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Characterisation of alkaline protease from newly isolated *Bacillus* strain NBB 04

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ABSTRACT

Bacterial isolate NBB04 producing alkaline protease was obtained from the waste of the paper industry. Gram Positive strain shows avtivity in alkalime medium with maximum activity abtained after 48 hours of incubation. The crude enzyme shows optimum temperature of 40ºC and oprimum pH of 10.0. The enzyme has stability of 24 hours at pH 10 at temperature of 40 and 50ºC. in the temperature range of 37-45ºC the temperature remain 100% stable thereby can be potential candidate for various application of this enzyme in various industries involving protease.

1. Introduction

Group of enzymes catalyzing hydrolysis of peptide bonds of proteins are referred to as protease. They vary in their capacity to hydrolyze variety of peptide bonds with specificity towards breaking peptide bonds (Sudipta 2010). On the basis of their site of action, proteases are categorized into two major categories i.e. exopeptidases and endopeptidases. Further, proteases are classified into four prominent groups namely, serine proteases, aspartic proteases, cysteine proteases and metalloproteases. This classification is based on active site analysis.

Proteolytic enzymes are of great significance as they aid in the digestion process by metabolizing the protein content of foods to release the amino acids which are vital for the body. Moreover, these proteolytic enzymes find their use in numerous forms of therapy. Their medicinal use has gained popularity as many research reports and clinical studies depicted their benefit in oncological experimentation, inflammatory conditions, control of blood rheology and regulation of immune responses. Commercially, proteases dominate enzyme market because of their uses in pharmaceutical, dairy/food and textile industry. Proteases have been isolated from plants, animals, bacteria and fungi and commercially available for various applications (Rao et al., 1998)

Most of the detergents used either in household laundering or cleaning agents for various devices contain proteases as one of the regular ingredients. Majorly, 25% of the global sales of proteases is due to its use in laundry detergents. Leather quality has been improved by employing enzymes instead of chemicals with significant reduction in environmental pollution. Selective hydrolysis of non-collagenous components of the skin along with elimination of nonfibrillar proteins (albumins and globulins) have been achieved via proteases. The enzyme has been extensively used in the dairy industryin cheese manufacturing and ripening process. It aids in flavor development too. Wheat gluten modifications have been done using Endo- and exoproteinases from *Aspergillus oryzae* via limited proteolysis to produce gluten free products (Souza et al., 2015). Bacterial protease has been used to improve the extensibility and strength of dough. Proteases are diverse possessing different specificities, thus providing opportunity in development of effective therapeutic agents (Chanalia et al., 2011). The use of immobilised alkaline protease from *Bacillus subtilis* posses various therapeutic properties for the development of soft gel medicinal formulas, non-woven tissues, and new bandage material properties. There are wide sources of protease from bacteria, fungi, plant and animals (Oseni 2011). A number of bacteria and fungi produce proteases which is extracellular. Predominantly, *Bacillus* species produce the enzyme including *B. subtilis B. sphaericus*, *B. licheniformis*

2. Material and methods

2.1 Isolation of bacteria producing alkaline protease enzyme:

Different samples of soil were collected from dumping sites in paper industry. Samples were diluted using saline water. The diluted samples were plated on medium plates containing beef extract (0.15%), peptone (0.5%), yeast extract (0.15%), skim milk (1%) and NaCl (5%). pH pf the medium was adjusted to 10.0 using NaOH (0.1N). The plates were incubated for 12-24 hrs at 45^{0}C. Protease producing colonies were selected depending upon the clearance zone.

2.2 Production of Alkaline Protease:

Production of the protease from the selected strain as Bacillus sp NBB04 was carried out in a medium (pH 10.0) containing beef extract (0.15%), peptone (0.5%), yeast extract (0.15%), skim milk (1%) and NaCl (5%) at 150 rpm at incubation temperature of 45ºC. Samples were taken out in duplicate at regular interval of 6 hours. Biomass and enzyme activity was determined. After 48 hours, fermentation broth was centrifuged at 10,000 rpm at 4^{0}C and supernatant was used as crude enzyme for further characterization.

