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Research Article

INFLUENCE OF VARIOUS ENVIRONMENTAL PARAMETERS ON PROTEASE SECRETION FROM *BACILLUS SUBTILIS* DKMNR

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ABSTRACT
A protease producing microorganism was isolated from soil collected from garden soil samples around the department of Chemical Engineering, Andhra University, Vizag, A.P, India and identified as bacillus species. The isolate DKMNR produced alkaline protease the optimum conditions for protease activity was 33^{0}C at pH 9 with 2% inoculum in the medium after 24 h of incubation with an agitation speed of 200 rpm and EDTA was found to be inhibitor of alkaline protease. The extracellular production of the enzyme, its alkaline nature and compatibility with most commercial detergents are features which suggest its application in the detergent industry.
KEYWORDS: Protease, *Bacillus subtilis*, screening, optimization, submerged fermentation

INTRODUCTION

Protease, a hydrolytic enzyme accounts 60% of total worldwide sale of industrial enzymes[1] proteases are commonly classified according to their optimum pH: acidic protease, neutral protease and alkaline protease[2-3]. One particular interest is the production of alkaline protease from bacillus for applications in detergent industry[4-5].

The microorganisms from diverse environments were considered as an attractive source for protease as they can be cultured in large quantities in less period. Furthermore, microbial protease is extracellular which simplifies the downstream processing and have longer self time[6]. As a rule, the wild strains usually produce limited qualities of the desired enzyme to be useful for commercial applications[7]. Some extracellular enzymes are used in the food, dairy, detergent, pharmaceutical, and textile industries and are produced in large amounts by microbial synthesis[8-9].

The amounts of protease produced by the microorganisms vary greatly with strain and the media used. Thirty to fourty percent of the production cost of industrial enzymes is estimated to be the cost of the growth medium[10]. In order to obtain high and commercial viable yields of protease it is essential to optimize fermentation media for the growth of biomass and production of protease[11].

Optimization of medium components was done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Research effort is mainly to Evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes, Requirement of metal ions in the fermentation medium and optimizes the environmental and fermentation parameters such as pH, temperature, age, size of inoculums and agitation.

In addition, no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism has its own special conditions for maximum enzyme production. So, it is important to know the suitable nutrients and cultural conditions required to achieve higher productivity[12].

In the preliminary stage, it was planned to formulate a suitable production medium for alkaline protease production from isolated *Bacillus subtilis* DKMNR by studying the effect of various culture and environmental factors on alkaline protease production in a conventional methodology.

MATERIALS AND METHODS

Microorganism and culture conditions

The *Bacillus subtilis* culture used in this study was isolated from garden soil samples around the department of Chemical Engineering, Andhra University, Vizag, A.P, India. The production media was composed of (YPD) yeast extract-peptone-dextrose medium consisting ($g.l^{-1}$) of Glucose 10.0; peptone, 7.5; yeast extract, 7.5; K_2HPO_4, 0.50, $MgSo_4$ 0.05 and $CaCl_2$ 0.02 and pH 8

after inoculation with 1%(v/v) culture media, incubated at 30^0C on rotary shaker at 120 Rpm. Initially the fermentation was carried out for 48 hrs, the culture media was separated by centrifugation and the supernatant was used for assaying enzyme activity.

Estimation of protease Activity

The protease was assayed according to the method of modified Auson-Hagihara method[13]. One unit of alkaline protease activity was defined as 1 ug of tyrosine liberated ml $^{-1}$ under the assay conditions.

Optimization of culture parameters

Effect of incubation temperature on Biomass growth and Protease activity

Incubation temperature had shown effect on biomass and protease activity. To study the effect of incubation temperature for maximum protease activity and biomass, the flasks with the production medium were inoculated and incubated at a range of temperatures from 24 to 46°C with an increment of 2 °C for 24 h. The general procedure mentioned earlier was followed for estimating protease activity and biomass.

