

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.510.020>

Alkaline Protease Production by *Aspergillus terreus* BAB-346 using Poultry Litter Waste

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ABSTRACT

Keywords

Alkaline Protease,
Aspergillus terreus,
Poultry litter,
Agro-industrial
wastes.

Article Info

Accepted:
12 September 2016
Available Online:
10 October 2016

Aspergillus terreus BAB-346 was isolated from various agro-industrial wastes dumping site of Anand region and screened for alkaline protease enzyme production. A comparative study was carried out on the production of Alkaline protease using different type of agro-industrial waste viz. Paper pulp waste, banana stem waste and poultry litter waste as substrate in submerged medium. Amongst the various isolates, *A.terreus* BAB-346 produced the highest enzyme activity at 120 h of incubation as 330.0 U mg⁻¹ using Poultry litter as the sole carbon source. However, produced enzyme was partially purified as 2.5 fold by Ammonium sulphate fractionation. The purified enzyme has temperature and pH optimal of 60°C and 8.0, respectively. Purified alkaline protease is tharmostable in nature and retained fully active even at end of 20 minute of incubation at 60°C. The Alkaline protease was completely inhibited by Hg⁺², Ca⁺², with little increase (22 %) in the activity of upon addition of Mn⁺² metal ions. It is envisaged that the isolate can be a potential source of Akaline protease for use as additive in industrial application like detergent industry.

Introduction

The Poultry industry has seen phenomenal growth worldwide. This fast growth has inevitably resulted in various problems especially in the environmental side because rapid transformation in environmental management is not possible (Guru, 2003). In India, there are about 3430 million population of Poultry with waste generate of

3.30 million tons per year (Lakshman *et al.*, 2005). The localization nature of poultry production also means that it can represent a large percentage of the agricultural economy in many states or regions. Although economical and successful, the Poultry industry is currently facing with a number of highly complex and challenging

environmental problems, many of which are related to its size and geographically concentrated nature from an agricultural perspective, poultry wastes play a major role in the contamination of ground water through nitrate nitrogen. Also the surface eutrophication of surface water due to phosphorus, pesticides, heavy metals and pathogens present in the poultry wastes applied to solid are the central environmental issues at the present time. Among the animal manures, poultry droppings have higher nutrient content. This component upon microbial action can be converted to value added compost with high nutrient status. In poultry dropping nearly 60 % of nitrogen which is present as uric acid and urea is lost through ammonia volatilization by hydrolysis (Mahimairaja *et al.*, 2008). Different enzymes such as Amylase, Protease, Xylanase, Lipase, Phytase, Cellulase, Pectinase, Keratinase, Invertase, Carboxypeptidase, Hydrolase, β – galactosidase & Gelatinase, has been produced using such waste. Proteases constitute a group of enzymes that cleaves the peptide bonds of proteins and peptides. Proteases have a wide range of function in nature. Extracellular microbial proteinases contribute to the nutritional well being of producing organism by hydrolyzing large polypeptide substrata into smaller molecules that the cell can absorb, in mammalian pancreatic proteases and intestinal and stomach peptidases generally perform a similar nutritional role in the digestion and absorption processes of these species. Proteases are involved in the modulation of gene expression, and in enzyme modification and secretion. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce proteases (Madan *et al.*, 2002). Molds of genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for production of proteases, as several species

of these genera are generally regarded as safe Microorganisms (Sandhya *et al.*, 2005). By keeping the above points in mind present work presents the production of alkaline protease using poultry litter. Hence produced enzyme was partially purified and kinetic properties were characterized.

Material and Methods

Isolation of fungi

Proteolytic fungi were isolated from various soil samples, procured from three main regions: 1) Paper mill area, 2) Banana Farm, 3) Poultry Farm area. Each sample were collected in U.V. sterilized sample bag and stored at 4°C before use. Appropriate dilutions from each sample were prepared and 0.1 mL of each diluted sample was spreader on to the following media to get different fungal isolates on the plate.

Malt Extract agar

Malt Extract.....30 gm
 Peptone.....05 gm
 Agar powder.....30 gm
 Distilled water.....1000 mL
 pH.....6.0

Screening of proteolytic fungi

Proteolytic fungi were directly screened out by observing the zone of hydrolysis surrounding colonies on Skim Milk Agar plate. All screened fungal isolates were picked up and pure culture of each isolates was prepared by repeated transferring it on to Malt Extract agar. After getting pure culture of each fungal isolates it was stored at 4°C and used further for identification.

