Optimization of process parameters for production of alkaline protease by OVAT method using isolated strain *Alternaria alternata* TUSGF1

ABSTRACT

Aim: The current study aimed at studying the optimum fermentation conditions for alkaline protease production by submerge fermentation using *Alternaria alternata* TUSGF1, isolated from poultry farm soil.
Study Design: Statistical analysis origin 6.1 software was study to optimize the environmental and nutritional parameters for protease production by OVAT method.
Place and Duration of Study: Department of Food technology and Biochemical Engineering, Jadavpur University, Kolkata, West Bengal, India between March 2017 and May 2017.
Methodology: A protease producing microorganism was isolated from from a poultry farm soil and identified as *Alternaria alternata* TUSGF1. Various environmental and nutritional process parameters such as volume of medium, fermentation time, temperature, age of inoculums, agitation and supplementation of different starch sources and nitrogen sources were standardized for the maximum yield of alkaline protease.
Results: The optimum conditions for protease activity was 30°C at volume of medium 60 ml with 7 days age of inoculum in the medium containing 168 h of incubation and 120 rpm agitation rate. Peptone, casien, skim milk, urea and yeast extract were good nitrogen sources whilst maltose, fructose, starch, and sucrose were appropriate for enzyme production by submerge fermentation.
Conclusion: Alkaline protease production by a newly isolated *Alternaria alternata* TUSGF1 from poultry fram soil was studied in shake flask conditions by submerge fermentation. It was establish that the optimum protease production was recorded at 30 °C, 60 ml volume of medium leaves and incubation time of 168 h. The best carbon and nitrogen sources for protease production were fructose and casein, respectively.

Keywords: Alkaline protease; casein; culture media; optimization; submerge fermentation.

1. INTRODUCTION

Alkaline proteases are one of the most broadly studied group of enzymes since of their wide utilize in various industrial applications such as food, detergent, pharmaceutical and leather and with two-third of distribute in detergent industry alone [1]. Microbial proteases are gaining new significance than conventional chemicals that cleave peptides since of the cheaper production rate and utilize of renewable resources. Microbial proteases can be produced from bacteria, fungi and yeast using several processes such as solid-state fermentation, submerged fermentation [2]. Filamentous fungi, such as *Aspergillus*, have been the organism of choice for large scale production of bulk industrial enzymes, as the fungi can be grown on relatively inexpensive media and the fungi can secrete bulk quantities of enzymes [3]. A proteolytic enzyme that had been isolated from *Aspergillus tamarii* was used to dehair goat skins [4].
It is also largely dependent on higher oxygen mass transfer and lesser shear forces on microorganisms. For aerobic fermentation, oxygen transfer is a key variable and is a function of aeration and agitation. Therefore, it is necessary to establish optimum combination of airflow and agitation for maximum yield. It is well known to alkaline protease production by microorganisms is significantly enhance by media components, physical factors like, aeration, agitation, temperature, inoculum density, dissolved oxygen and fermentation time [5, 6]. Designed for productions of enzyme for industrial apply isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process [7].

This paper reports the results of a study carried out to investigate the high-production of protease enzyme from isolated strain and optimization of cultural conditions such as carbon sources, nitrogen sources, initial medium volume, fermentation time, temperature, age of inoculums and agitation for maximum production of protease.

2. MATERIALS AND METHODS

2.1 Microorganisms

Alternaria alternata TUSGF1 (strain accession number MF401426) strain was originally isolated from poultry farm soil [8] and maintained on Potato Dextrose Agar (PDA) media and stored at 4^0C.

2.2 Optimization of Protease Production

A loop full of culture was added into 50 ml of modified basal medium (pH 9.0) containing glucose 30%, casein 1%, KCl 0.5%, $FeSO_4$ 0.01%, $MgSO_4$ 0.5, K_2HPO_4 1% into 250 ml Erlenmeyer flask. The medium was incubated at 30 ^{0}C for 7 days at 120 rpm [9]. At the end of fermentation period, the culture medium was centrifuged at 4000 rpm for 10 minutes and the culture supernatant was used as a crude enzyme.

