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THERMOACTIVE, EXTRACELLULAR AND ALKALINE PROTEASE FROM *BACILLUS SP.* : PURIFICATION, BIOCHEMICAL CHARACTERIZATION AND POTENTIAL APPLICATION AS A DETERGENT ADDITIVE IN LAUNDRY.

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ABSTRACT
Proteases are one of the most widely used industrial enzymes. A thermo stable, extracellular, alkaline protease was isolated from bacteria. Bacterial protease was purified by using ammonium sulphate precipitation, membrane filtration, DEAE cellulose and Sephadex G-200 chromatography. Protease activity was assayed using the casein as a substrate. The molecular weight of purified protease was estimated approximately 45kDa using casein 12% SDS PAGE Zymography. The optimum temperature and pH of this enzyme was 45^0C and 10 respectively. Purified enzyme was found detergent compatible.

KEYWORDS: *Bacillus sp*, Detergent additives, .Purified enzymes, Zymography.

Introduction

Amylases, celluleses, proteases and lipases enzymes are widely used in detergent formulation industry to improve their detergency quality. Proteases are more important and widely used industrial enzymes produced by molds, yeasts and bacteria (Walsh and Wilcox, 1970).Bacterial proteases are more significant (Fujiwara *et al.*, 1991).Most of the bacterial proteases are extracellular in nature, thermostable, wider pH stability rangeand easily producible in large scale. Bacterial proteases are widely used in the industrial application as bakery, food, bioremediation process, laundry detergent industries and peptide synthesis (Rao *et al.*, 1998; Anwar and Saleemuddin, 1998; Gupta *et al.*, 2002; Banik and Prakash, 2004; Jaswal and Kocher, 2007). Among the bacteria, *Bacillussp*. is specific producer of extracellular proteases (Priest, 1977).

The major limitation of enzyme recovery from sources are their instability at wider pH range as well as thermo stability (Griffin *et al.*, 1992; Lee *et al.*, 1996). So there is need to screen a bacterial strain which having wider pH range as well as thermostable. In laundry detergent formulation process various alkaline proteases producing *Bacillus* strains have been reported as *Bacillus stearothermophilu* (Dhandapani and Vijayaragvan, 1994),*Bacillus brevis* (Banerjee et al., 1999), *Bacillus sp*. SSR1 (Singh *et al.*, 2001). *Bacillus tequilensis* (Shah and Birmole, 2014). Thus the industrial demand for more active forms of proteases with suitable specificity and stability of pH, temperature continues to stimulate the search for new enzymes. The objective of this study was to purify and biochemically characterize alkaline protease produced by *Bacillus sp.* isolated from ant nest soil.

MATERIALS AND METHODS

Screening of alkaline proteases from bacteria

The soil bacteria with protease activity were isolated with the help of selective screening method mentioned in Gatson et al. (2006) and Bonala and Mangamoori (2012)**.**The bacterium was isolated from an ant nest soil under selective isolation conditions. The nutrient media containing 1% skim milk powder was used for the screening of protease producing bacteria at an alkaline pH 9.0. The potential isolate was selected among the many strains showing different proteolytic activities as a clear zone of skim-milk hydrolysis. The isolate was used for further study of morphological and gram characteristics.

Enzyme production and culture conditions

The production of alkaline protease was performed in the GYP (Glucose Yeast Peptone) media;1% glucose, 0.5% yeast extract, 0.5% peptone and 0.3% sodium chloride supplemented with 2% casein at pH 9.5. The inoculum was prepared by adding a loop full of cells from 24h pure active slant culture into 25 mL of autoclaved above mentioned medium containing glucose as a sole carbon source and supplemented with additional casein (enzyme hydrolysed) in

250 mL Erlenmeyer flask. The culture flask was incubated at 37^{0}C and 180 rpm in an orbital shaker incubator for 24h. A 10% of seed culture inoculum was added to 100 mL of the production medium in 250 mL Erlenmeyer flasks and incubated in an orbital shaker incubator at 37^{0}C and 180 rpm. The cultures were centrifuged at 5000 rpm and the supernatants were used for determination of protease activity.