Skim Milk Agar (pH-10)

Skim milk powder..... 30 g/L
 Nutrient agar..... 1000 mL
 Distilled water..... 1000 mL

Both the Skim Milk agar powder and Nutrient agar were separately sterilized in autoclave at 121°C for 20 min and aseptically mixed after it. After inoculation all the plates were incubated at 37°C for 24hr.

Identification of Alkaline protease producing fungi

Potent alkaline protease producing fungal isolate was identified on the basis of colonial and morphological characteristic by growing them on to the Malt extract agar plate as well as the selected fungal isolate was identified using FAST Microseq® D2 LSU rDNA fungal identification kit. (Applied Biosystems, Foster city, CA, USA). DNA extraction was carried out using Prepman™ ultra sample preparation reagent and D2 LSU rRNA gene was amplified and cycle sequencing was carried out as per the kit instructions. Amplification was carried out in a thermal cycler (9800, Applied Biosystems, Foster city, CA, USA) with reaction profile: initial denaturation at 95°C for 10 s followed by 35 cycles of denaturation 95°C for 30 s, annealing at 64°C for 15 s, extension at 72°C for 1 min and finally extension at 72°C for 5 min. The purified PCR product was sequenced and the phylogenic relationship of the isolate was determined by comparing the sequence data with the existing sequences available through the gene bank database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

Production media

For the production of protease enzyme the waste residue cleaned, ground and sieved to get 2 mm particle size and each following 100 mL production medium set were prepared in 250 mL conical flasks.

- I. Gelatin (1%) + Vogel minimal salt media
- II. Casein (1%) + Vogel minimal salt media
- III. Paper pulp waste (1%) + Vogel minimal salt media
- IV. Banana stem waste (1%) + Vogel minimal salt media
- V. Poultry litter waste (1%) + Vogel minimal salt media

Vogel minimal salt media use for protease production.

Tri Sodium Citrate.2H ₂ O	: 0.25 g
KH ₂ PO ₄ Anhydrous	: 0.5 g
NH ₄ NO ₃	: 0.2 g
MgSO ₄ .7H ₂ O	: 0.01 g
CaCl ₂ .2H ₂ O	: 0.01 g
Trance Element Solution	: 0.01 mL
Biotin Solution	: 0.005 mL
Distilled Water	: 100 mL
Yeast extract	: 0.25g
pH	: 8.0

Trance element (100 mL)

Citric acid	: 5 g
ZnSO ₄ .7H ₂ O	: 5 g
Fe (NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	: 1 g
CuSO ₄ .5 H ₂ O	: 0.25 g
MnSO ₄ . H ₂ O	: 0.05 g
H ₃ BO ₃	: 0.05 g
Na ₂ MoO ₄ .2 H ₂ O	: 0.05 g

For enzyme production, a 1×10⁶ spores/mL was inoculated into 250 mL conical flask containing 100 mL of the Vogel minimal medium (pH-8) with 1 % of different waste as following and incubates 28 °C in an orbital shaker (140 rpm).

After 24, 48, 72, 96 and 120 hours time interval samples were withdraws, centrifuged to remove biomass and supernant were taken for different estimation.

Enzyme assay

Protease activity was assayed by a modified method of Tsuchizla *et al.*, (1986) by using casein as substrate. The enzyme reaction carried out using 100µl of enzyme solution was added to 900µl of substrate solution (2 mg/ml (w/v) casein in 10 Mm Tris-HCl buffer (pH 8). The mixture was incubated at 45°C for 30min. Reaction was terminated by the addition of an equal volume of 10% (w/v) chilled trichloroacetic acid then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble protein. The supernatant was separated by centrifugation at 10,000 rpm for 10 min at 4 °C. The acid soluble product in the supernatant was neutralized with 5ml of 0.5 m Na₂CO₃ solution. The color developed after adding 0.5 mL of 3-fold-diluted folin-ciocalteau reagent was measured at 660nm. One unit of protease activity was defined as the unit of enzyme required to liberate µg of product equivalent to tyrosine per min per ml under the standard assay condition. The specific activity was expressed in unit per mg of protein.

