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

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ABSTRACT

Aim: The current study aimed at studying the optimum fermentation conditions and nutritional conditions for alkaline protease production by submerge fermentation using *Alternaria alternata* TUSGF1, isolated from poultry farm soil.
Study Design: The results of environmental and nutritional parameters for protease production by OVAT method was analyzed by origin 6.1 software.
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 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 carbon 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, skimmed 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 farm soil was studied in shake flask conditions by submerge fermentation. It was established 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.

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1. INTRODUCTION

Alkaline proteases are one of the most broadly studied group of enzymes because of their wide application in various industries including food, detergent, pharmaceutical and leather with two-third of distribute in detergent industry alone [1]. Utilization of renewable resources and cheaper production rate makes microbial proteases more significant than conventional chemicals sources that cleave peptide bonds. 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 to be significantly enhance by media components, physical factors like, aeration, agitation, temperature, inoculum density, dissolved oxygen and fermentation time [5, 6]. Isolation and characterization of new potent strain for enzyme production using cheap carbon and nitrogen source is a continuous process [7].
This paper report 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 determined according to the method described by Kembhavi [10] using casein as substrate. The enzyme activity of the crude enzyme was estimated spectrophotometrically at 280 nm. The proteolytic unit was defined as 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 were 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 days 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, skimmed 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 (Fig 1) 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. Ganguly and Banik, also reported maximum L-glutamic acid production by mutant of *Micrococcus glutamicus* in the flask [13].

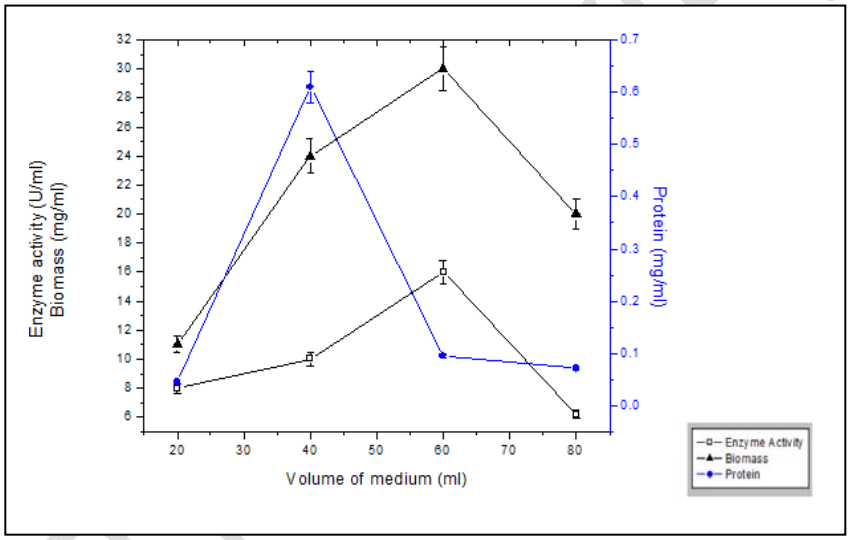

Fig.1. Effect of various volume of medium on protease production by *Alternaria alternata* TUSGF1.

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 h to 216 h. 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 2). As the fermentation period increases from 168 h 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 h to 216 h depending upon the strain and substrate used as reported in several cases [14].

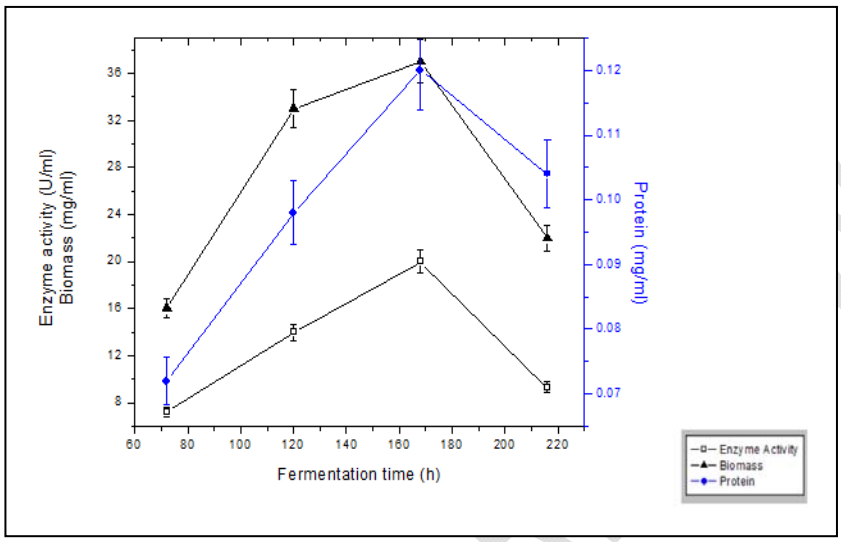

Fig.2. Effect of various fermentation time on protease production by *Alternaria alternata* TUSGF1.

