

## RESEARCH ARTICLE

## Compatibility with commercial detergents and stain removal capability of *Aspergillus versicolor* protease

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### Abstract

An alkalophilic fungal strain, *Aspergillus versicolor* PF/F/107 isolated from poultry farm produced alkaline protease at 40°C and 9.0 pH. Protease showed compatibility with commercial detergents and retained 50-76% of its original activity at 40°C in the presence of detergents in the following order: Ghadi, Ujalla, Surf excel, Ariel and Tide indicating its suitability for application in detergent industry. Its efficiency as a detergent additive to remove stains like blood, grease and soil was found satisfactory. Alkaline protease when used alone was also capable to remove the stain, but its efficiency increased when mixed with detergents. The enzyme worked both on different fabrics and colors. On mixing with detergent, it was found to retain its activity up to one month.

**Keywords:** *Aspergillus versicolor*, alkaline protease, detergents, Ghadi, compatibility.

### Introduction

Alkaline proteases are used in various industrial processes viz., baking, brewing, detergent, leather processing, pharmaceutical, meat tenderization, cosmetic, peptide synthesis and medical diagnosis (Kumar *et al.*, 2002; Kumar and Bhalla, 2005; Najafi *et al.*, 2005; Tari *et al.*, 2006; Bhaskar *et al.*, 2007; Mukhtar and Haq, 2008; Dodia *et al.*, 2008; Sindhu *et al.*, 2009; Pawar *et al.*, 2009; Arasu *et al.*, 2010). Other potential industrial applications include their utilization in peptide synthesis, in the resolution of the racemic mixture of amino acids, in the hydrolysis of gelatin layers of X-ray films and in the recovery of silver (George *et al.*, 1995; Singh *et al.*, 1999; Nakiboglu *et al.*, 2001; Gupta *et al.*, 2002). The food industries are the major protease using industries. However, they have also found widespread application in laundry detergents (Maurer, 2004; Prakash *et al.*, 2005; Jaswal and Kocher, 2007).

The thermo stability and their activity at high pH and the alleviation of pollution characteristic have made proteolytic enzymes an ideal candidate for laundry applications (Gupta *et al.*, 2002; Kirk *et al.*, 2002). Alkaline proteases are supplemented in different brands of detergents for use in home and commercial establishments. Enzymes have been added to laundry detergents since last 50 years to facilitate the release of proteinaceous material in stains such as those of milk and blood. The proteinaceous dirt coagulates on the fabric in the absence of proteinases as a result of washing condition (Maurer, 2004). The enzyme removes not only the stain, such as blood, but also other materials including proteins from body secretion and food such as milk, egg, fish and meat.

An ideal detergent enzyme should be stable and active in the detergent solution and should have adequate temperature stability to be effective in a wide range of washing temperature (Bhosale *et al.*, 1995). Out of the vast pool of enzymes protease from microorganism constitute a major group which has been widely exploited in detergent industries (Kumar and Takagi, 1999). Amongst the detergent proteases producing *Bacilli*, *Bacillus licheniformis* is one the predominant sources of alkaline protease (Gupta *et al.*, 2002). However, the fungal alkaline proteases are advantageous due to the ease of downstream processing to obtain microbe free enzyme. An alkaline protease from *Conidiobolus coronatus* was found to be compatible with the commercial detergents used in India (Phadatare *et al.*, 1993). It was reported to retain 43% of its activity at 50°C for 56 min in the presence of 25 mM Ca<sup>2+</sup> and 1M glycine (Bhosale *et al.*, 1995).

Other detergent protease have been isolated from *Aspergillus niger* (Kalpana Devi *et al.*, 2008), *A. terreus* (Usama Ali, 2008), *A. flavus* (Mulimani *et al.*, 2002), *Penicillium godlewskii* (Sindhu *et al.*, 2009) etc. New interest in properties such as low temperature performance and the complexity of the patent situation has led to renewed interest in screening for novel enzyme in nature. The development of new detergent composition will also have an important impact on the development of new proteases and other types of detergent enzymes. In view of the above facts, alkaline protease produced by *Aspergillus versicolor* PF/F/107 was checked for its compatibility with commercial detergents.