Protein determination

Total soluble protein was estimated according to Bradford's method (Bradford, 1976) employing bovine serum albumin as a standard.

Partially purification of alkaline protease enzyme

All steps were done at 4°C

Step 1: filtration. After 120 hrs of production all the content of flask were centrifuged at 1000 rpm for 10 min following by supernatant was again passed through Whatman filter paper to remove biomass and unutilized media particles.

Step 2: Ammonium sulphate fractionation. To enzyme solution, solid ammonium sulphate was slowly added to 60 % saturation. After the mixture was left for 30 min on stirrer, the precipitate was collected by centrifugation and dissolved in minimum volume 10 mM Tris-HCL buffer pH 8, and then dialyzed against the same buffer.

Effect of pH and temperature on the activity and stability of alkaline protease enzyme

To determine the effect of temperature on the alkaline protease enzyme activity, the standard enzyme assay was followed except with various incubation temperatures (30-80°C). When the tests of thermal stability were performed, the enzyme was pre-incubated in 10 mM Tris-HCL buffer (pH 8.0) at 60°C over a period of 5-60 min. The residual enzyme activity was then determined under the standard condition. For the determination of pH effect on alkaline protease enzyme activity, the standard assay was used over a pH range of 6.0-12.0. The residual enzyme activity was then determined under the standard condition.

Effect of various metal ions on alkaline protease enzyme activity

The effect of various metal ions viz. Ca⁺², Mn⁺², Zn⁺², Hg⁺², Cu⁺² and Mg⁺² (Chloride and Sulphate salts @ 5mM) on enzyme activity were investigated by adding 1 ml of each metal solution into the reaction mixture incubated for 20 min at 60°C (pH 8). The activity of enzyme was measured.

Effect of organic solvents on the alkaline protease stability

Organic solvents used were methanol, ethyl acetate, benzene, glycerol, toluene, acetone

and isopropanol. In the stability test 1.0 mL of organic solvent (100% v/v) was added to 1mL of the reaction mixture and pre-incubated at 60 °C for 20 min (pH 8). The remaining proteolytic activity was measured.

Determination of kinetic parameters

The kinetic parameters, Michaelis constants (K_m) and maximal velocities (V_{max}), of Alkaline protease were determined in reference to the Casein substrate. A linear regression was used to analyze and obtain Lineweaver-Burk plots and K_m and V_{max} also were calculated. This investigation was carried out by the concentration range of 0.2 to 2 mg at the optimal pH 8 and temperature (60°C).

Results and Discussion

Isolation, screening and identification of the fungal isolate

For the isolation of proteolytic fungi different soil samples were collected from the Banana farm, Paper mill area and poultry farm surrounding Anand region. Pure cultures of each isolates were selected for screening purpose. Screenings of proteolytic fungi were easily carried out by growing the isolates on 1 % Skim Milk agar plate (pH 10) and observing the zone of hydrolysis of casein protein surrounding colony (Fig.1).

Best alkaline proteolytic producer shows 6 mm zone of hydrolysis whereas, other fungal isolate shows zone of hydrolysis in the range of 1 to 5 mm which were further used for production of enzyme. Potent fungal isolate was further identified at molecular level. The sequence had 97% homology with *A. terreus* showing 435 maximum score having 3e-118 e-value. The sequence was deposited in the NCBI gene bank bearing the Accession no. JN662343.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.077 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 218 positions in the final dataset. The phylogenetic tree was drawn using bioinformatics software MEGA 5.05 (Fig.2).

Production of alkaline protease from various agro-industrial wastes using *A. terreus* BAB-346

Various reports has been published for the production of alkaline protease production using crude agro-industrial wastes as a carbon and nitrogen source such as Rice mill waste for *Aspergillus niger*, Poultry waste for *Pseudomonas thermaerum*GW1, Soybean for *Bacillus sp.*, Molasses, Wheat flour and Wheat bran for *Alkaliphilic actinomycete* and Rice bran for *Aspergillus oryzae*, (Paranthaman *et al.*, 2009; Gaur *et al.*, 2010; Saurabh *et al.*, 2007; Mehta *et al.*, 2006; Chutmanop *et al.*, 2008). In present work also we selected various cheap and easily available waste materials such as banana stem waste, paper pulp waste and poultry litter waste for the production of alkaline protease enzyme. Production of alkaline protease enzyme varies from organisms to organisms and using different substrate such as rice bran by *A. oryzae* and *A. niger*, wheat bran *Bacillus sp.* and *actinomycete* (Paranthaman *et al.*, 2009; Mona *et al.*, 2006; Mehta *et al.*, 2006).

