Production And Optimization Of Production Of Bacterial Protease

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Abstract

Bacillus subtilis RKR-14 is utilized to produce protease. The activity of protease was measure at 37 C and 5 pH. Growth curve was plotted and it was seen that the organism achieved its log phase at about 6 hrs which was then utilized to harness active culture. The organism was found to be gram positive rod. This actively growing culture was optimized for temperature and pH conditions for production of protease and it was revealed that pH 5 and 50 C were the best among the tested states for highest enzyme activity. Further a two-way ANOVA technique was utilized to access the combinatorial effect of both the factors on enzyme activity. It was seen that while temperature has a profound effect on the enzyme activity, pH was found to affect it not so significantly. In all, the organism was found to be a potent candidate for the production of protease.

1. Introduction

Proteases are type of enzymatic proteins. These are produced by a variety of microbes to sustain their functions of life and other components [1]. They exert their function like other enzymes and help the organism to cope the condition prevailing for life [2]. They mostly help the organism to grow under high protein sources. These protein sources are degraded by these enzymes and then assimilated as basic components of food for microorganism. Proteases are protein degrading enzymes. They cleave the peptide bonds between amino acids and release free residues are then taken up as food material [3].

These proteases are not only useful for the organism itself, but human have also exploited these group of enzymes for commercial purposes. A lot of review has already been done over application of bacterial proteases and has been published elsewhere [4]. Most common applications are therapeutic [5], laundry [6], food industry [7], biosensing [8], leather processing [9], waste management [10]. So, protease becomes an important industrial enzyme whose demand is bound to increase in future.

Although there are several organisms reported already which are able to produce and secrete extracellular protease, only few meet the industrial requirements. Several organisms are being reported to produce proteases with better yields as compared to previous and with better activities [11]. Genetic engineering and system biological tools are being used to improve the characteristics of organisms and enzymes [12]. With these tools it is becoming possible to produce a new protease in a host producer. Genetic barriers have been lifted up with the help of these tools of molecular biology.

Not only molecular biology, but bioprocess engineering is also playing an important role in development of technologies for achieving even higher yields [13]. New type of cheaper and economic reactor systems [14], inexpensive raw material and new states of system parameters are altogether giving impetus to the development of this industry. Here we have utilized an already isolated and characterized bacterial strain of Bacillus group for production of protease and have optimized its production with statistical design of experiments.

2. Materials and methods:

2.1 Bacterial strain procurement and characterization: *Bacillus sp.* RKR-14 (BSR) has been obtained as a generous gift from somewhere else. It was characterized by gram staining for shape and size. Growth characteristics were obtained by plotting growth curve by standard way.

2.2 Protease production: Pure culture of BSR was grown on nutrient medium supplemented with 2% albumin (Sigma) to induce protease production. 1 ml of liquid culture of actively growing bacteria was taken from log phase. The OD of bacterial culture at 600nm as measured as a control parameter. This was added to nutrient medium (Sigma) and supplemented with albumin as reported. The culture was kept in a shaker incubator at 37 C for 12 hours at 100 RPM. The culture was withdrawn and supernatant was collected after centrifugation (REMI-NDLS) at 1000 RPM for 5 min to remove cells. This supernatant was used as sample to measure enzyme activity. This was kept at 4 C till further use.

2.3 Determination of protease activity: The protease activity was measured as given. Five dilutions of the above stated supernatant were taken and diluted upto 3 ml. 1ml of casein powder (0.5 g/ml, Sigma) was added in each tube which acts as the substrate for protease. The time of addition was carefully noted and these tubes were incubated at 37 C for 20 min. If the time of addition of casein was different, then it was adjusted in further steps to make reaction time 20 min. The reaction was stopped by adding 1 ml of 10% TCA and tubes were kept at 4 C for 60 hrs. The solution was filtered and digested peptides were measured using Lowry's method. Standard curve was prepared with BSA which was used to measure unknown concentrations of

proteins. One unit of enzyme activity is defined as amount of digestion production produced per unit volume of supernatant used at a given incubation period

2.4 Optimization of enzyme activity

2.4.1 Temperature: For determining the proper temperature for enzyme production, bacteria were cultured at five different temperature viz. 20, 30, 40, 50, 60 C. pH was kept at 5 for these treatments. After growth, the supernatant was taken to measure enzyme activity.

2.4.2 pH: Culture was then grown at pH 5, 8, and 10 for determining the best pH for enzyme production. For this 50 C temperature was used as it was optimized earlier. It was grown at given pH and supernatant was used to measure enzyme activity.