2.3 Enzyme Assay:

100 ul of enzyme was added to 100 ul of azocasein (1% w/v) in 0.2M glycine NaOH buffer and incubated at 45ºC for one hour. After incubation 1 ml of 10% trichloroacetic acid (TCA) was added. Mixture tubes were kept on ice for 20 minutes followed by centrifugation at 8000 rpm for 15 min. 1 ml

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of supernatant was mixed with 0.5ml of NaOH (1.8N). Absorbance of samples were measured at 420 nm with appropriate blanks.One unit (U) enzyme activiy was defined as the amount of enzyme required to produce an increase in absorbance equal to 0.01 in one hour.

2.4 Protein Assay:

Concentration of protein in different samples was estimated by the method of Lowry *et al.* (1951). 0.5 ml of protein sample and 2.5 ml of working solution was mixed followed by incubation for 10 minutes at 25ºC. To the mixture, 0.25 ml of Folin reagent was added, followed by incubation at 25ºC for 30 minutes. Absorbance was measured spectrophotometrically at 750 nm against suitable blank. Standard curve of protein was prepared using BSA solution (0.02-0.2 mg).

2.5 Characterization of crude enzyme

2.5.1 Determination of optimum pH

To study the effect of various pH on alkaline protease activated from selected isolate substrates were prepared in different buffers (0.05 M): phosphate (pH 6.0-8.0), glycine NaOH (pH 9-11). Reaction mixtures of substrate and enzyme were incubated at 45^{0}C and the activity of the enzyme was measuredas described earlier. Relative protease activity was determined at different pH.

2.5.2 Determination of pH stability :

To determine pH stability, enzyme was diluted with different pH buffer (pH 6-11) and incubated at 40 and 50ºC for 12 hours. Residual enzyme activity was estimated as per assay protocol.

2.5.3 Determination of optimum temperature:

Reaction mixture containing enzyme and asocasein (as per protocol) was incubated different temperature range (30 - 70^{0}C) followed by determination of enzyme activity.

2.5.4 Determination of temperature stability :

To determine the temperature on enzyme stability crude enzyme was incubated at differnet temperatures (37-60^{0}C) and relative activities were determined at per standard conditions.

2.5.5 Effect of different metal ions on enzyme activity:

To reaction mixture containing crude enzyme and substrate, added different metal ions (40mM each) including Ca^{2+}, Mg^{2+}, Fe^{3+}, Na^{+}, Zn^{2+}, Cu^{2+} and relative protease activity was estimated.

3. Results and discussion

3.1 Screening of protease producing bacteria:

Nine bacterial strains producing alkaline protease were isolated from samples collected from paper industry and slaughter house. Depending on the clear zone obtained and production of Protease under alkaline conditions Table 1. Strain NBB04 was selected for further work.

Table 1
Isolated strain with optimum pH for protease activity

Strain Name.	pH optimum	Enzyme Activity (U/ml) (After 12 hr)
NBB 01	9.0	640
NBB 02	9.0	710
NBB 03	8.0	140
NBB 04	**10.0**	**890**
NBB 05	10.0	660
NBB 06	9.0	587
NBB 07	9.5	322

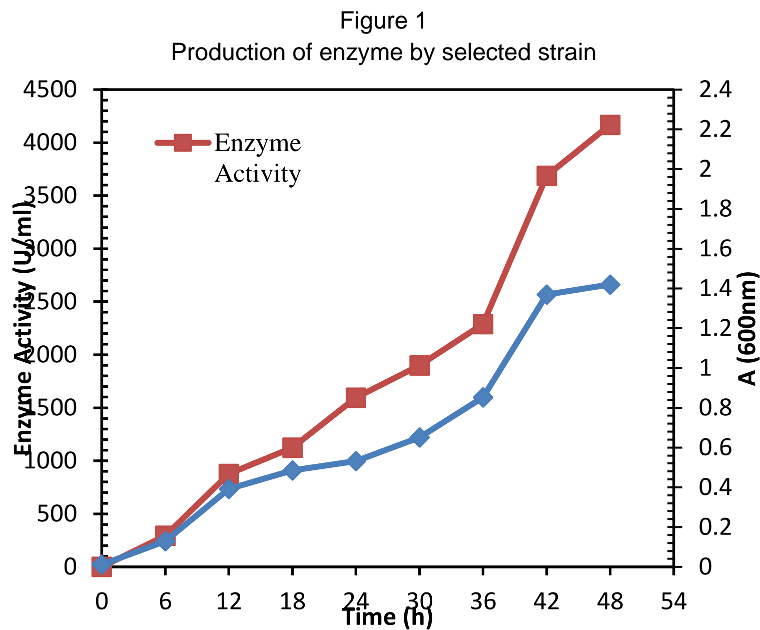
3.2 Strain characterisation:

The isolated strain was characterized for different structural and physiological properties. The selected strain was gram positive bacilli and able to grow in NaCl (0.2-5.0 %), Temperature (20-55ºC) and pH (6-11).

3.3 Production of enzyme by selected strain:

Bacillus strain, was grown on medium containing skim milk (1%), NaCl (5%), Beef Extract (0.15%), Yeast Extract (0.15%), Peptone (0.5%) at 45ºC and 150 rpm. The pH of the medium was adjusted to 10.0 with 0.1 N NaOH. Maximum enzyme production was observed after 48 h (Figure 1).

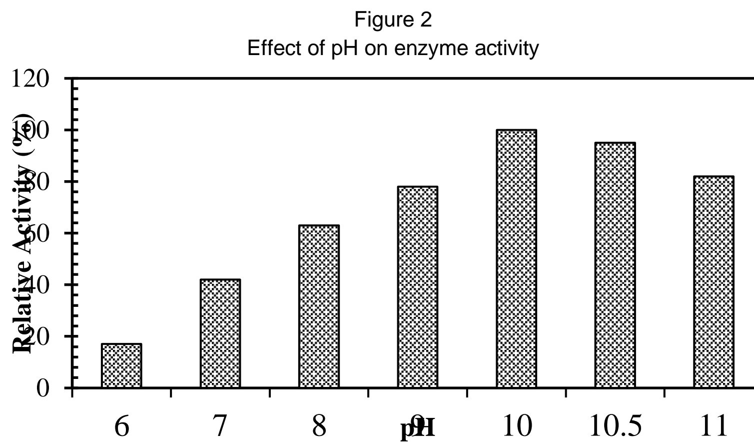
Figure 1
Production of enzyme by selected strain



3.4 Effect of pH on enzyme activity

A broad pH activity range of 7.0-10.5 and pH optima of 10.0 was observed. Based on this observation, the selected strain could be classified as an alkaline protease producing bacteria. About 100% activity was retained at pH 10 (Figure 2). Similar studies, reported optimum enzyme activity in the range of pH 7- 11(Kocabiyik *et al.*,2006, Miyaji *et al.*,2006, Setvormi *et al.*,2006, Vidvasagar *et al.*,2006, Cha *et al.*,2005).

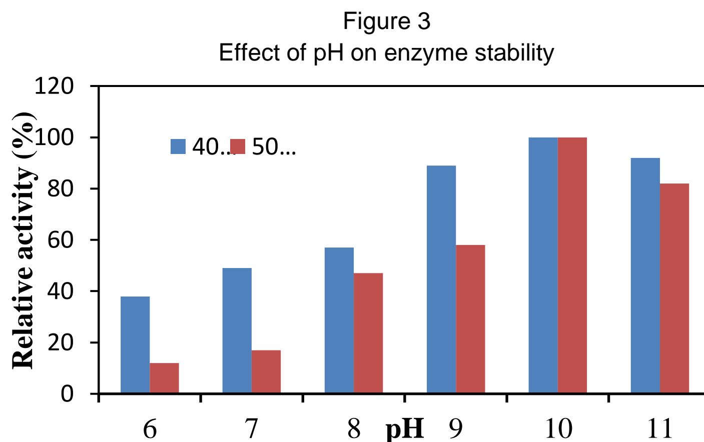
Figure 2
Effect of pH on enzyme activity



3.5 Effect of pH on Enzyme stability:

The enzyme was stable at alkaline pH and more than 90% activity was retained after incubation (40ºC and 50ºC) for 24 hrs at pHs between 7.0 and 9.0 (Figure 3). Our result differs from a *Bacillus pumilus* alkaline protease that was stable over the pH 8-11 at 50º C. (Miyaji *et al.*,2006)