Poultry litter is the good source for the production of enzyme compared to other substrate and shows the production in the range of 0.76 to 330.0 IU mg⁻¹(Table 1) However, amongst all the isolated fungi, *A.terreus* BAB-346 shows maximum enzyme production using poultry litter that is 330 IU mg⁻¹ at 120 h of incubation. Due to higher amount of total protein and nitrogen content in poultry litter compared to other substrate provides precursors for the synthesis of protease enzyme. Our results are also in tune with Paranthaman *et al.*, 2009.

Partial purification of alkaline protease enzyme

After the selection of potential substrate and isolate for the production of enzyme, produced enzyme was purified on the basis of protein solubility using ammonium sulphate fractionation.

The main draw back with production of bacterial protease is the requirement of cost intensive procedure for separation of enzymes from cells. On the other hand enzyme from fungal origin offers an advantage of extracellular enzyme which is easily separated from mycelium by simple

filtration. At 60-65 % saturation concentration of ammonium sulphate significant level of protein was pooled and was dialyzed against 10mM Tris-HCL buffer (pH-8). Purification of alkaline protease resulted in 2.5 fold and purification with 85 % recovery (Table 2). Similar observation was also reported by Gaur *et al.*, (2010). Purified enzyme solution was stored at 4°C and further used to study the kinetics of it.

Alkaline protease enzyme characterization

Temperature optimization and Thermal stability

Temperature is a critical factor for maximum enzyme activity and it is a prerequisite for industrial enzyme to be active and stable at higher temperature. Assay mixture was incubated at different temperature ranging from 10 to 80°C and maximum enzyme activity was found at 60°C (Figure 3). Lowering or rising the temperature from 60°C resulted in reduction of alkaline protease activity. Alkaline protease of *A. terreus*BAB-346 was most active at 60°C resembling alkaline protease of *A. usami* (Morimarvet *et al.*, 1994).

Table.1 Effect of various agro-industrial wastes on production of alkaline protease enzyme using *A.terreus* BAB-346.

Time Interval (h)	Specific activity IU mg ⁻¹				
	Paper pulp	Banana stem	Poultry litter	Casein	Gelatin
24	0.76	30.1	37.5	1.43	0.76
48	8.32	35.4	55.3	1.72	1.34
72	12.0	38.0	59.0	16.18	1.52
96	33.2	40.6	60.6	7.55	3.29
120	141.6	11.1	330.0	3.35	20.1
144	1.2	2.32	12.7	0.82	10.8

Table.2 Partial purification of alkaline protease enzyme

Fractionation Steps	Unit Activity (IU/ml)	Protein (mg/ml)	Specific activity (IU/mg)	Purification (fold)	Recovery Yield(%)
Culture filtration	20	0.060	333.3	1	100
Ammonium sulphate fractionation	17.09	0.020	854.9	2.5	85.45

Fig.1 Zone of hydrolysis of casein protein surrounding screened fungus colony



Fig.2 Phylogenetic relationships on the basis of homology index for a proteolytic fungal isolate *A. terreus* BAB-346