## Materials and methods

**Protease production:** Medium I containing Glucose-2.0 g/L, casein-0.5 g/L, peptone-0.5 g/L, yeast extract-0.5 g/L,  $\text{KH}_2\text{PO}_4$ -1.0 g/L,  $\text{MgSO}_4$ -0.5 g/L,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -0.1 g/L was used as cultivation medium. Autoclaved Erlenmeyer flasks containing 50 mL of medium I were inoculated with 1 mL of spore suspension (containing  $2 \times 10^6$  spores/mL) of *Aspergillus versicolor* PF/F/107. The flasks were incubated at 35°C for 4 d at 150 rpm in rotary shaker. The contents were then filtered through Whatman No. 1 filter paper and the filtrate was used as a source of enzyme. The enzyme activity was determined by the method of Takami *et al.* (1989).

**Effect of pH on enzyme activity:** The effect of pH on alkaline protease was determined by incubating enzyme sample in appropriate buffers (pH 6.0-11). For this following buffers were used:

pH 6-7	-	0.1 M Sodium phosphate buffer
pH 8-9	-	0.1 M Tris-HCl buffer
pH 10-11	-	0.1 M Glycine-NaOH buffer

Alkaline protease activity was carried out at different pH using different buffers following the method of Takami *et al.* (1989). Effect of different pH on enzyme activity was recorded in terms of relative activity by considering maximum activity as 100%.

**Effect of temperature on enzyme activity:** For determination of optimum temperature for alkaline protease activity at 6 different temperatures i.e. 30, 40, 50, 60, 70 and 80°C were tested using casein as substrate. The purified enzyme sample was diluted in Glycine-NaOH buffer (pH 9). Both enzyme sample and substrate were kept for 10 min at test temperature to avoid temperature variation. Alkaline protease activity was then carried out. Effect of different temperatures on enzyme activity was recorded in terms of relative activity by considering maximum activity as 100%.

**Compatibility with various commercial laundry detergents:** In order to confirm the potential of *Aspergillus versicolor* PF/F/107 protease as a detergent additive, its compatibility and stability towards some commercial laundry detergents available in local market such as Surf excel and Wheel (Hindustan Lever Ltd., India), Tide and Ariel (Proctor and Gamble, India), Ujala (Jyoti Laboratories Ltd., India) and Ghadi detergent (Ghadi Industries Pvt. Ltd., India) were examined. Aqueous solutions of detergents (7 mg/mL to stimulate washing condition) were heated at 100°C for 60 min to denature the indigenous protease activity (if any). The detergent solution and protease enzymes were mixed in a ratio of 1:1 (v/v) incubated at 40°C for 20 min followed by measuring the residual protease activity by standard assay procedure as mentioned previously and compared with the control (Enzyme diluted to 1:1 in tap water without detergent). The relative activity was expressed as percentage activity considering the activity of control as 100%.

**Effect of incubation on stability of protease activity in presence of Ghadi detergent:** The stability of protease activity in presence of Ghadi detergent was tested by preincubating the Ghadi detergent (7 mg/mL) and enzyme (1 mL) at different time intervals (10-60 min) at 40°C. The residual activity was measured by standard assay procedures as mentioned previously. The activity of control (in the absence of detergent) was considered as 100%.

**Effect of alkaline protease on wash performance of detergents:** Crude enzyme obtained from *Aspergillus versicolor* PF/F/107 was used for the experiment. The application of protease as a detergent additive was studied on white fabric pieces (4 × 4 cm) stained with blood samples. In the present study, three different types of fabrics were used i.e., cotton, polyester and silk mix. Local Ghadi powder was used as a detergent. The fabric pieces were pre-stained with blood so as to obtain 21, 14 and 7 d old blood stains on test fabric pieces. Detergent solution having 7 mg/mL was prepared. Following sets of experiments were prepared for present study using the standard protocols by Adinarayana *et al.* (2003) and Malathu *et al.* (2008).

