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DESTAINING EFFECT OF ALKALINE PROTEASE PRODUCED BY PD4 STRAIN OF *BACILLUS SUBTILIS*

N. Vanitha Manonmaniam Sundaranar University, Tirunelveli, TamilNadu, India.

S. Rajan* PG And Research Department Of Microbiology, M.R. Government Arts College, Mannargudi-614001, TamilNadu, India. *Corresponding Author

AG. Murugesan SPKCES, Alwarkurichi, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India.

ABSTRACT The demand for alkaline proteases in industries is increasing worldwide. The present research describes the production of alkaline protease in a lab scale fermentor from PD4 strain of Bacillus subtilis isolated from effluent. The production of alkaline protease was enhanced by optimization of cultural condition. Of all proteases, alkaline proteases are of great importance in detergent industry due to their high thermal stability and pH stability. Ability of the alkaline protease to destain different stains on the cotton fabric samples were evaluated using four different stained clothes. Destaining was done using 10 ml of crude preparations. Crude alkaline protease showed good destaining ability when compared to that of other three test fabrics treated with distilled water, distilled water with detergent, enzyme extract with detergent respectively and it was revealed that the purified enzyme can be used as an additive in detergent industry.

KEYWORDS : *Bacillus subtilis,* alkaline protease, submerged fermentation, detergent.

INTRODUCTION

Proteases, one among the three largest groups of industrial enzymes, and places pivotal niche accounting for 60% of the total worldwide sale of enzymes. Alkaline protease alone makes up 89% of total protease market. They possess desired characteristics for their industrial applications (Adinarayana *et al*., 2003). Alkaline proteases are widely used in detergent, pharmaceutical, leather processing, organic synthesizing industries as well as bioremediation process (Mukherjee *et al.,* 2008). Industries are always in search of new and cheaper enzymes from microorganisms (Mukherjee *et al*., 2008). The use of cost effective growth medium for the production of alkaline proteases from an alkalophilic microorganisms is especially important (Gessesse, 1997; Das and Prasad, 2010). Alkaline proteases of microbial origin posses considerable industrial potential due to their biochemical diversity and wide applications in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, and chemical industries, as well as tannery waste treatment (Vijay *et al*., 2010). Microbes like *Bacillus, Pseudomonas, Serratia, Paenibacillus* isolated from natural sources are needed to be explored for better usage. Among these microorganisms *Bacillus sp.,* produced effective alkaline protease enzymes in terms of their stability in high pH and temperature. In the present study, alkaline protease ability was assessed for its dye removing capability on different stained cloth preparations like blood, planitain juice, turmeric and kum kum (Plate I) using alkaline protease crude preparations.

MATERIALS AND METHODS

Samples: Three different solid samples (leather Industry waste, food industrial waste and slaughter house waste) were collected in sterile container and shifted to the laboratory for the isolation of alkaline protease producing bacteria.

Screening of protease procedures and characterization: Samples were individually inoculated on skim milk agar plates, incubated for 48hrs at 37˚C. Protease producers were selected by size of the hydrolytic zone (Genkal *et al*., 2006). Good hydrolytic colonies were selected and subjected for characterization. Isolate PD4 was found to be a best protease producer. This organism was subjected for microscopy, biochemical test and also 16SrRNA analysis and identified as *Bacillus subtilis*. This organism thrives at alkaline pH of 9. Hence this organism producing protease in alkaline medium could be considered as alkaline protease.

Alkaline protease Production: PD4 strain of *Bacillus subtilis* was subjected for alkaline protease production in medium consisting of beef extract (3 g/L), peptone (10 g/L), and NaCl (7 g/L), with pH 9. The media were autoclaved for 20 min at 121°C. Fermentation processes were completed on a rotary shaker at 37°C for 24 h at 150 rpm, with 50 mL working volume in 250 mL triangular flasks. At the end of each fermentation period, the culture broth was centrifuged at 10,000 rpm for 15 min to remove the cellular debris and the clear supernatant was used for enzyme assay and destaining.

Quantitative assay of protease activity: The total protein contents of the samples were determined according to the method described by Lowry's method using Bovine Serum Albumin (BSA) as standard. Enzyme activity was determined using culture supernatant collected by centrifuging culture broth at 10, 000 rpm for 15min. Protease activity was measured by standard assay procedure proposed by Akcan and Uyar, (2011). About 0.5ml of 0.5% casein and 1.25ml of tris buffer (pH-8.0 to 14.0) was added into 0.2ml of each of the culture supernatant separately. Mixture was incubated for 30 min at 370C. About 3ml of trichloroacetic acid was added and incubated at 400C for 10 min to form precipitate. The mixture was centrifuged at 10,000rpm for 15min and 0.5ml of supernatant was collected. Reagent containing sodium carbonate, copper sulphate, sodium potassium tartarate was mixed with 1ml of Folin-phenol reagent. The mixture was incubated at dark for 30 minutes to form blue colour. The absorbance was read at 660 nm to determine the optical density of each sample. The obtained OD was extrapolated in the standard graph. The standard curve was obtained for series of known concentrations of bovine serum albumin. From the graph, the amount of protein liberated due to the action of enzyme protease in the supernatant was determined. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/ml tyrosine under the experimental conditions. Enzyme activity = OD value X amount of protein released (µg)/ concentration of substrate X time of incubation X weight of the sample.