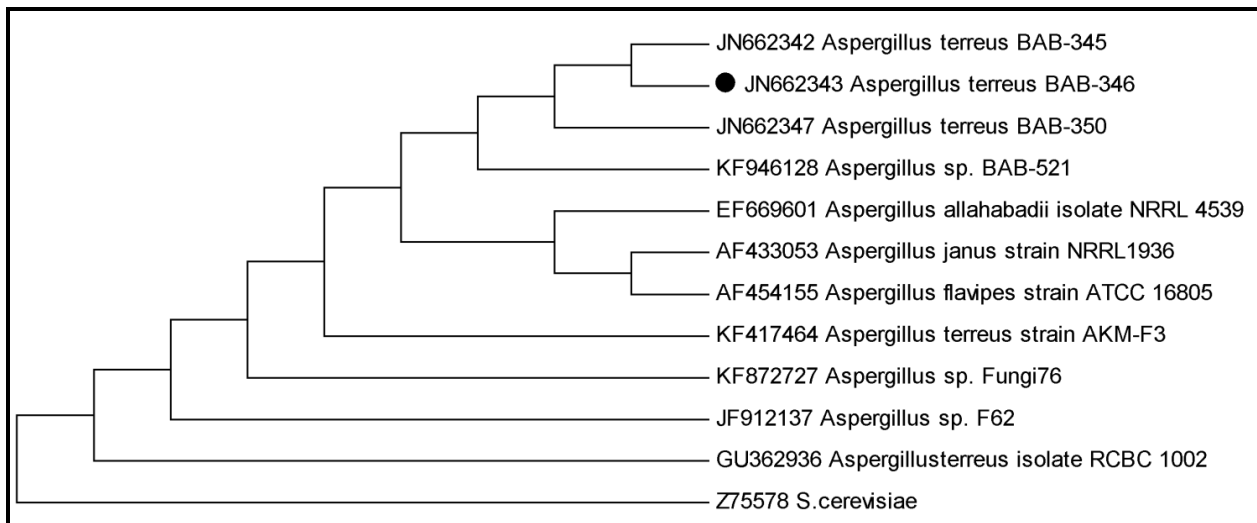


Fig.3 Effect of Temperature on the relative activity of alkaline protease enzyme

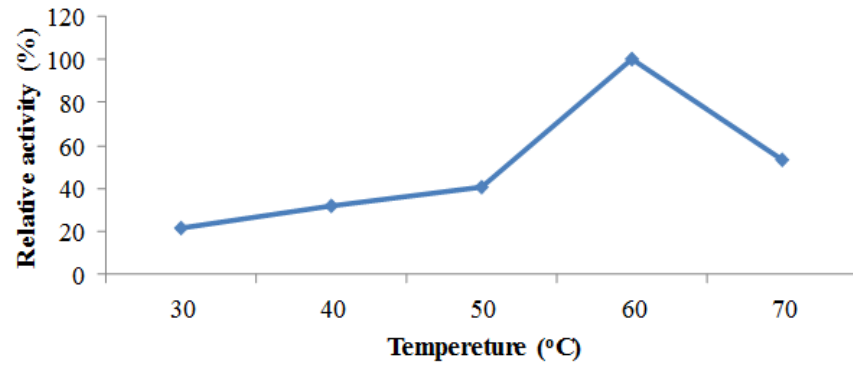


Fig.4 Thermo stability of alkaline protease at 60°C

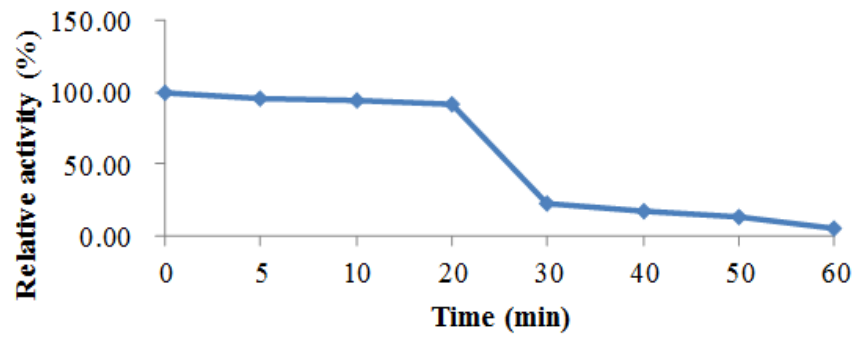


Fig.5 Effect of pH on the relative activity of alkaline protease enzyme