Effect of pH on Biomass growth and Protease activity

The pH of the medium has shown effect on protease activity and the growth of biomass. Thus, the effect of pH on alkaline protease activity and biomass growth was studied. The production medium was adjusted at various levels of pH (5.0 - 12.0). General procedure mentioned earlier was followed for protease activity and biomass growth determination as described earlier.

Effect of age of inoculum on Biomass growth and Protease activity

In order to understand the impact of culture age on protease production, the fermentation experiments were conducted using cultures having different age (12 to 36 hrs old culture as inoculum). The flasks with the production medium having pH 9 were inoculated using cultures of different age at 2% level. The above mentioned procedure was followed for estimating protease activity and biomass growth.

Effect of inoculum level on Biomass growth and Protease activity

The effect of level of inoculum was studied for optimal alkaline protease activity and biomass growth. Experiments were carried out using 0.5 - 3.5% inoculum volume each, containing O.D of 1.0. The flasks with the production medium (pH 9) were inoculated as above and incubated for 24 hrs. The above mentioned procedure was followed for estimating protease activity and biomass growth.

Effect of agitation speed on Biomass growth and Protease activity

Fermentation experiments were carried out at different agitation speed (80, 100, 120, 150, 200, 220 and 250 rpm) conditions at 33 °C with a pH of 9. The inoculum used was 2% (v/v) culture grown for 24 hrs.

Effect of inhibitors on Biomass growth and Protease activity

In the present study, an attempt has been made to study the effect of some metabolic inhibitors on protease production. Inhibitors Silver nitrate ($AgNO_3$), Ethylene Diamine Tetra Acetate (EDTA), potassium iodide (KIO_3), Isoniazide (INH), 2,4-Dinitrrophenol (2,4-DNP), Sodium fluoride (NaF), Mercuric chloride ($HgCl_2$) and Potassium permanganate ($KMnO_4$) in solution form were separately sterilized and added individually at concentrations of 0.05% to the sterile basal medium and fermentations were conducted along with a control without inhibitors.

RESULTS AND DISCUSSION

Effect of Incubation Temperature

To study the effect of various temperatures on the growth of biomass and alkaline protease production, different temperature ranges (24 - 46 °C) were used. The fermentations and assays were carried out in triplicate as described earlier.

From the temperature 24 to 33°C the biomass growths was observed and from 33 to 46 °C protease production and biomass were decreased. It was occurred due to at the higher temperatures the organism was unable to grow as well as produce the protease. . The maximum biomass growth of 0.8 g/L was observed at 33 °C.

Increase in incubation temperature from 24 °C to 33 °C the yield increased to 650 U/ml and further increase in temperature, decreased the yield of protease to the least amount. Hence, the optimum incubation temperature for protease production by this organism is 33 °C. The results are shown in Fig 3.

Effect of pH

The effect of medium pH on biomass growth and protease yield was studied. Different initial pH values (5.0-12.0) were used to study their effect on the biomass growth and protease production. The fermentations and assays were carried out in triplicate as per the general procedure and the results are shown in fig 2. The growth of organism was very low in acidic media. As the pH increased from neutral to alkaline 9.0 the growth of the biomass was excellent. Further increase in pH from 9.0 to 12.0, a gradual decrease was observed as the former increased. The maximum growth observed was 0.85 g/L. The organism produced low amounts of protease in acidic media. The measure of protease was increased in

neutral and alkaline conditions up to 9.0 with highest yield of 690 U/ml, where it had maximum growth. The protease production decreased as the alkalinity increased in the media. So the optimum pH for protease production and biomass growth was found to be 9.0.

Effect of age of inoculum

Fermentation experiments were carried out using cultures of different age (12, 18, 24, 30 and 36 hrs). From the figure 3 it was observed that highest biomass growth of 0.91 g/L was observed at 24 hrs when varied from 12 hrs to 36 hrs. Fair amounts of protease was produced, when the culture of different ages were used. In that the maximum protease yield (735 U/ml) was obtained at the culture age of 24 hrs.