Figure 3
Effect of pH on enzyme stability



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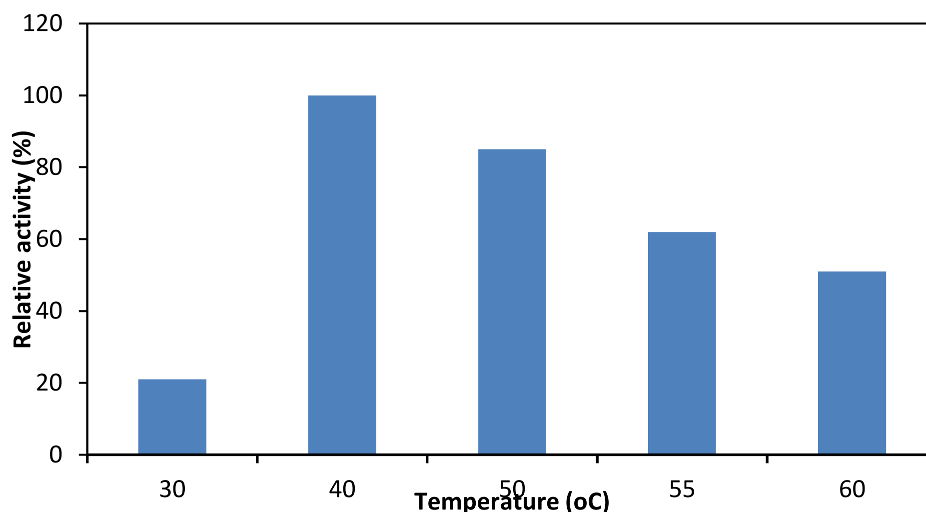
3.6 Effect of Temperature on Enzyme Activity:

The protease activity of the crude enzyme was measured at temperatures ranging from 37-60ºC. Temperature optimization studies at pH 10, showed 60 ºC to be the optimum temperature. The optimum catalysis reported is in the range of 55ºC (Huang *et al.,* 2003).

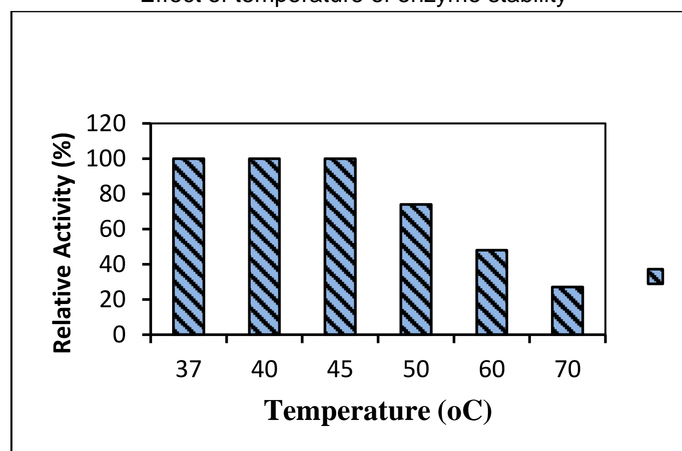
Figure 4
Effect of temperature on enzyme activity



3.7 Effect of Temperature on Enzyme Stability:

Maximum stability of strain D protease was reported at temperature of 60 ºC. A reduction of 50% in enzyme activity was observed at 50 ºC (Figure 5). This range of stability is quite broad as compared to some reports for alkaline proteases where the stability is maximum at 50ºC (Chu, 2007), 55ºC (Wang *et al.,* 2006), 60ºC (Hiraga *et al.,* 2005).

Figure 5
Effect of temperature of enzyme stability



3.8 Effect of Metal Ions:

The effect of various metal ions on protease activity was studied in 40 mM Glycine-NaOH buffer. Among the metal ions tested, Zn^{2+} acted as strong enhancer of enzyme. Further activation effect were shown by Na^{+} (more than 80%) & Cu^{2+} (80%). Fe^{3+} showed an activity of about 60%. 80% activity was shown in the presence of Mg^{2+}.

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