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Research Article

PRODUCTION, CHARACTERIZATION AND PURIFICATION OF ALKALINE PROTEASE FROM *ALCALIGENES sp.,* AND ITS APPLICATION IN DETERGENT INDUSTRY

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ABSTRACT

Proteases are enzymes well known for their wide range industrial applications such as food, detergent, pharmaceutical etc. The aim of the present work was to study the production, purification and application of alkaline protease from the strain *Alcaligenes sp.* (MTCC 9730). Batch and fed batch fermentation were used as the production strategies. The optimum temperature and pH for the production of enzyme was found to be 55°C and pH 9 respectively. Ca^{2+} increased the activity, Cu^{2+} and Mg^{2+} mildly inhibited and by Zn^{2+} strongly inhibited the activity. The protease enzyme was purified using Ammonium sulphate precipitation and maximum activity of 60 U/ml was found at 70% saturation. Alkaline protease is known to be an active ingredient of the commercial detergents. The enzyme was used to study its compatibility with other detergents and its ability to remove the protein stain.

Keywords: Alkaline protease, *Alcaligenes sp,* fermentation, purification, detergent.

INTRODUCTION

Proteases are one of the most important classes of enzymes and are expressed throughout the animal kingdom, plant and as well as microbes .The proteases obtained from plant and animal sources were unable to meet current world demands has led to an increased interest in microbial proteases [1,2]. In addition, proteases from microbial sources are preferred than other sources, since they possess almost all characteristics desired for their biotechnological applications [3]. There are acidic, alkaline and neutral proteases, but of all these, alkaline proteases are used primarily as cleansing additives. One of the most physiologically and commercially important groups of enzymes is alkaline protease also known as Subtilisins, E.C. 3.4.21.14 used as additives in detergent industries. They play a major catalytic role in the hydrolysis of proteins.[4-7]. Biodetergents account for about 30% of the total worldwide enzyme production. Protease is used in detergents to remove protein-based stains by hydrolyzing them into small peptides which are readily dispersed in the washing liquor [8].

MATERIALS AND METHOD

Microbial species and cultivation

An alkaline stable bacterium *Alcaligenes sp* (MTCC 9730) was obtained from microbial type culture collection (MTCC), Chandigarh India that produced an extracellular alkaline protease. The strain was grown in nutrient media: Yeast Extract 2.0g, Beef extract 1 g, Peptone 5 g, NaCl 5 g, distilled water 1 L[9].

Protease production and assay

Using the nutrient media (Yeast Extract 2 g, Beef extract 1 g, Peptone 5 g, NaCl 5 g, Distilled water 1L) the enzyme was produced. Using the 5% (v/v) of 5 days old culture, the media was inoculated and incubated at 37ºC in shaker for 16 hours. The culture was centrifuged at 4ºC at 8000 rpm for 15 min and the supernatant was used for assay. The reaction mixture consist of 0.25ml of 50mM sodium phosphate buffer (pH 7.0) which consisted of 2.0% (w/v) of casein and 0.15 ml of enzyme solution. The mixture of enzyme-substrate was incubated at 34ºC for 10 min. To stop the reaction, 1.2 ml of 10% (w/v) TCA was added and then incubated for 10 minutes at room temperature. The precipitate was removed by centrifugation at 8,000 rpm for 5 min. To 1.2 ml of the supernatant, 1.4 ml of 1M NaOH was added and the absorbance was then measured at 440nm. The release of amino acids (tyrosine) from casein was used to determine the activity. The standard curve constructed with tyrosine is used to calculate the amount of tyrosine. One protease unit is defined as the amount of enzyme that releases 1mg of tyrosine per ml per minute under the above assay condition.[10, 11]

CHARACTERIZATION OF ENZYME ACTIVITY

Effect of temperature on protease activity

The effect of temperature on extracellular enzyme was studied by assaying the enzyme maintained at pH 7 with the temperature range from 35ºC to 75ºC using casein as substrate incubated for 1 hour. After this the enzyme activity was assayed using the above procedure.[10,12]

Effect of pH on protease activity

The effect of pH on the protease activity was determined by incubating the reaction mixture for one hour mixed with 150 µL of phosphate buffer solution of pH range from pH 7 to 10.[10,13]

Effect of cations on enzyme activity

A stock solution of 0.01 M of each salt was prepared. The effects of some salts/cations ($CaCl_2$, $CuSO_4$,$MgSO_4$, and $ZnCl_2$) on enzyme activity was also determined. The mixture of substrate - salts was incubated at room temperature for 5 minutes before it was used for enzyme assay.[14,15]