Effect of level of inoculum

Initial microbial load to a medium does affect the growth of biomass and secondary metabolite production. To study the effect of inoculum level the experiments were conducted using 0.5 - 3.5% of inoculum. The fig 4 depicts that the biomass growth had increased sequentially to 1.3 g/L with the increase in the level of inoculum up to 3% and there the growth was stationary. Thus the maximum growth was reached when 3% of inoculum was added to the production media.

The protease production was also increased as the level of inoculum was increased till 2%, at this point the maximum protease 751 U/ml was produced. The production of protease started to decline as the level of inoculum increased above 2% of inoculum. The optimum level of inoculum was 2% for the maximum production of protease.

Effect of agitation speed

Fermentation experiments were carried out at different rpm ranging from 80 to 250 conditions at 33°C with a pH of 9.0. The inoculum used was 2% (v/v) culture grown for 24 hrs. The data indicated that the agitation speed at the time of incubation played vital role in microbial growth and enzyme production by this isolate. The biomass growth was very formidable and reached to the maximum of 0.9 g/L at 200 rpm and thereafter the drop of growth was observed (Fig 5). The enzyme titer values improved with increase in rpm up to 200 and further increase revealed no noticeable improvement. Maximum protease activity of 720 U/ml and biomass growth was noticed with cultures incubated at 200 rpm.

Effect of inhibitors

Proper growth and metabolic activity increase the effective production of protease. The substances inhibitory for the growth of the organism inhibit the production of protease also. No reports are available on the effect of metabolic inhibitors for protease production. It is evident from fig 6 that all the inhibitors used for the inhibition of biomass growth and protease production were at 0.05% level. In those all inhibitors EDTA and $KMnO_4$ were least effective inhibitors. But the rest all, silver nitrate, mercuric chloride, INH, NaF and 2, 4-DNP inhibited strongly.

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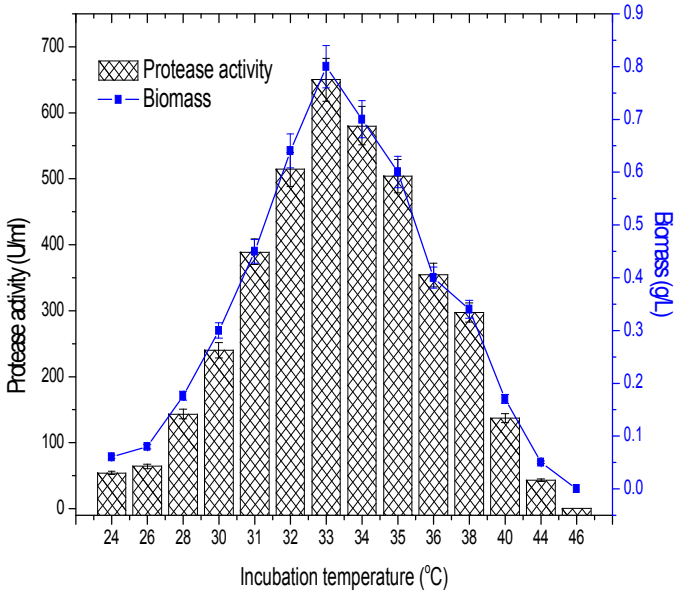

Figure 1: Effect of incubation temperature on protease production and biomass growth