2.4.3 Statistical Design: We utilized statistical design of two-way ANOVA for finding the combined effect of temperature and pH on enzyme activity. For this a set of experiments have been created as given in table 1. The experiments are run and combinatorial effect of temperature and pH was accessed. The results were analyzed by two-way ANOVA with MSExcel.

Temp pH	42 C	47 C	52 C
3			
5			
8			

3 Results and discussion

3.1 Organism is found to be gram positive as shown in Fig 1. These were found to be rods with tendency for forming small chains. The growth curve of the organism is depicted in Fig 2. It was found to achieve active growth after 6hours of growth.

3.2 Protease activity: The preliminary results on enzyme activity are shown in Fig 4. The enzyme activity was tabulated in table 2. Standard curve of BSA used in the study in depicted in Fig 3. From this curve it was found that the sample contained 0.566 mg/ml of total protein.

Table 2 consolidates the results of experiment done for enzymatic activity measurement. It can be seen from there that the average enzymatic activity was found to be 0.889±0.1 ml (Fig 4).

3.3 Effect of temperature and pH: Fig 5 depicts the effect of temperature on enzyme activity. It can be seen that out of the points of temperatures used, enzyme activity was found to be best at 50 C. Also, as shown in Fig 6, best pH from the group was found to be 5 for producing enzyme with best activity.

3.4 Combinatorial effect: Results of observations of combinatorial tests are given in table 3. The results of ANOVA are shown in table 4. It can be interpreted from the results that temperature is having profound effect on enzyme activity ($P<0.05$) while pH does not affects the activity very significantly with change in temperature. Both the factors are responsible to produce an effect on enzyme activity but when temperature is changing effect of pH is diminished.

4. Conclusion: actively growing culture was optimized for temperature and pH conditions for production of protease and it was revealed that pH 5 and 50 C were the best among the tested states for highest enzyme activity. Further a two-way ANOVA technique was utilized to access the combinatorial effect of both the factors on enzyme activity. It was seen that while temperature has a profound effect on the enzyme activity, pH was found to affect it not so significantly. In all, the organism was found to be a potent candidate for the production of protease.