ENZYME PRODUCTION: FED BATCH FERMENTATION

Fed batch fermentation was carried out with two feeding strategies: constant and linear feeding. The microorganism was inoculated in nutrient medium. Constant feeding: 200µl of nutrient media was added at the end of every one hour. Linear feeding: Incremental 200µl of the nutrient media was added at the end of every one hour respectively. The sample was withdrawn for every one hour and was analyzed for cell growth and protease activity.[16]

PURIFICATION

The fermented broth, incubated at optimized conditions, was centrifuged at 4°C (8000g, 15 min) and the extracellular protease in cell free culture supernatant was obtained by ammonium sulphate precipitation method. The protein precipitate was dissolved in 0.05 M potassium phosphate buffer (pH 7). This precipitate was dialyzed against the same buffer for 24 hours. During this period there were 6-8 changes of the buffer solution was made. The next subsequent steps were carried out at 4°C. The purified samples were analyzed for cell growth and protease activity. Among the samples the one with high protease activity was concentrated by lyophilization.[17, 18]

APPLICATION

To study the application of protease as a detergent additive white cloth pieces (4 cm × 4 cm) stained with blood was used. The de-staining property of the enzyme was studied by dipping pieces of stained cloth in the following set of flasks after which it was incubated 15 minutes at 34°C. The following are the set of flasks prepared:

Sample 0 : Water + stained cloth

Sample 1: Local detergent (Jill) [boiled]+ enzyme + stained cloth

Sample 2 : Jill+ stained cloth

Sample 3 : Jill + enzyme + stained cloth

Sample 4 : Rin[boiled] + enzyme + stained cloth

Sample 5 : Rin + stained cloth

Sample 6 : Rin + enzyme + stained cloth

The stained cloth is removed after every 5 minutes and the turbidity of the solution is checked using turbidity meter (in terms of Nephelometric turbidity units). Simultaneously the stained cloth was examined quantitatively and the washing efficiency of the enzymes from both the strains were studied.[19, 20]

RESULT AND DISCUSSION

Optimization of enzyme production

Effect of temperature and pH on enzyme activity

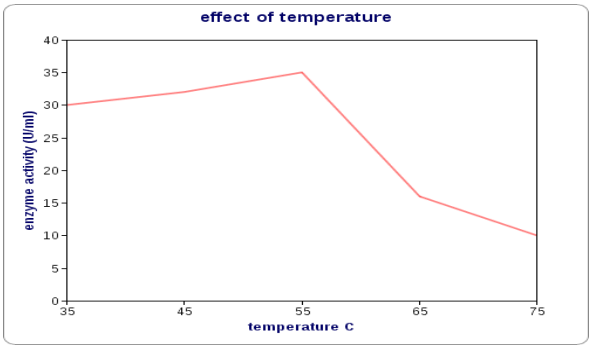
Temperature has profound influence on production of microbial enzymes. The maximum protease production (35 U/ml) was obtained at 55ºC and the activity was found to decrease above 55ºC (as shown in table 1 and graph1).This indicates that the enzyme was thermoprotease. The enzyme was found to grow in wide range of pH 7 to 10 but the maximum enzyme production was achieved at pH 9 (35 U/ml) at 1 hr incubation and 34°C. (as shown in table 2 and graph 2).

Table 1: Effect of temperature on protease activity

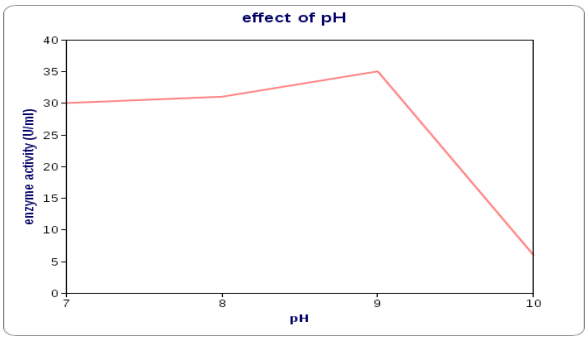
TEMPERATURE(°C)	ENZYME ACTIVITY(U/mL)
35	30
45	32
55	35
65	16
75	10

Table 2: Effect of pH on protease activity

pH	ENZYME ACTIVITY(U/mL)
7	30
8	31
9	35
10	6



Graph1: Effect temperature on protease activity



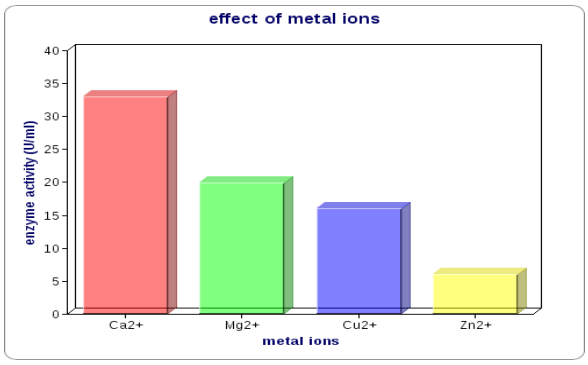
Graph 2: Effect of pH on protease activity

Effect of metal ions on protease activity

The metal ions that were used are categorized into activators (Ca^{2+}), mild inhibitor (Mg^{2+},Cu^{2+}) and strong inhibitor (Zn^{2+}).(as shown in table 3 and graph 3).Ca^{2+} increases the protease activity by preventing it from denaturing at higher remperature.