- Flasks having distilled water (100 mL) + stained cloth with blood (Cotton cloth piece/polyester cloth pieces/silk mix cloth piece).
- Flasks having distilled water (100 mL) + stained cloth (above mentioned cloth pieces) + 1 mL Ghadi detergent (7 mg/mL).
- Flasks having distilled water (100 mL) + stained cloth (as mentioned above) + 1 mL Ghadi detergent (7 mg/mL)+ 2 mL enzyme solution.

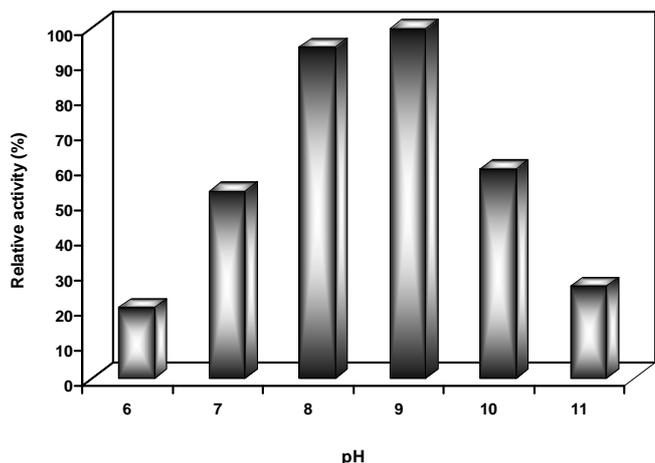
The above flasks were incubated at 50°C for 5 min in water bath and after incubation cloth pieces were taken out, rinsed with water and dried. Performance of enzyme addition in detergents was then visually observed. Untreated cloth pieces stained with blood were taken as control. After protein purification, the partially purified enzyme was also used to exhibit its effect on stain removal by following the above procedure.

## Results and discussion

**Effect of pH on enzyme activity:** The effect of pH on alkaline protease activity of test fungi is expressed in terms of % relative activity (Fig. 1) at different pHs. Alkaline protease activity was observed at pH 6 to pH 11. The enzyme showed optimum activity at pH 9 and found to retain residual activity at pH 8. At pH 10, the activity reduced significantly and only 59.95% residual activity was noted at this pH, which was further reduced at pH 11 showing only 26.31% residual activity. At neutral pH 7, the enzyme retained 53.44% activity. Higher activity of the test enzyme from *Aspergillus versicolor* PF/F/107 indicates its possible use in detergent formulations as a detergent additive.

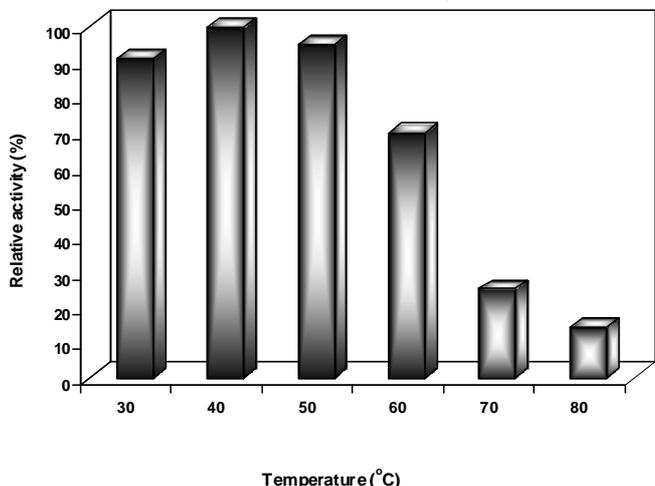
Alkaline protease from *Microbacterium luteolum* showed maximum activity at pH 7.5 with 71% residual activity at pH 10 and pH 10.5 (Malathu *et al.*, 2008). Abbas *et al.* (1989) showed that protease enzyme complex produced by *Penicillium Charlesii* degraded casein optimally are pH 7-9. The optimum activity of protease of *Aspergillus oryzae* was found to be at pH 8-9 (Samarntarn *et al.*, 1999).

Fig. 1. Effect of different pHs on alkaline protease activity.