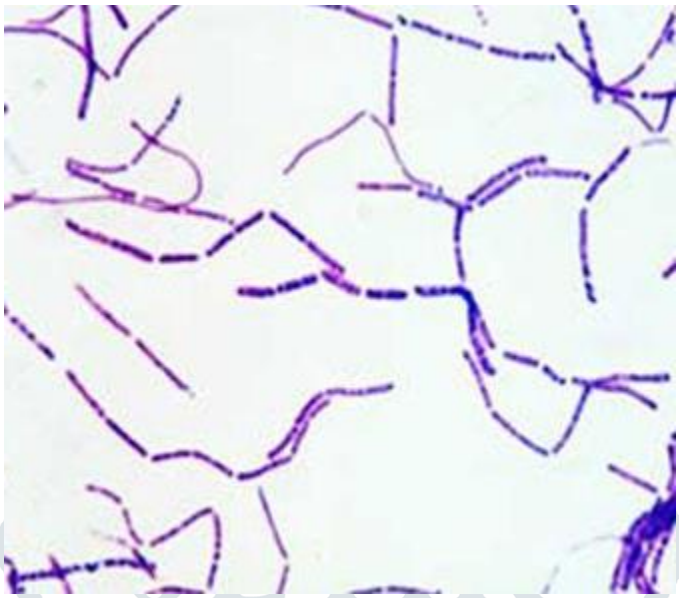

Fig 1. Gram staining of bacteria

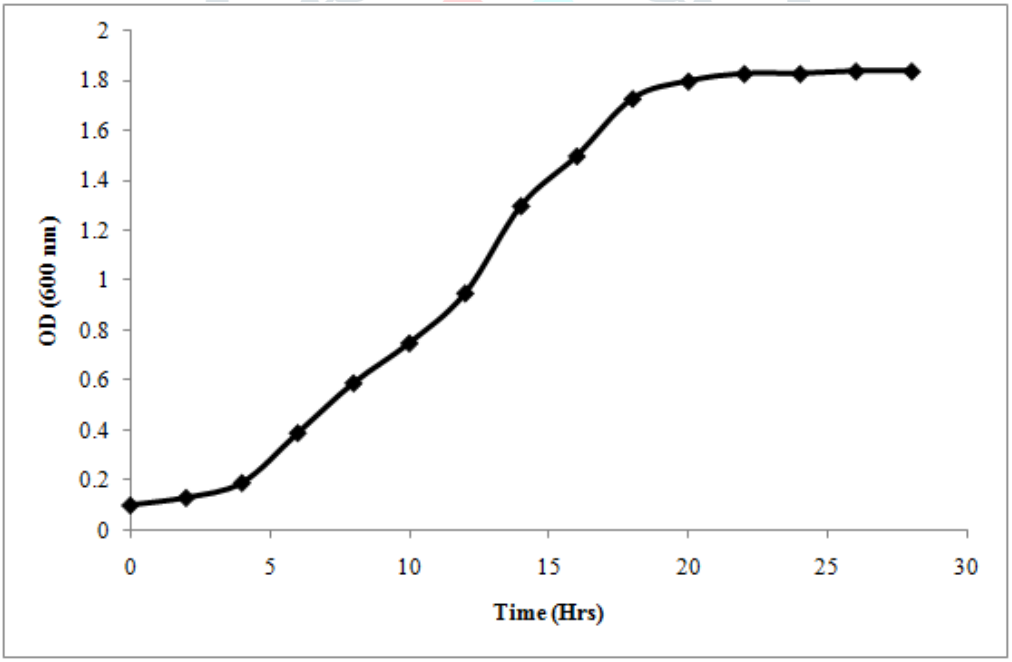

Fig 2. Growth curve of bacteria

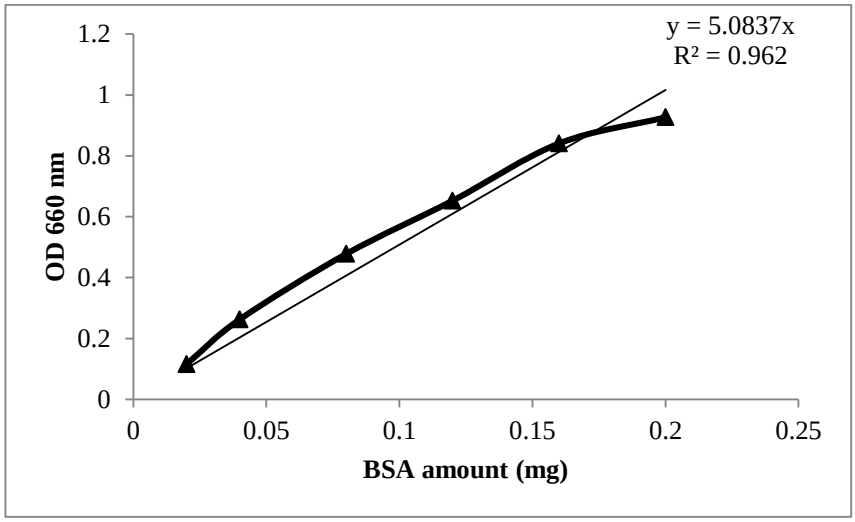

Fig 3. Standard curve for BSA.

Volume (ml) of supernatant added	Absorbance (660nm)	Amount (mg) of protein in supernatant	Amount (mg) of protein produced per ml of enzyme supernatant added	Volume (ml) required to produce 1 mg of protein
0.1	0.51	0.10033445	1.003344	0.996667
0.2	0.77	0.15148534	0.757427	1.32026
0.3	1.37	0.26952587	0.89842	1.113066
0.4	1.83	0.36002361	0.900059	1.111038