Protease activity assay

Protease activity was assayed using the casein as substrate. In case of caseinolytic activity reaction mixtures were consisted of 1 ml 1% casein (according to Hammerstein) dissolved in 0.1N NaOH, then subsequent dilution was performed with 50mM of Tris HCl buffer pH 9.0). Before reaction substrate was incubated in water bath at 37^{0}C for 10 min. Then 0.1 ml enzyme sample was added in the test tube. The incubation was in water bath at 37^{0}C for 30 min. The reaction was terminated with 2 ml 5%TCA solution. The absorbance was measured at 660 nm. One unit of protease activity was defined as the amount of the enzyme required to produce one microgram tyrosine per milliliter per minute under the specified conditions.

Purification of alkaline protease

Ammonium sulfate precipitation

Ammonium sulfate was added to the clear supernatant with constant stirring and incubated overnight. The fractionation was performed as 0-40, 40-80 and 80-100%. Maximum protease activity was observed with the fraction precipitated at 40–80% saturation. The precipitate was collected by centrifugation at 10,000g for 30 min and dissolved in a minimal amount of 50 mM Tris–HCl buffer (pH 8.8) and dialyzed against the same buffer for 24 h.

Membrane filtration

The enzyme solution was dialyzed through a 10 kDa cut-off Millipore centrifugal membrane filter devices. This was repeated after each purification process to concentrate the sample.

DEAE-cellulose chromatography

The concentrated enzyme sample was loaded on 50mM Tris-HCl buffer (pH 8.8) pre-equilibrated diethylaminoethyl (DEAE) cellulose column. The column was washed with three column volume of the equilibration buffer. The adsorbed protein was eluted using a linear gradient of NaCl (0–500 mM) in the same buffer at a flow rate of 1ml per 5 minutes. The active fractions obtained after the ion exchange chromatography were pooled, dialyzed and concentrated with 10 kDa cut-off Millipore centrifugal membrane filter devices.

Sephadex G-200 chromatography

The active concentrated fraction of ion exchange chromatography was applied to a Sephadex G-200 column. It was preequilibrated with a 100mM Tris-HCl buffer (pH 8.3). The protein elution was carried out with the same buffer at a flow rate of 6 ml/min. The fractions were collected using a Bio-Rad 2110 fraction collector. It was assessed for protein at 280 nm and the enzyme activity. The active fractions were pooled and concentrated using Amicon® Ultra - 15Millipore centrifugal membrane filter devices.

Zymographic visualization of protease activity

In gel activity analysis of the alkaline protease was performed in 12 % SDS polyacrylamide gel electrophoresis. The electrophoresis was carried out according to the procedure of Laemmli (Laemmli, 1970). 12% SDS acrylamide gel was polymerized with addition of 0.1% casein (according to Hammerstein). After electrophoresis the renaturation was carried out by washing the gel with 2.5% triton-X 100 for 1h. Then the gel was extensively washed with deionized water followed by incubation in activation buffer (glycine NaOH 50mM, pH 10.0) at 37 ^{0}C for overnight. The gel was stained with Coomassie brilliant blue R-250 and the zones of proteolysis were detected.

Biochemical characterization

Effect of pH on protease activity

The optimum pH for alkaline protease activity was determined over a pH range of 3–11. The buffers, acetate (pH 3.0 – 5.0), potassium phosphate (50 mM, pH 5.5–7.5) and Tris–HCl (50 mM, pH 8.0–10.5) were used for this study. The enzyme preparations were incubated at pH of 3–11 and activity was determined under assay conditions.

Effect of temperature on protease activity

The optimum temperature range for protease activity was determined by incubating the assay mixture at different temperatures ranging from 25 to 90 ^{0}C. The activity was determined under assay conditions (Glycine NaOH, pH 10.0, 50 mM).