2.3 Protease Assay

Protease activity was measured using the method described by Kembhavi et al. (1993) [10]. The enzyme activity of the crude enzyme was estimated spectrophotometrically at 280 nm. One unit of protease activity was definite since the amount of enzyme that released 1µg of tyrosine per minute under the assay condition.

2.4 Protein Assay

Protein estimation was done by the method of Lowry *et al* (1951), with bovine serum albumin (BSA) as standard [11].

2.5 Fungal Biomass Measurements

Culture media were filtered using Whatman No. 1 filter paper and dried at 70^0C overnight [12].

2.6 Statistical analysis

All the data was statistically evaluated by origin 6.1 software to optimize the process parameters for protease production.

2.7 Optimization of different growth conditions

A range of process parameters influencing enzyme production were optimized independently and individually of the others and the optimized conditions were used in all subsequent study in sequential array. Effect of different volume of medium (20ml, 40ml, 60ml, 80ml), various temperature ranging from 20 to 50 (°C), fermentation time periods up to 216 hours, effect of different agitation rate (80-140), various age of inoculum ranging from 3-9 day and different cheap carbon sources 1% (Glucose, maltose, fructose sucrose and starch) were also evaluated for optimum production of alkaline protease by *Alternaria alternata* TUSGF1. To study the effect of different nitrogen sources on protease production, casein in the basal medium was substituted with (0.5% w/v) of peptone, yeast extract, skim milk and urea.

3. RESULTS AND DISCUSSION

3.1 Effect of volume of medium on alkaline protease production

To study the effect of different volume of medium on alkaline protease production various volume ranges (20 ml, 40 ml, 60 ml and 80ml) were used separately for all fermentation media. The maximum alkaline protease production (30 U/ml, protein 0.095 mg/ml and biomass 16 mg/ml) was observed at 60 ml of volume of medium.

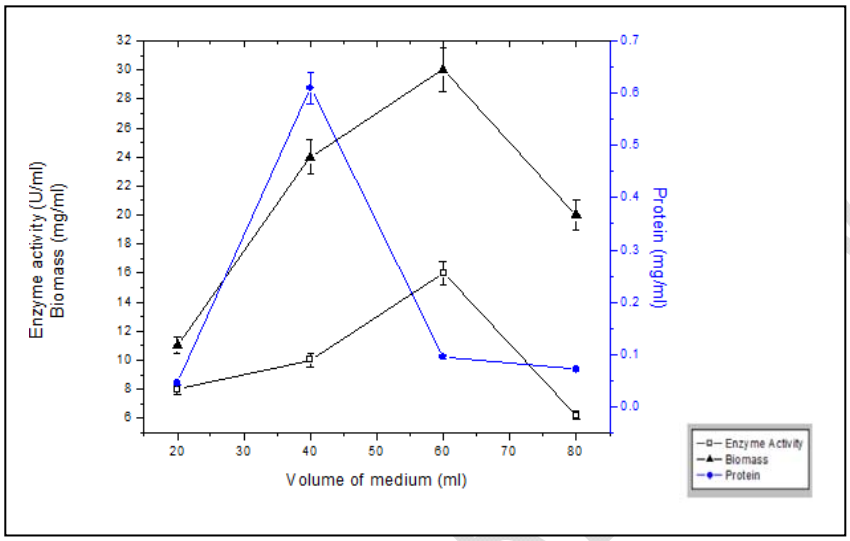

Fig.1. Effect of various volume of medium on protease production by *Alternaria alternata* TUSGF1.

3.2 Effect of incubation time on protease production

To investigate the effect of fermentation period on the production of protease enzyme was incubated at 30°C for different time periods from 72 hrs to 216 hrs. It was found that maximum enzyme activity, total protein and cell biomass were found to be (37 U/ml), (0.120 mg/ml) and (20 mg/ml) respectively (Fig 4). As the fermentation period increases from 168 hrs the enzyme activity, total protein and cell biomass was started to decrease. The fermentation period is fixed designed for the maximum protease production by bacteria or fungus may vary from 48 hrs to 9 days depending upon the strain and substrate used as reported in several cases [13].