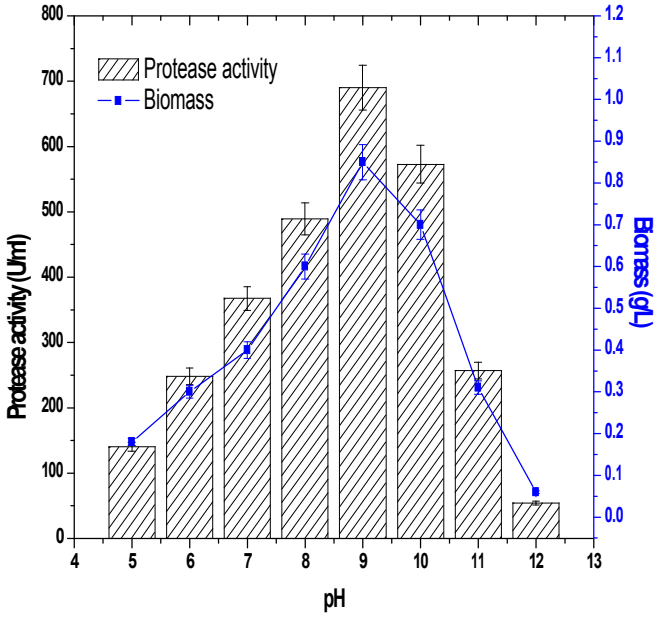

Figure 2: Effect of pH on protease production and biomass growth

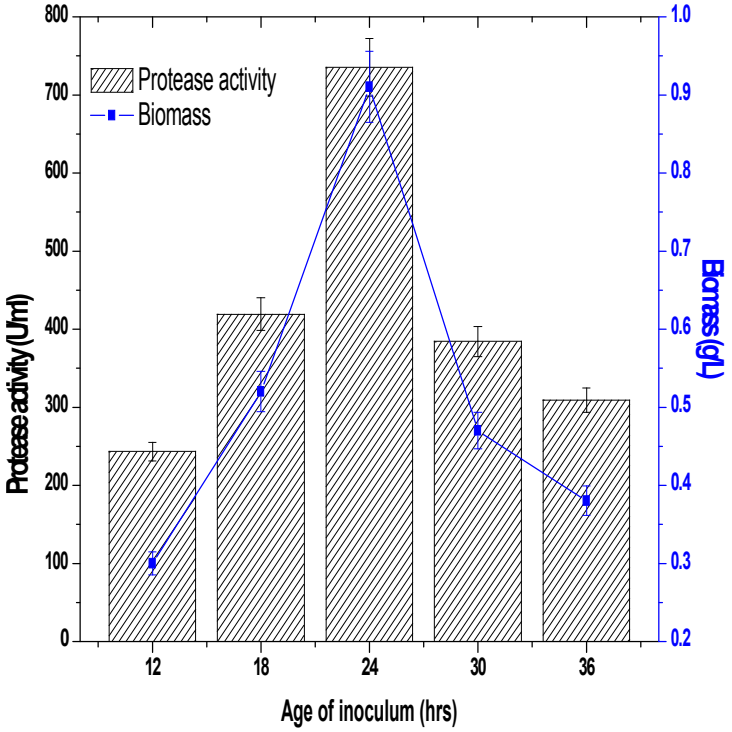

Figure 3: Effect of age of inoculum on protease production and biomass growth

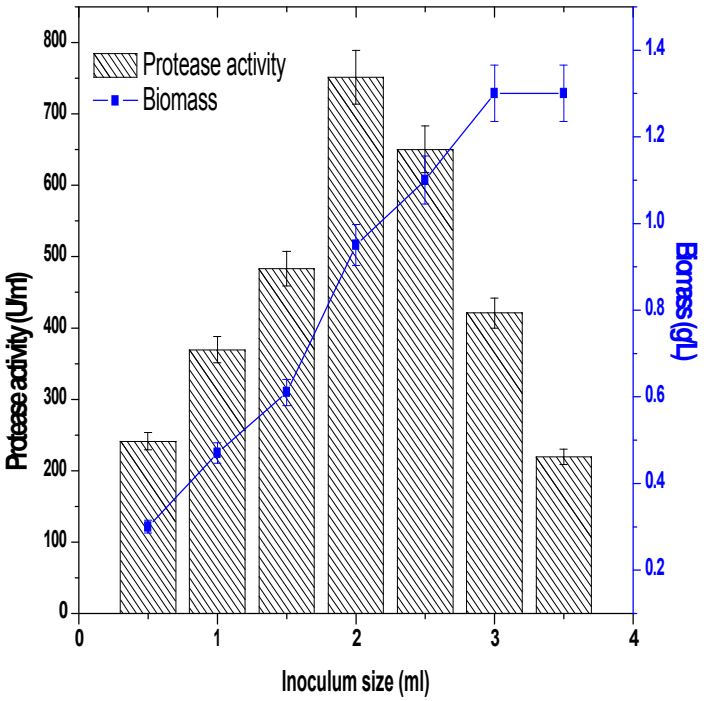

Figure 4: Effect of level of inoculums size on protease production and biomass growth