Destaining activity (Anonymous, 2000): Stain release test was performed according to standard AATCC 130 method. Cotton cloth was selected as the test specimen. It was cut into 150 mm (6 inch) square for each swatch. The fabrics were then piled side up on a non absorbent surface for 24 hours at standard condition of 21 ± 1˚C and 65 ± 2% RH. Contamination by contact with extraneous materials was avoided. Natural stains like turmeric, kumkum, blood and plantain stem juice was selected. About 50 mm diameter staining ring was then placed in the center of the test specimen. The stained cloths were taken in separate flask. The following sets were prepared and studied as follows:

S. No	Test No	Stain	Test
Set I	I	Turmeric	Flask with distilled water (100 ml) + stained cloth
	II		2% surf excel detergent (7mg/ml) + stained cloth
	II		1% surf excel detergent (7 mg/ml) + 5 ml of enzyme + stained cloth
	IV		10 ml of enzyme + stained cloth
Set II	I	Plantain Juice	Flask with distilled water (100 ml) + stained cloth
	II		2% surf excel detergent (7mg/ml) + stained cloth
	II		1% surf excel detergent (7 mg/ml) + 5 ml of enzyme + stained cloth
	IV		10 ml of enzyme + stained cloth
Set III	I	Plantain Juice	Flask with distilled water (100 ml) + stained cloth
	II		2% surf excel detergent (7mg/ml) + stained cloth
	II		1% surf excel detergent (7 mg/ml) + 5 ml of enzyme + stained cloth
	IV		10 ml of enzyme + stained cloth
Set IV	I	Plantain Juice	Flask with distilled water (100 ml) + stained cloth
	II		2% surf excel detergent (7mg/ml) + stained cloth
	II		1% surf excel detergent (7 mg/ml) + 5 ml of enzyme + stained cloth
	IV		10 ml of enzyme + stained cloth

The stained specimen was kept at 24º±4ºC in a flat position, piled side up at standard condition of 21º±1ºC (70º±2ºF) and 65º2RH. Stained specimen were rinsed with running water at 21º±6ºC until the rinsed water was colorless which indicated the removal of all unfixed following stains, turmeric, plantain stem juice, blood and kumkum.

RESULTS AND DISCUSSION

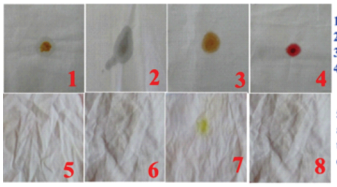
Alkaline proteases has considerable industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, feeds and chemical industries, as well as tannery waste treatment. At present, the largest part of the hydrolytic enzyme market is occupied by the alkali proteases. Extreme environments are important sources for isolation of microorganisms for novel industries and enzymes production. Hence, in this present study the protease producing bacteria were isolated from tannery, foodindustry; and slaughter house industrial effluent discharge site. The zone of hydrolysis was due to protease enzyme produced by the isolates on Skim milk agar media. Narendra *et al.,* (2012) reported that about 25 organisms were recovered from different fields near to Ravulapalem village, East Godavari district, Andhra Pradesh. In this study various strains indicated the production of alkaline protease. Among these PD4 strains showed better hydrolytic power. Characterization of PD4 strain showed, it is *Bacillus subtilis* strain.

Alkaline protease production of *Bacillus subtilis* (PD4) was carried out under controlled cultural conditions in a stirred bed reactor. The selected optimized conditions favourable for the growth oforganism and production of protease were made during the fermentation process. At the end of each fermentation period, the culture broth was harvested to remove the cellular debris and the clear supernatant was used for destaining activity.

Destaining of 100% cotton fabric was studied using the crude enzyme samples as the first industrial application after harvesting process. Ability of the alkaline protease to destain different stains on the cotton fabric samples was evaluated. In all the four tests, fabric treated with 10 ml of crude alkaline protease showed good destaining ability when compared to that of other three test fabrics treated with distilled water, distilled water with detergent, enzyme extract with detergent respectively. All the test results were separately indicated in Plates I to IV. Plate I showed the comparative analysis of the removal of turmeric stain on cotton using enzyme extracts and detergents. The action of alkaline protease to remove plaintain stem juice and blood stains was presented in comparison with the other washing solutions like detergents and distilled water. The action of enzyme on kumkum to remove completely was presented in Plate-I. The application of enzymes on distaining was clearly proved and showed in plates.

Plate I

Destaining of Different Stained Cloth with Alkaline Protease



1. Cloth Stained with Turmeric.
2. Cloth Stained with Plantain Juice.
3. Cloth Stained with Blood.
4. Cloth Stained with Kumkum.

5, 6, 7, 8 - Destained Cloth after Alkaline protease treatment 10ml of crude enzyme preparation

Similar stains were used by different researcher for the destaining ability of protease enzymes. These reports were illustrated to compare the obtained results in order to prove the mode of action of these enzymes. Reports by Venugopal and Saramma (2007) proved the efficiency of protease in the removal of blood stain from cloths. Nadeem et al. (2009) studied the high capacity of blood stain removal by B. *licheniformis* N-2. Jabeen and Qazi (2011) reported that alkaline protease expressed capability for removing blood stain and partially purified enzyme showed higher efficiency. The researchers also suggested that enzyme treatment required relatively less time in removing the blood. Najafi and Deobagkar (2005) concluded that proteases from Pseudomonas aeruginosa PD 100 and B. *licheniformis* were reported for destaining blood stains from cloth without detergent use. Tambekar and Tambekar (2013) reported that alkaline protease produced by Bacillus *pseudofirmus* exhibited better action by showing faintness of the chocolate spots, sauce spots and turmeric powder on the cloth. The isolate, Bacillus pseudofirmus showed efficient removal of dirt from cloth by all the three protease produced. Present study confirms the effectiveness of alkaline protease in destaining process.

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