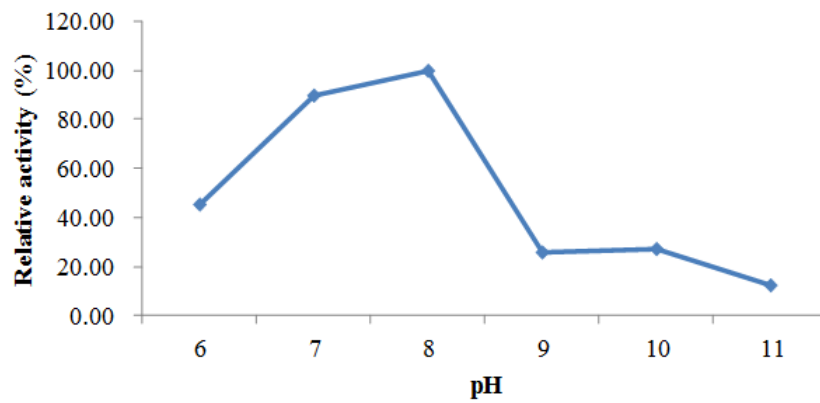


Fig.6 Effect of Metal ions on alkaline protease enzyme activity

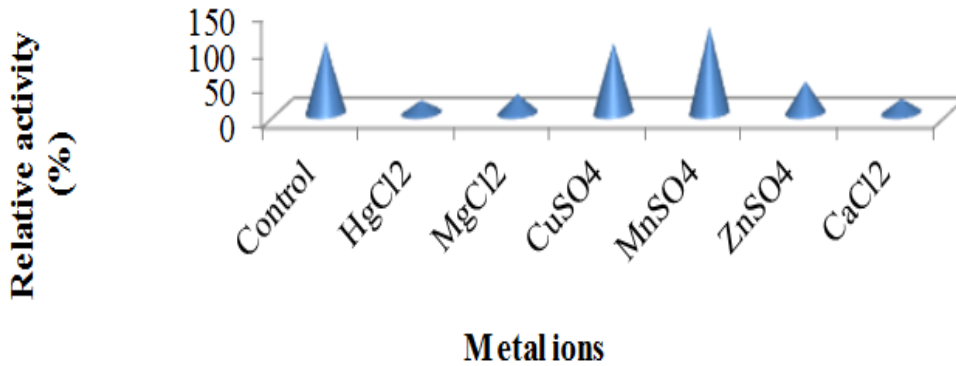


Fig.7 Effect of various organic solvents on alkaline protease enzyme activity

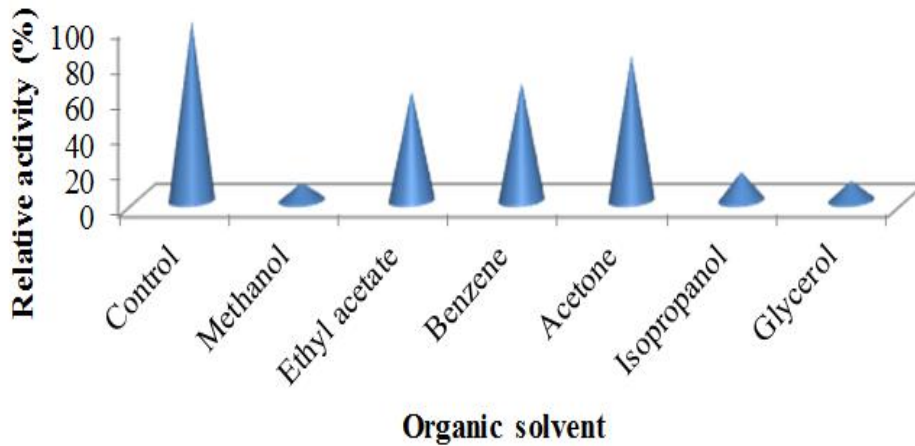
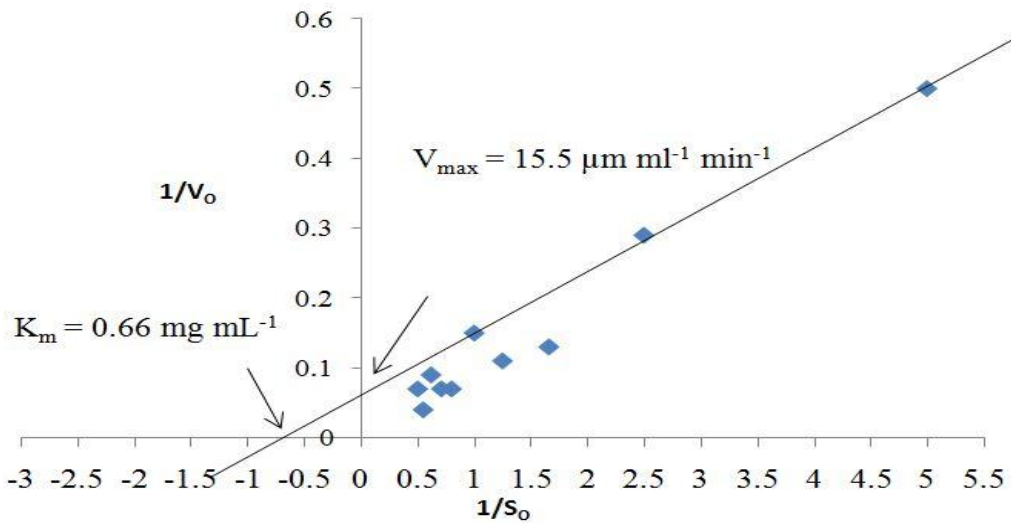