Table 2. Observations of enzyme activity measurement

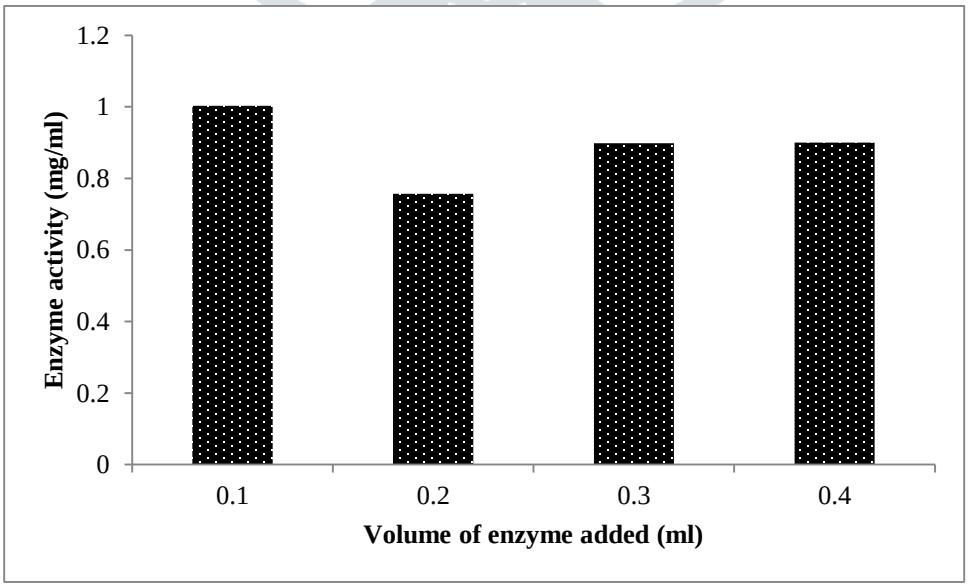

Fig 4. Variation in enzyme activities of sample

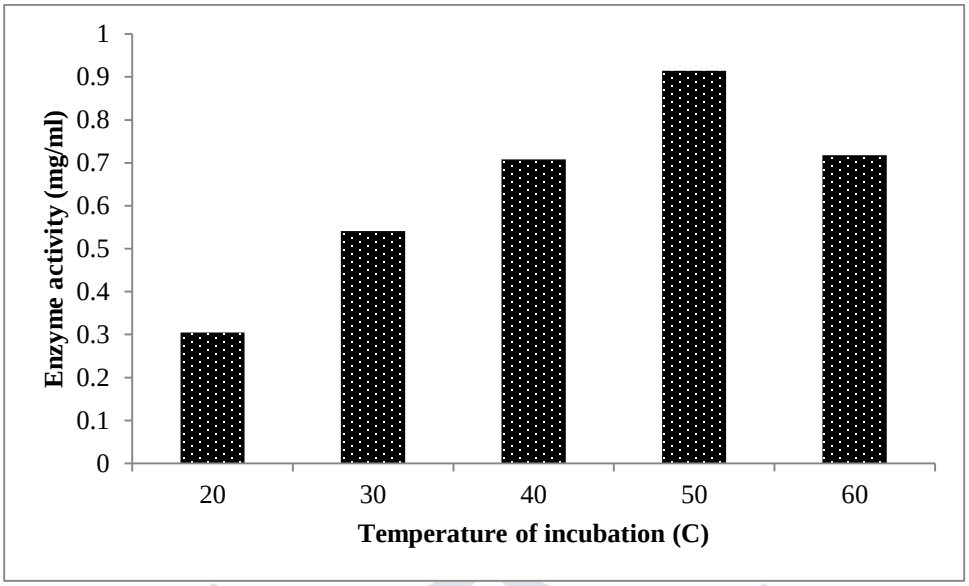

Fig 5: Bar graph of activity at different incubation temperatures

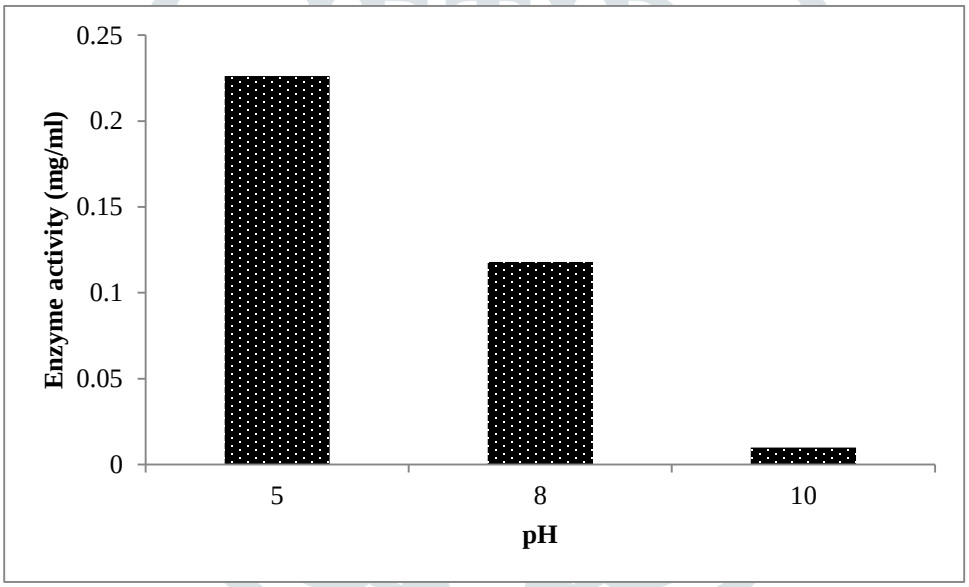

Fig 6: Bar graph of activity at different pH

Temp pH	42 C	47 C	52 C
3	0.335	0.420	0.718
5	0.428	0.565	0.912
8	0.375	0.510	0.625

Table 3. Observation for combinatorial experiment

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
pH	0.038224	2	0.019112	3.874775	0.115898	6.944272
Temp.	0.216971	2	0.108485	21.99426	0.006948	6.944272
Error	0.01973	4	0.004932			
Total	0.274925	8				

Table 4. ANOVA table of combinatorial experiment

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