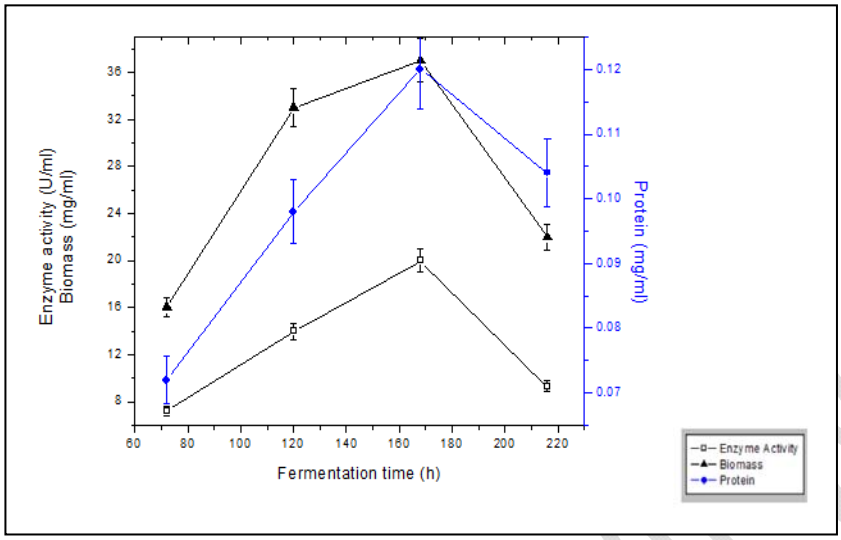

Fig.2. Effect of various fermentation time on protease production by *Alternaria alternata* TUSGF1.

3.3 Effect of incubation temperature on protease production

The fermentation flasks were incubated at different temperature ranges (20 °C, 30 °C, 40 °C and 50°C) were used individually for protease production. The optimal fermentation temperature for the *A. alternata* protease production was 30 °C for submerged fermentation (Fig. 3). So, fermentation temperature must always be considered a significant parameter although carrying out the fermentation experiments [14].

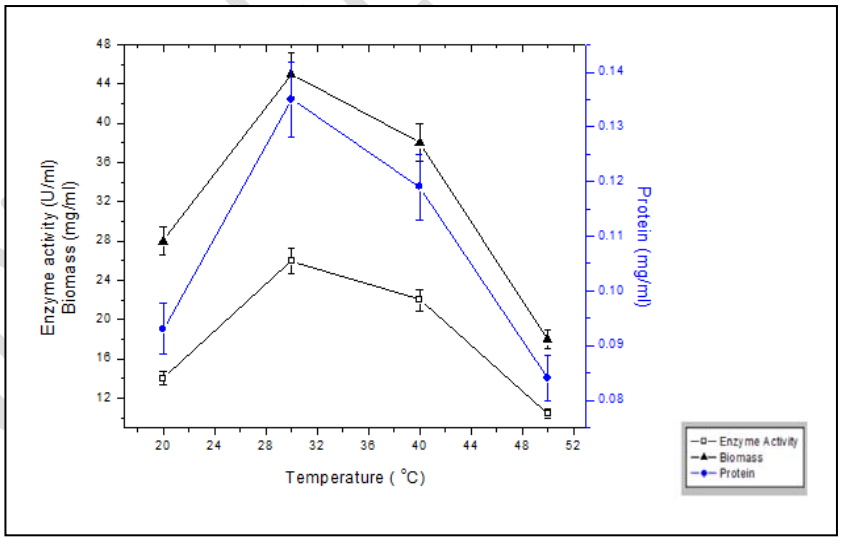

Fig.3. Effect of various temperature on protease production by *Alternaria alternata* TUSGF1.