Washing performance of protease

The cotton cloth pieces (4cm x 4cm size) stained with human blood and washed under different condition as Water, Detergent, Enzyme and Detergent + enzyme. The Surf Excel detergent was used for this study. The performance was observed after 15 min of interval

RESULTS AND DISCUSSION

Screening of alkaline protease producing strain

The soil bacteria with protease activity were isolated with the help of selective screening method mentioned in Gatson et al. (2006) and Bonala and Mangamoori (2012)**.**The cultures were maintained on nutrient agar slant and sub-cultured once in a week and preserved at 4-7 ^{0}C. The screening was performed by incorporating skimmed milk powder in to the nutrient agar. The potential colonies exhibiting zone of proteolysis were then isolated and used for fermentation. The four isolates were obtained and one giving major activity was studied further (isolate 2) shown in Fig. 1 and Fig.2.

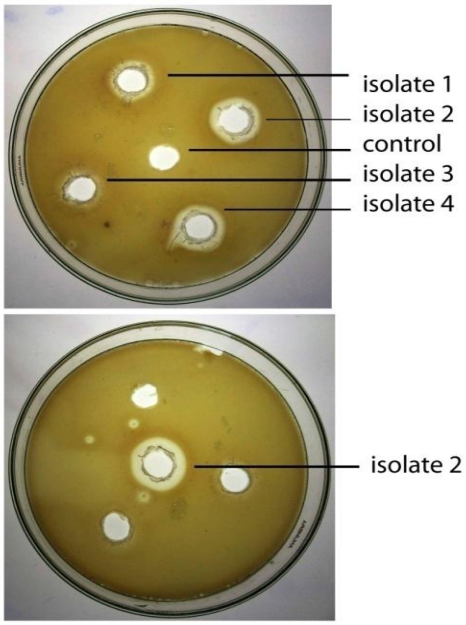

Figure 1. Screening of alkaline protease producing strain.

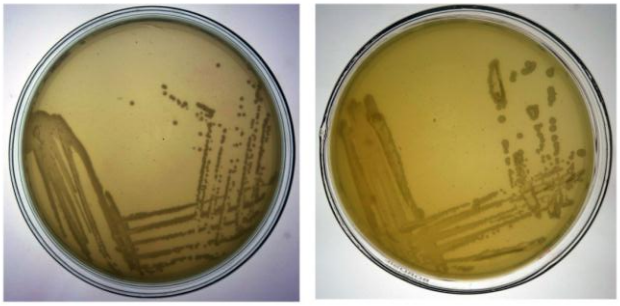

Figure 2: Isolation of potential bacterial strain producing alkaline protease.

Enzyme production fermentation medium and culture conditions

The crude enzyme was obtained after incubation of media at shaking conditions of 180 rpm at room temperature (approximately 30^0C). The maximum production of proteases after 48-72 hrs of fermentationwas reported by Hoshino et al. (1995) and Shumi et al. (2004). Ward (1995) also reported that bacillus species usually secrete more proteases during last exponential phase. Similarly our results reports that maximum enzyme production was obtained after 48 hrs.

Purification of enzyme

Purification of the extracellular alkaline protease was carried out by a combination of conventional purification procedures. The stepwise data is summarized in Table 1.

Table 1: Stepwise purification of enzyme.