Table 3: Effect of metal ions on protease activity

METAL IONS	ENZYME ACTIVITY(U/mL)
Ca^{2+}	33
Mg^{2+}	20
Cu^{2+}	16
Zn^{2+}	6



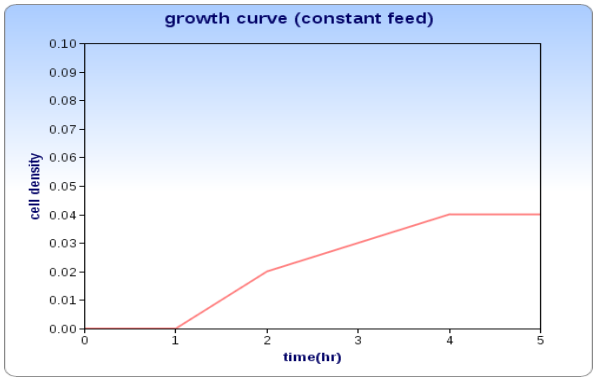
Graph 3: Effect of metal ions on protease activity

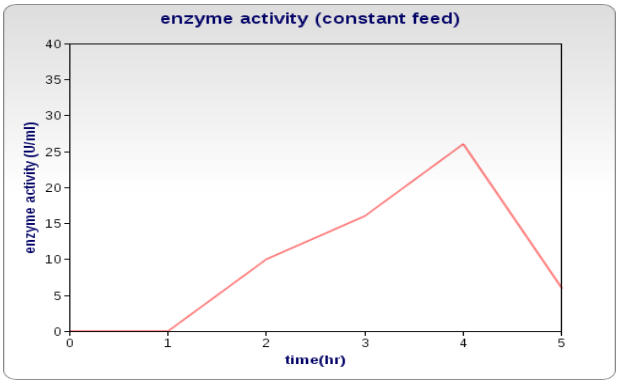
Enzyme production

Fed batch fermentation was performed till the strain reached the stationary phase. Growth curve and enzyme activity for both the feeding strategies were plotted (as shown in table 4 and graph 4 for constant feed, table 5 and graph 5 for linear feed).

Table 4: Cell density and enzyme activity for the constant feed in fed batch fermentation constant feed

TIME(hr)	CELL DENSITY	ENZYME ACTIVITY(U/mL)
0	-	-
1	-	-
2	0.02	10
3	0.03	16
4	0.04	26
5	0.04	6

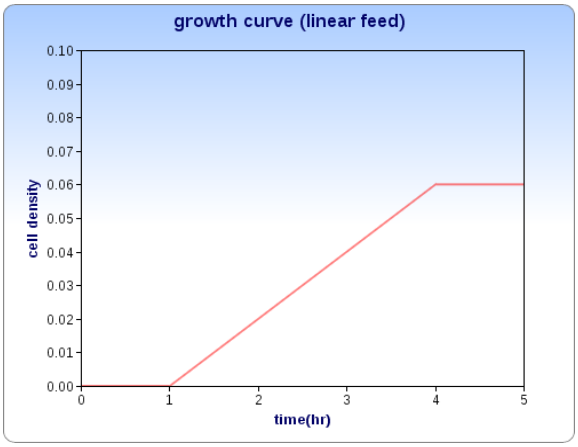


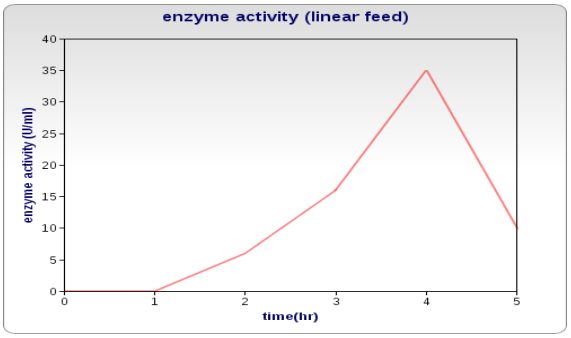


Graph 4: Plot of cell density and enzyme activity for the constant feed in fed batch fermentation

Table 5: Cell density and enzyme activity for the linear feed in fed batch fermentation Linear feed