3.4 Effect of age of inoculum on protease production

The age of inoculum is one of the key factors for microbial growth and activity for submerge fermentation (Fig. 4). The optimal protease production occurred with a 168 hrs age of inoculum. It was observed that 7

days age of inoculum gave highest enzyme activity, total protein and cell biomass (49 U/ml), (0.142 mg/ml) and (28 mg/ml) respectively.

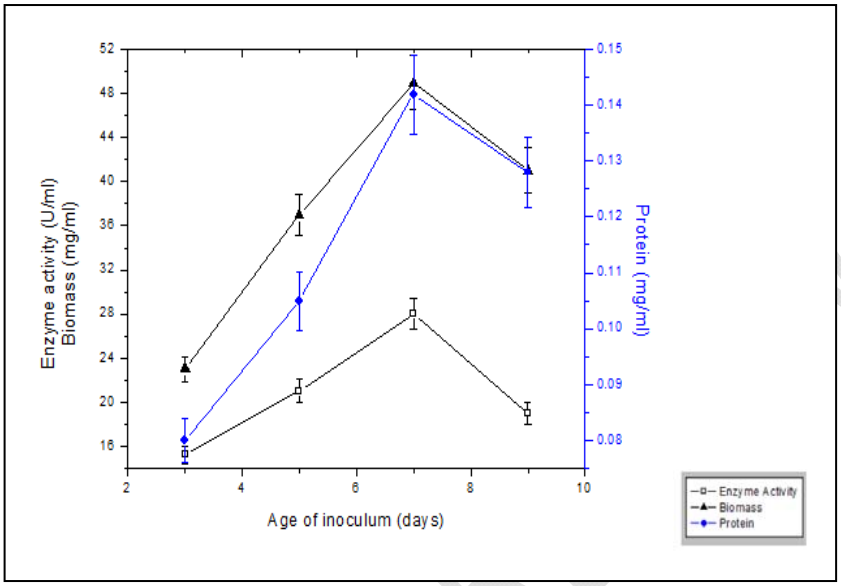

Fig.4. Effect of various age of inoculum on protease production by *Alternaria alternata* TUSGF1.

3.5 Effect of agitation rate on protease production

The effect of various agitation rates on alkaline protease production various agitation rate (80, 100, 120 and 140) were used. The results depicted that the highest protease production was 53 U/ml, protein 0.168 mg/ml biomass 35 mg/ml (Fig.5). As the agitation rate was increased above 120 rpm, the enzyme production was decreased.

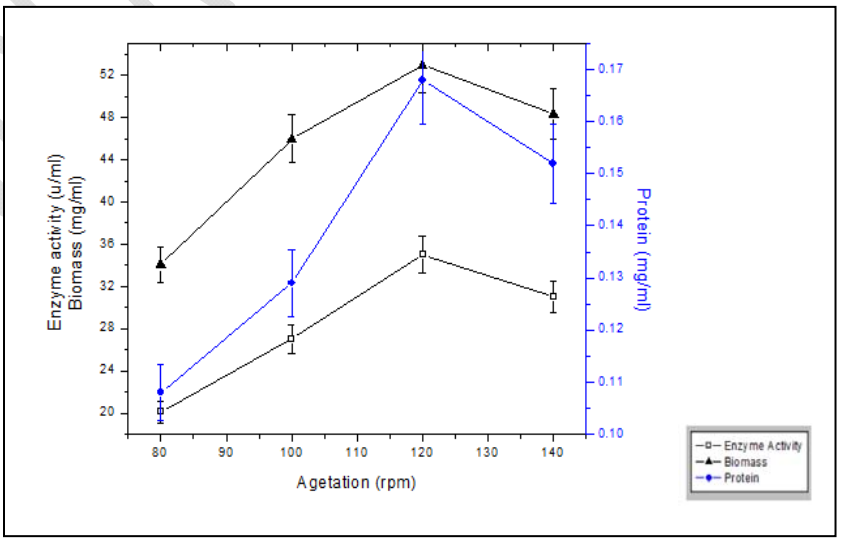

Fig.5. Effect of various agitation on protease production by *Alternaria alternata* TUSGF1.