3.3 Effect of incubation temperature on protease production

The fermentation flasks incubated at different temperature ranges of (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 as a significant parameter when carrying out the fermentation experiments [15].

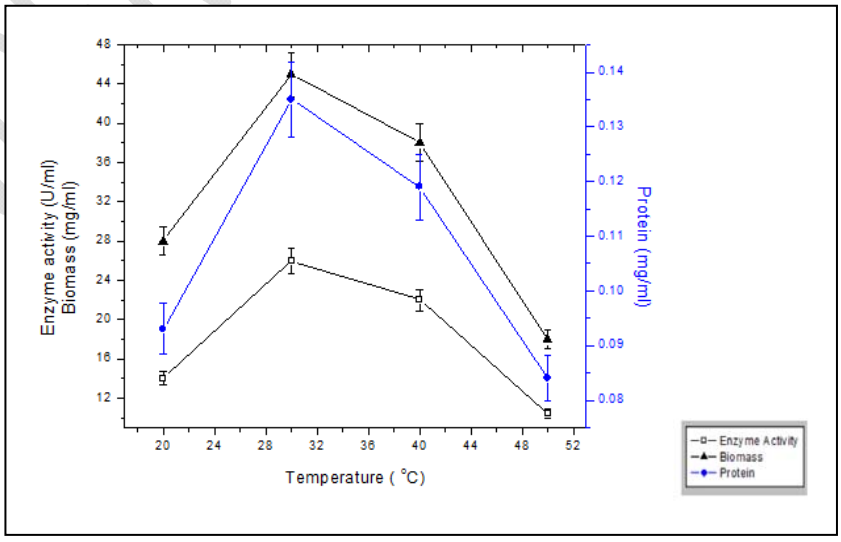

Fig.3. Effect of various temperature on protease production by *Alternaria alternata* TUSGF1.

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 7 days 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. The best age of inoculum was found at 30 h [16].

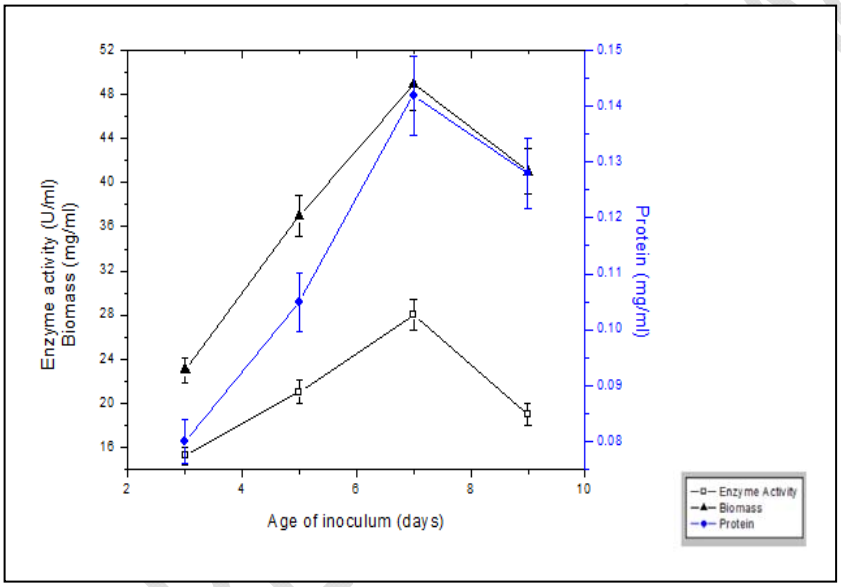

Fig.4. Effect of various age of inoculum on protease production by *Alternaria alternata* TUSGF1.

3.5 Effect of agitation rate on protease production

To study the effect of agitation 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 decreased. At this speed, aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media [17].