TIME(hr)	CELL DENSITY	ENZYME ACTIVITY(U/mL)
0	-	-
1	-	-
2	0.02	6
3	0.04	16
4	0.06	35
5	0.06	10





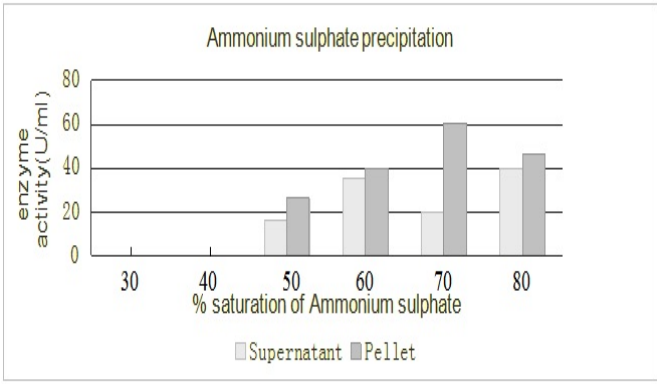
Graph 5: Plot of cell density and enzyme activity for the linear feed in fed batch fermentation

Purification

The enzyme was partially purified using centrifugation and ammonium sulphate precipitation. At 70% saturation of ammonium sulphate the activity was found to be maximum.(table 6 and graph 6) at lower percentage saturation of ammonium sulphate no precipitation was observed and at higher percentage saturation of ammonium sulphate there was excess of salt due to which precipitation was negligible.

Table 6: Ammonium sulphate precipitation on enzyme activity

% SATURATION OF AMMONIUM SULPHATE	ENZYME ACTIVITY (U/mL)	
	Supernatant	Pellet
30	-	-
40	-	-
50	16	26
60	35	40
70	20	60
80	40	46



Graph 6: Ammonium sulphate precipitation on enzyme activity

Application

Alkaline protease is primarily employed as cleansing additives. Application studies for protease from the strain was carried out and its efficiency was analysed using Turbidity measurement and visual analysis.(table 7 and figure 1)

The measurement of turbidity is indicative of the amount of dirt (here the blood stain) removed from the samples. From table 7 it was found that maximum turbidity was obtained in sample 3 and 6. From figure 1 it was found that sample 3 and 6 showed maximal removal of blood stain. This indicates that enzyme from the strain is stable as an ingredient in the presence of detergent. The turbidity values of sample 1 and 4 (as shown in table 7) and Samples 1 and 4 from figure 1 indicate that the enzyme is effective in degrading the protein (blood sample) in absence of detergent, while sample 2 and 5 indicate that the detergent solution alone could not remove the strain as effectively.

Table 7: Effectiveness of enzyme measured in terms of turbidity

SAMPLE	TURBIDITY(NTU)		
	5th MINUTE	10th MINUTE	15th MINUTE
1	20.4	23.7	29.5
2	16.9	21.8	25.7
3	28.4	33.5	39.8
4	29.6	36.2	47.1
5	20.2	26.3	29.1
6	31.6	35.4	50.1

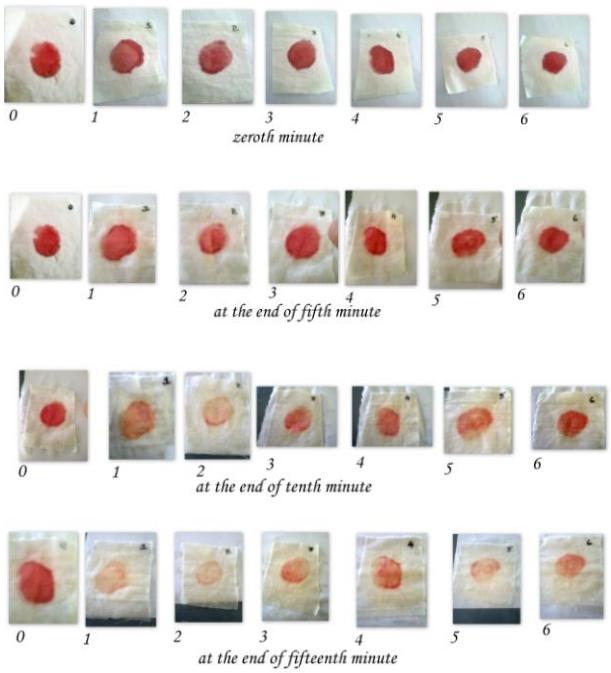


Figure 1: compatibility of alkaline protease in presence of commercial detergents.

CONCLUSION

The protease from *Alcaligenes sp* was optimized for its maximum production. The effect of metal ions on its activity was also studied. Purified enzyme was obtained using ammonium sulphate precipitation. It can be effectively used with the commercial detergents.

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