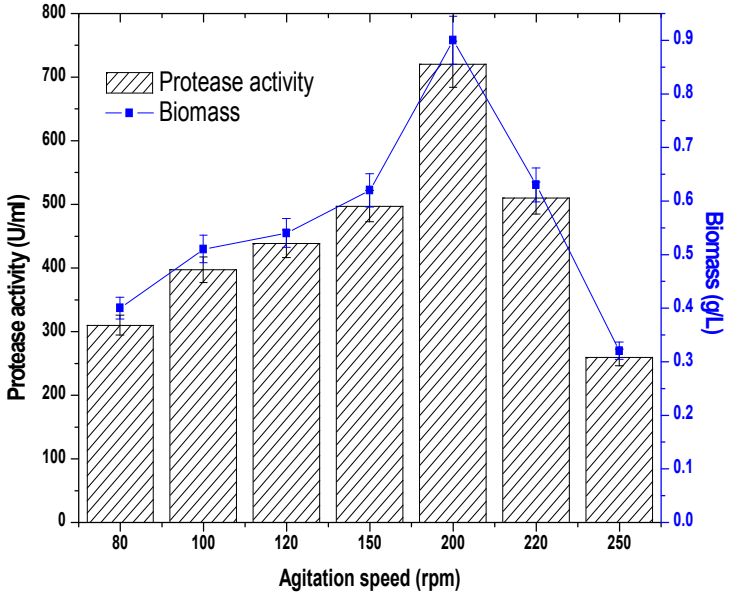

Figure 5: Effect of agitation speed on protease production and biomass growth

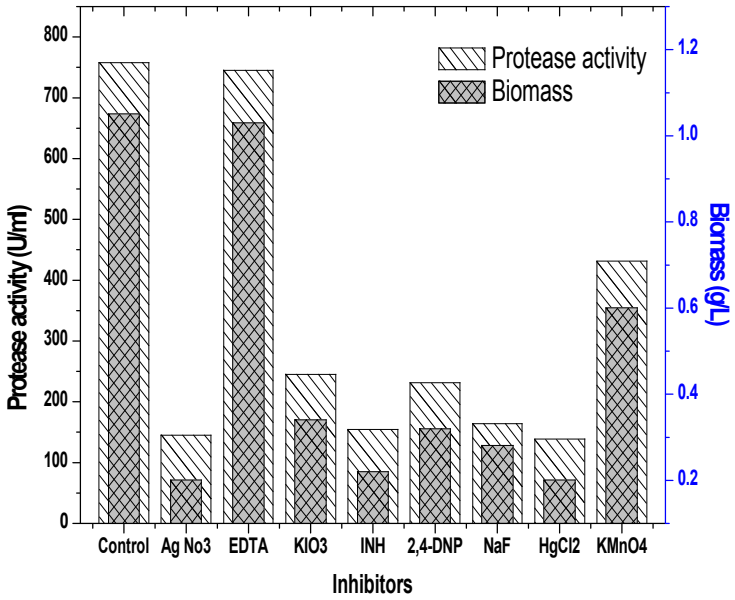

Figure 6: Effect of inhibitors on protease production and biomass growth

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