Purification steps	Volume	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Crude Extract	1000	2127.13	600	3.55	1.00	100
Ammonium sulfate	345	1568.44	181	8.67	2.44	74
DEAE Cellulosecolumn	130	1371.46	10	137.15	38.68	64
Membrane Filtration	55	970.8	5	194.16	54.77	46
Sephadex G- 200	5	139.41	0.5	278.82	78.65	7

Alkaline protease (1000 ml) in the crude extract was separated by ammonium sulfate fractionation. The maximum activity was obtained in the 40 -80% fraction. After this precipitation, the enzyme attained 2.44-fold purification and 74% enzyme yield. The precipitated fraction was dialyzed against 50 mM Tris–HCl buffer, pH 8.3 and then subjected to ion exchange chromatography on diethylaminoethyl (DEAE) cellulose column. The elution yielded enhanced specific activity 137.15 (IU/mg) with 38.68 fold purification. The fraction of major activity was thereafter subjected to centrifugal membrane filtration of 10 kDa cut-off resulted in to 54.77 purification fold and 46% yield. During the last step of gel filtration the concentrated sample was loaded on to Sephadex G 200 gel filtration column pre equilibrated with 50 mM Tris–HCl buffer, pH 8.3. Fractions containing major activity were pooled and concentrated. The following figure shows the protein and activity profile of membrane filtration fraction purified on Sephadex G-200 gel filtration column chromatography(Fig.3). Although this fraction contained different protein molecules, in the final purification step a sharp distinctive peak was obtained correlating with the peak of protein concentration. The last purification stage increase specific activity 278.82 with 78.65 fold purification.

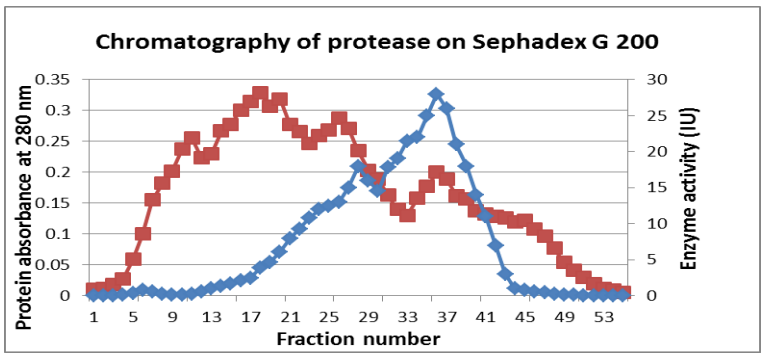

Figure 3. Graph of Gel filtration chromatography

Effect of pH on enzyme activity

The effect of pH on alkaline protease activity was studied at various pH values, at constant 37^{0}C temperature. Alkaline protease is active in a wide range of pH (6.0–12.0), with an optimum activity at pH 10.0. Enzyme activity decreased slowly under increasing acidic conditions (Figure 4).

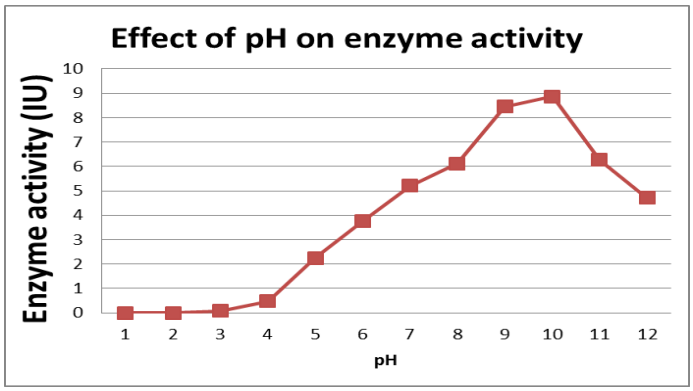

Figure 4. Determination of optimum pH of the alkaline protease

Effect of temperature on enzyme activity

Alkaline protease was active between temperature ranges 35to65^{0}C. Activity increased rapidly from 25 to 40^{0}C, reaching a maximum at 45^{0}C. Thus the optimum activity was found to be at 45^{0}C, when Glycine-NaOH (50 mM, pH 10.0) was used for this study. The alkaline protease was found to be denaturing above 70^{0}C (Figure 5). Earlier researchers reported that optimum temperature for protease activity is differing from 40 to 70^{0}Cfor different bacterial species (Joo *et al.*, 2002, Singh *et al.*, 2001, Kumar and Takagi, 1999, Gupta *et al.*, 1999).

