration of 1×10^7 spores/mL.

Fermentation Medium

The selected *Aspergillus awamori* KGSR 12 were cultured on production medium. The production medium consists (g/L of distilled water) Sucrose-30.0; Sodium nitrate-2.0; K₂HPO₄-1.0, MgSO₄. 7H₂O-0.5; KCl-0.5; FeSO₄-0.01; pH 6.8 for 96 -120h.

Fermentation Kinetic studies for the biosynthesis of Protease

Initial pH

The 250 ml Erlenmeyer flasks containing 100 ml of production medium were prepared by mixed with acid/alkali solution to obtain required initial pH. The pH was adjusted in the range of 3-7 with increments of 1.0. Thus prepared flasks were cotton plugged and autoclaved at 121°C for 15 min. The flasks were inoculated and incubated.

Temperature

The 100ml of the production medium was separately taken in 250 ml Erlenmeyer flasks and prepared for submerged fermentation. Thus prepared flasks were incubated at different temperatures like 25-40°C with increments of 5°C.

Inoculum size

The inoculum was prepared separately by reviving the 168h old culture of *Aspergillus awamori* KGSR12 at different levels i.e., 0.25, 0.50, 0.75, 1.0 and 1.25 ml and then fermentation studies were carried out.

Extraction of protease from production medium

The culture broth samples of volume approx. 5 mL were withdrawn periodically at 24 hrs in aseptic condition. The extract was filtered through Whatman filter No.1. The clear extract 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.

Assay of Protease

The protease activity was determined by the modified method proposed by Keay et al.(1970). 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°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 Na₂CO₃ and 0.5 ml of Folin Ciocalteu reagent (FCR) was added, mixed thoroughly and incubated at 75°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 1 µg of tyrosine per ml per minute under the above assay conditions.

RESULTS AND DISCUSSION:

The Fungal isolates were identified tentatively as *Aspergillus awamori* in the laboratory which produced clear zone around colony in casein (0.5%) plate medium; those were selected from the soil samples. Of the thirty isolates, *Aspergillus awamori* KGSR 12 was considered to be the best and high protease producing strain. It showed 1.12 cm of cleared zone around the colony and 41.3% of hydrolysis. The results obtained in the present study on the effect of pH and temperature on submerged fermentation is represented in (Fig. 1 and 2) which reveals that the production of protease increased with the increase in pH of the medium up to pH 6.0 (1.96 IU), with increase in temperature 35°C (2.12 IU) at 72nd hours of fermentation period and thereafter the decrease of protease was observed.

Figure 1: Effect of pH on Protease production

Figure 2: Effect of temperature on Protease production

The results reviewed that Francois *et al.* (2011), they showed that pH 6 was the suitable for maximum protease production by using *Aspergillus carbonarius* and it shows 0.14 IU. Karthic *et al.* (2014) were reported screening and production were carried out by using *Aspergillus oryzae* and they reported 1.7 IU at 72 hrs of fermentation period. Vishwantha and Appu (2010), who reported pH 5.0 as the best initial pH for the production of protease from *Aspergillus oryzae* MTCC 5341. Our results are good agreement with Karthic *et al.*, (2014).

The 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 (Satynarayana *et al.*, 1994). Similar observation were observed by Karthic *et al.*, (2014) were reported that the production were carried out by using *Aspergillus oryzae* and they reported 1.36 IU at

72 hrs of fermentation period. Similar observations were reported by Francois *et al.*, (2013), reported that the maximum production of protease was observed at temperature 37°C by using *Aspergillus terreus* and it showed approximately 7 IU/ml. Our results are coincides with Karthic *et al.*, (2014).

The importance of inoculum size on microbial fermentation process is widely accepted. Out of five inoculum size tested (0.25, 0.50, 0.75, 1.0 and 1.25 ml) and 0.5 ml inoculum was found to be the most suitable for high production of protease by *Aspergillus awamori* KGSR 12 in submerged fermentation at 72 hrs of fermentation and it showed 2.23 IU. From Fig. 3, it is clear that the protease production steadily increased with the increasing in the size of the inoculum until it reaches to the magnitude when enzyme productivity became maximum, thereafter no appreciable change in production of protease with high inoculum size could be observed.

Figure 3: Effect of inoculums size on Protease production

Francois *et al* (2013) reported that 2% of fungal spores as an inoculum. The maximal protease production (9.021 PU/ml) was observed when an inoculum concentration of 2% fungal culture was added. At lower and higher inoculum levels, poor protease

production was observed. Karthic *et al.*, (2014) reported that the maximum enzyme production (1.7 IU/ml) was observed in 1.0% of initial inoculum supplemented conditions. Our results are best agreements with the results of Karthic *et al.*, (1994).

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