**Effect of temperature on enzyme activity:** Alkaline protease activity in precipitated enzyme was assayed over a range of temperature i.e., 30-80°C and the optimum alkaline protease activity (i.e., 206.15 U/mL) was observed at 40°C. Figure 2 indicates that enzyme was active in broad range of temperatures i.e., 30-80°C. At 30°C, the activity of this enzyme was found to be 187.98 U/mL that increased with increase in temperature up to 50°C. At 60°C, the activity of alkaline protease was noted only 144.16 U/mL, this indicates the retention of 69.93% of the enzyme activity at this temperature. The enzyme had temperature optima at 40°C while it showed more than 91.19% activity at 30°C.

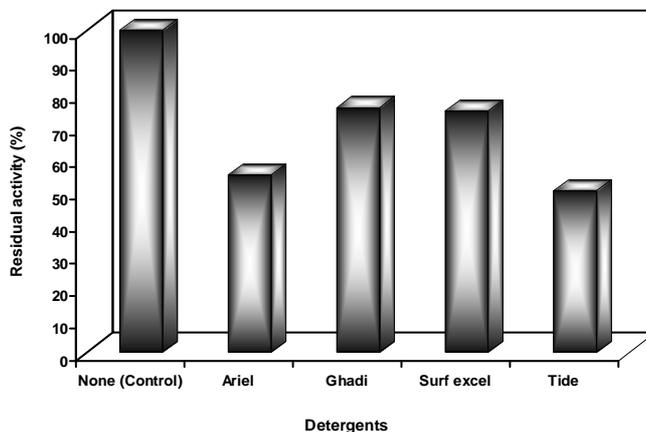
Fig. 2. Effect of different temperatures on alkaline protease activity.



A little more i.e., 95.05% of its maximum activity was noted at 50°C. Stability towards high temperature is very much desirable in some of the industrial enzymes. The enzyme was less active above 70°C (Fig. 2). Temperature optima of 35°C have been reported for protease from *Aspergillus nidulans* (Charles *et al.*, 2008). Similar results were recorded with proteases produced by *Aspergillus terreus* and *A. fumigatus* (Chakrabarti *et al.*, 2000; Wang *et al.*, 2005). Kalpana Devi *et al.* (2008) reported maximum alkaline protease activity at 45°C for *Aspergillus niger*. Protease of *Aspergillus versicolor* PF/F/107 also showed remarkably good activity at 50°C with optimum at 40°C thus indicates its thermal tolerance and application in those processes operating at high temperature.

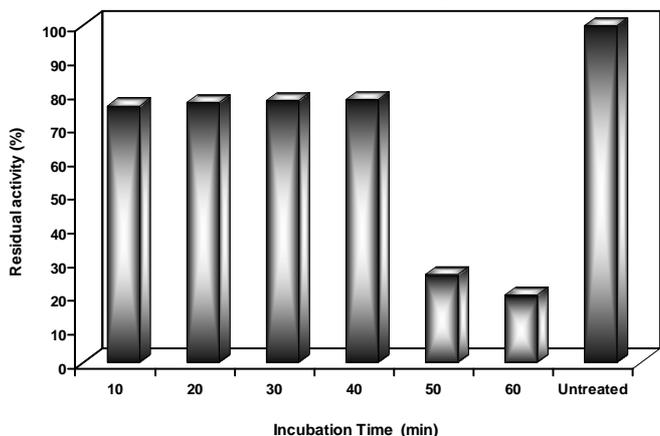
**Compatibility of protease with commercial laundry detergents:** Protease from *Aspergillus versicolor* PF/F/107 exhibited a significant stability and compatibility with all the tested commercial laundry detergents (Fig. 3). The enzyme retained 50-76% of its original activity at 40°C in the presence of detergents in the following order: Ghadi® > Ujalla® > Surf excel® > Ariel® > Tide®. Stability of protease for laundry detergents were reported by Venugopal and Saramma (2006) for alkaline protease produced by *Vibrio fluvialis* strain VM10. Phadatare *et al.* (1993) reported high activity of protease from *Conidiobolus coronatus* and showed its stability at 50°C in the presence of 25 mM CaCl<sub>2</sub> with variety of commercial detergents.

Fig. 3. Effect of detergents on alkaline protease activity.