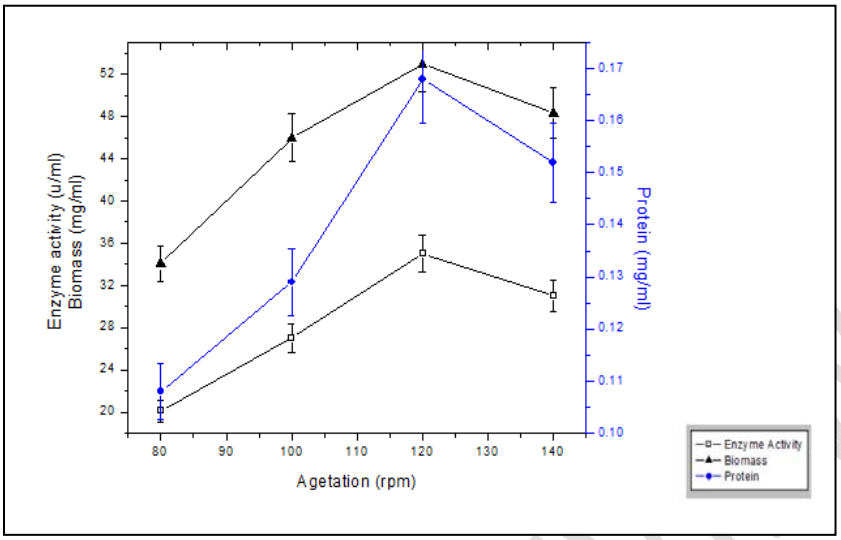

Fig.5. Effect of various agitation on protease production by *Alternaria alternata* TUSGF1.

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 [18] starch; raffinose, arabinose and fructose to be good carbon sources.

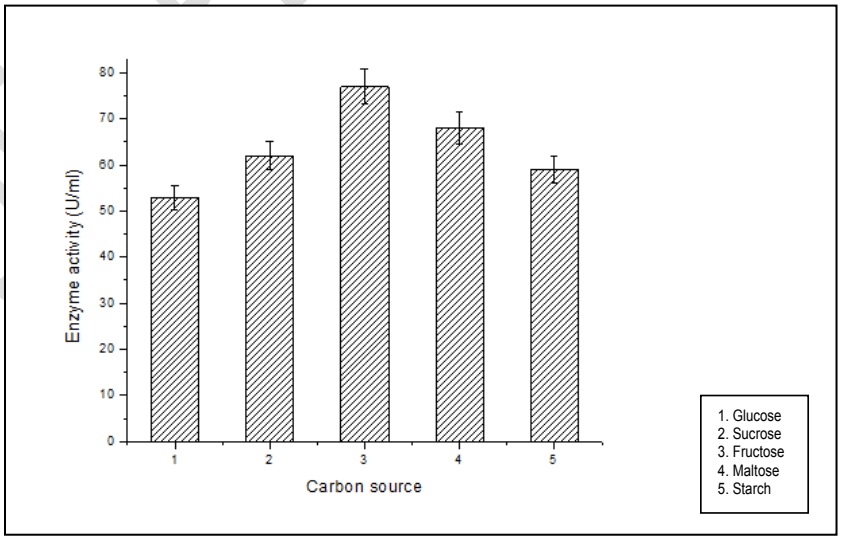

Fig.6. Effect of various carbon sources on protease production by *Alternaria alternata* TUSGF1.

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, skimmed milk, yeast extract, peptone and urea incubated at 30 °C for 168 hrs at 120 rpm. It was noted that skimmed 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*. [19] that nitrogen sources have significant enhancement on production of alkaline protease.

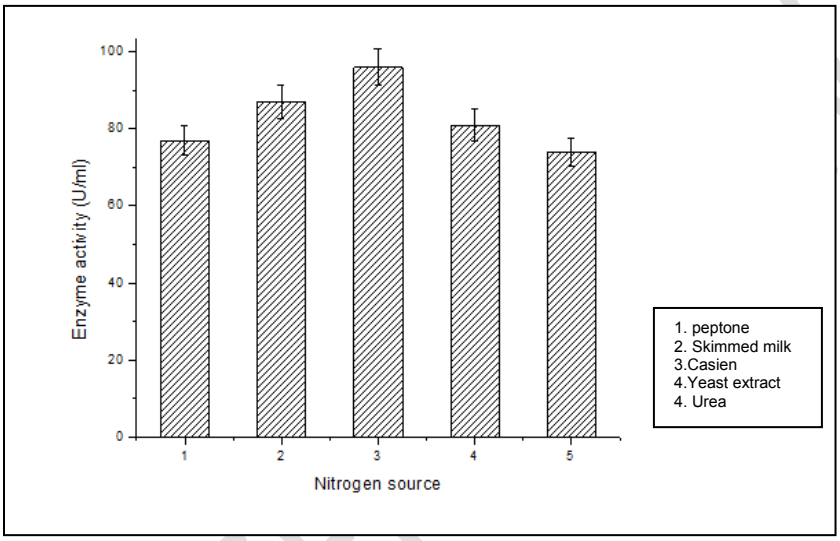

Fig.7. Effect of various carbon sources on protease production by *Alternaria alternata* TUSGF1.

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 available substrate selected 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 alternate* TUSGF1 when cultivated for 168 h at 7days age of inoculum, 30 °C and 120 rpm.

Acknowledgement

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