3.6 Effect of carbon sources on protease production

Different carbon sources have various impacts on the production of alkaline protease by OVAT method. Among a range of carbon sources tested, fructose was found to be the most excellent support protease production in culture medium (Fig. 6). Maximum enzyme activity was observed (77 U/ml). In addition, optimum protease activity was also found in the basal media supplemented by glucose, sucrose, starch and maltose into culture media. Johnvesly and Naik reported [15] starch; raffinose, arabinose and fructose to be good carbon sources.

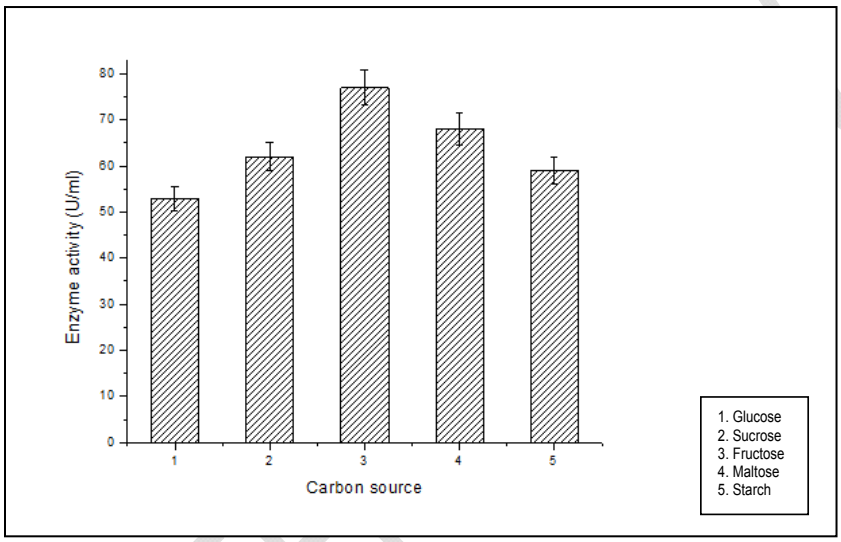

Fig.6. Effect of various carbon sources on protease production by *Alternaria alternata* TUSGF1.

3.7 Effect of nitrogen source on protease production

The effect of various nitrogen sources on the production of protease was checked out by inoculating a set of flasks with various nitrogen sources i.e. casien, skim milk, yeast extract peptone and urea incubated at 30 °C for 168 hrs at 120 rpm. It was noted that skim milk as a nitrogen source has a significant effect on protease production (Fig. 7) and shows optimum enzyme activity (96 U/ml). It has been previously reported by Shampa *et al*. [16] that nitrogen sources have significant enhance on production of alkaline protease.

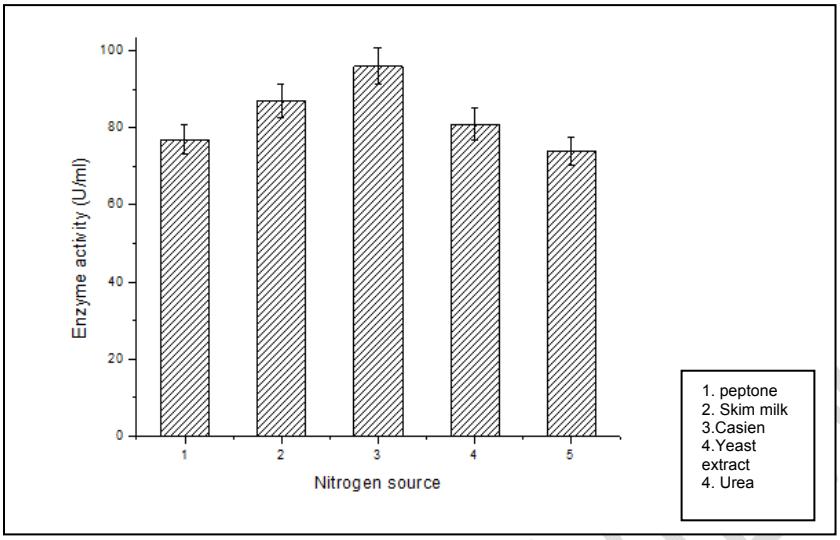

Fig.7. Effect of various carbon sources on protease production by *Alternaria alternata* TUSGF1.