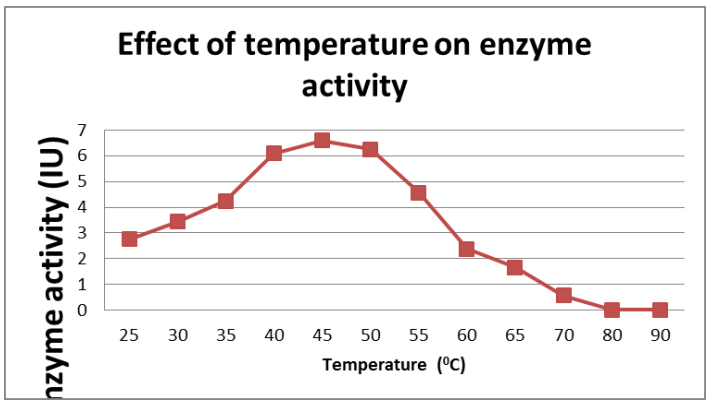

Figure 5: Determination of optimum temperature of the alkaline protease

Zymographic visualization of enzyme

In gel activity analysis of the alkaline protease was performed in 12 % SDS acrylamide gel. The white distinctive bands of proteolytic activitywere observed after overnight incubation in activation buffer Glycine-NaOH (50 mM, pH 10.0).

Washing performance of protease

The wash test was performed to analyse the detergent compatibility of the alkaline protease with tap water. The efficiency of alkaline protease was tested for removing bloodstain from cotton fabric with market detergent (surf excel). It required 15 min to remove the bloodstain from cotton cloth with tap water at room temperature. Fig.7 showed the removal of bloodstain by washing the cloth with tap water only as a control, washing with enzyme and detergent separately, and washing with enzyme detergent mixture. The addition of enzyme enhanced the detergent activity

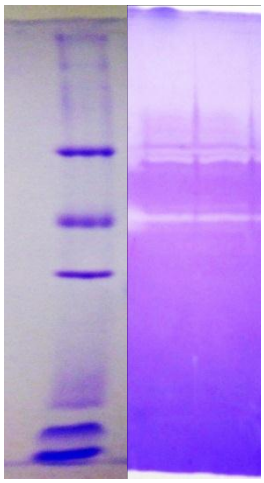

Figure 6: Zymographic visualization of enzyme

These results demonstrated that alkaline proteases isolated from *Bacillus* can be effectively usedas detergent additive. Similar recent reports also proved that alkaline proteases from *Bacillusmojavensis* A21 (Haddar *et al.,* 2010), *Bacillus cereus* (Kanmani *et al.*, 2011), *B.subtilis* WIFD5 (Sharma and Aruna, 2012), *Bacillus subtilis* (Ravishankar *et al.*, 2012), *Bacillus tequilensis* (Shah and Birmole, 2014) were used to study detergent compatibility. Therefore, in this study we report that extra cellular proteases produced by *Bacillus sp*. may be used as detergent additives.

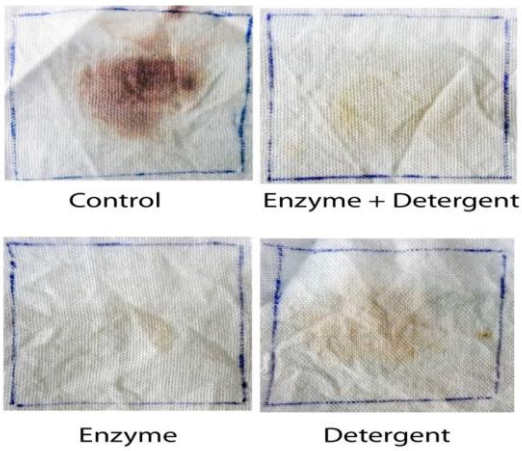

Figure 7. Wash performance analysis.

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