Fig.8 Lineweaver-Burk Plot showing the parameter K_m and V_{max} of alkaline protease



For test of the thermal stability, enzyme solution was kept at 60°C for 5 to 60 min. and the remaining enzyme activity was

determined. It was found that alkaline protease of *A.terreus* BAB-346 stable at 60°C (Fig.4). The half life ($T_{1/2}$) at 60°C was

20 min. On the other hand, the activity of the enzyme diminished rapidly after holding above 20 minute.

pH optimization

To determine the effect of pH on the enzyme activity, the enzyme activity was measured over a pH range of 6 to 11 (Fig. 5). The partially purified alkaline protease of *A.terreus*BAB-346 showed the maximum activity 916.0 IUmg⁻¹ at pH 8.0 coming close to those of *Bacillus thuringiensis* pH 8.0 (Chudasama *et al.*, 2010), *Pseudomonas thermaerum* pH 8.0 (Gaur *et al.*, 2010) and *Bacillus subtilis* pH 8.0 (Gitishree *et al.*, 2010) but in contrast optimal pH 10.0 and 10.5 for the Alkaline protease from *Aspergillus niger* (Kalpana Devi *et al.*, 2008) and *Vibrio metschikovii* (Durham *et al.*, 1987) respectively.

Effect of metal ions on the activity of alkaline protease enzyme

Effect of various metal ions (5 mM of chloride and sulphate salts) on the activity of alkaline protease from *A. terreus* BAB-346 is shown in (Fig.6).Of all the tested ions Hg⁺², Zn⁺², Mg⁺² and Ca⁺² inhibit the activity up to 82% , 80 % , 73 % and 55 %, respectively. The present study results are also in tune with Guar *et al.*, 2010 for Mg⁺² and Zn⁺² Metal ion using *Pseudomonas sp.* and Chudasama *et al.*, 2010 for Mg⁺² and Ca⁺² using *Bacillus sp.* Interestingly, Mn⁺² and Cu⁺² increased or stabilized the activity of the enzyme up to 122 % and 100 % respectively. Results confirming that may these cations take part in the stabilization of the protease structure and are required for protection against thermal denaturation (Paliwal *et al.*, 1994).

Effect of organic solvents on alkaline protease stability

Six organic solvent were used to study the

effect on alkaline protease activity using *A. terreus* BAB-346. As shown in figure 7 enzyme has ability to act in the present of solvent in reaction system. The enzyme retained 81%, 66%, 61% of activity in the presence of Acetone, Benzene and Isopropanol respectively whereas; enzyme lost its activity up to 90%, 89% and 84% of total activity in the presence of Methanol, Glycerol and Ethyl acetate respectively. Various investigators reported that the activity protease was affected by Glycerol (Gaur *et al.*, 2010).

Determination of kinetic parameters

Initial reaction rate was determined as the amount of casein hydrolysis (2mg) per min against various substrate concentrations (pH 8 and Temperature 60°C). The K_m value and maximal velocity for the reaction with casein was calculated form a Lineweaver-Burk plot. The K_m value was 0.66 mg ml⁻¹ which was comparable with the alkaline protease of *Aspergillus nigeras* reported by Kalpanadevi *et al.*, 2008. This work proved that alkaline protease could be produced from the agro-industrial wastes and hence poultry litter is the potent substrate for production of this enzyme.

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How to cite this article:

Gopalkumar G. Raol, Priyanka M. Patel, B.V. Raol and Rakeshkumar R. Panchal. 2016. Alkaline Protease Production by *Aspergillus terreus* BAB-346 using Poultry Litter Waste. *Int.J.Curr.Microbiol.App.Sci.* 5(10): 174-184. doi: <http://dx.doi.org/10.20546/ijcmas.2016.510.020>