4. CONCLUSION

In this experiment, we established that the culture broth of *Alternaria alternata* TUSGF1 grown on broth medium displayed the proteolytic activity. Among the different carbon and nitrogen parameters tested in the current study 1% fructose and 0.5% casien was found to be the most excellent inducer. These are cheap and readily accessible production it the substrate of selection for cost effective media formulations. Nutritional optimization showed an approximately 3.31-fold enhance in protease activity followed by environmental optimization, which showed a 1.83-fold enhance under the submerged fermentation. Therefore, based on the optimization studies, we achieved a yield of 96 U/ml (3.31-fold increase) with the *Alternaria alternata* TUSGF1 when cultivated for 168 h at 7days age of inoculum, 30 °C and 120 rpm.

References

1. Ravichandra Potumarthi, Subhakar Ch., Annapurna Jetty**.** Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. Biochemical Engineering Journal 34 (2007) 185–192.
2. Haki GD, Rakshit SK. Developments in industrially important thermostable enzymes: a review. Bioresour Technol. 2003; 89: 17–34.
3. Bergquist PL, Te'o VS Jr, Gibbs MD, Cziferszky ACE, DeFaria FP, Azevedo MO, Nevalainen KMH. Production of recombinant bleaching enzymes from thermophilic microorganisms in fungal hosts. Appl Biochem Biotechnol. 2002; 98–100:165–176
4. Dayanandan A, Kanagaraj J, Sounderraj L, Govindaraju R, Suseela Rajkumar G. Application of an alkaline protease in leather processing: an ecofriendly approach. J Clean Prod. 2003; 11:533–536.
5. Gupta R, Beg QK, Khan S, Chauhan B. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biot. 2002; 60: 381–395.
6. Celik E, Calik P. Bioprocess parameters and oxygen transfer characteristics in beta-lactamase production by *Bacillus* species. Biotechnol Prog. 2004; 20: 491–499.

7. Krishna Suresh Babu Naidu, Kodidheia Lakshmi Devi. Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. 2005; 4 (7): 724-726
8. Polley T, Ghosh U. Isolation and identification of potent alkaline protease producing microorganism and optimization of biosynthesis of the enzyme using RSM. Indian Chem Eng. 2018; 60 (3): 285-296.
9. Polley T, Ghosh U. Statistical optimization of alkaline protease production using isolated strain by submerged fermentation. JABB. 2016; 7(4): 2394-1081.
10. Kembhavi AA, Kulharni A, Pant AA. Salt- tolerant thermostable alkaline protease from *Bacillus subtilis* NCIM No. Appl Biochem Biotechnol. 1993; 38: 83-92.
11. Lowry OH, Rosebrough NJ, Farr AC & Randall RJ. Protein measurement with the folin-phenol reagent. J Biol Chem. 1951; 19: 265-275.
12. Abidi F, Limam F, Nejib MM. Production of alkaline proteases by *Botrytis cinerea* using economicraw materials: Assay as biodetergent. Process Biochem. 2008; 43:1202–08.
13. Mukherjee AK, Adhikari H, Rai SK. Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using Imperata cylindrical grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation. Biochem Eng J. 2008; 39:353-361.
14. Rao K, Narasu LM. Alkaline Protease from *Bacillus firmus* 7728. African Journal of Biotechnology. 2007; 6(21): 2493-2496.
15. Krishnaveni K, Mukesh kumar DJ, Balakumaran MD, Ramesh S and Kalaichelvan PT. Production and optimization of extracellular Alkaline Protease from *Bacillus subtilis* isolated from dairy effluent. 2012; 4 (1):98-109.
16. Shampa S, Dasu VV, Bishnupada M. Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1. Ann Microbiol 2009; 59(3):531-538.