**Stability of protease activity in presence of Ghadi detergent:** Stability of protease activity in Ghadi detergent formulation was tested for 10-60 min duration. The enzyme was found stable throughout the testing period but the residual activity was found to decrease with increase in time duration. The enzyme was found to retain more than 70% residual activity up to 40 min which was reduced to only 26% and 20% (Fig. 4).

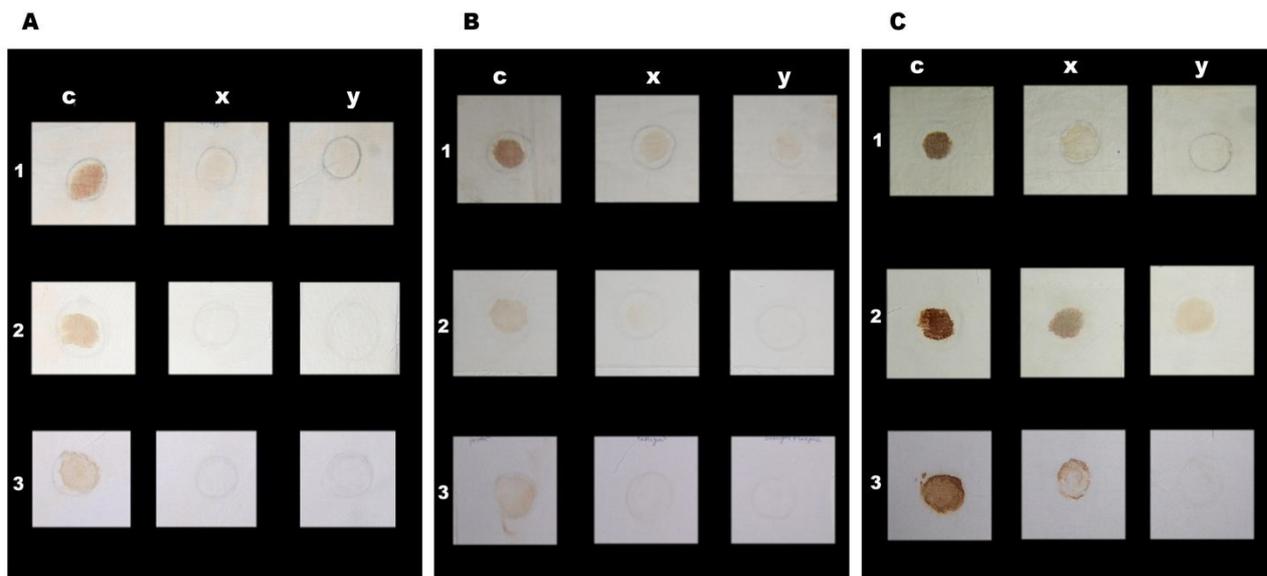
Fig. 4. Stability of alkaline protease activity in presence of Ghadi detergent.



**Effect of alkaline protease on stain removal:** The ability of proteases to remove proteinaceous stains can be measured either by performing wash test (Durham, 1987) or by testing their ability to act on insoluble substrates under conditions mimicking the wash conditions (Safarik and Safarikova, 1994). The compatibility of alkaline protease was studied with Ghadi detergent for a period of 60 min at 40°C (Fig. 4). The enzyme retained about 78% activity after 40 min in the presence of Ghadi detergent at 40°C and was found to be active up to 1 h (Fig. 4). As the protease produced by fungal isolate *Aspergillus versicolor* PF/F/107 was stable over a wide range of pH value and temperatures and also showed compatibility with various commercial detergents tested it was used as an additive in detergent, to check the contribution of the enzyme in improving the washing performance of the detergent. The efficiency of alkaline protease produced from *Aspergillus versicolor* PF/F/107 was tested for removing blood stains of 7 d, 14 d and 21 d old from test fabrics viz., cotton and silk mixed and polyester fabric. It took 5 min to completely remove the blood stains at 50°C (Fig. 5). It was clearly seen that as the enzyme could work efficiently on different fabrics (Silk fabric > polyester fabric > cotton fabric). The alkaline protease was capable of cleaning stains but its mixture with detergent proved more effective. On mixing with the detergent it was found to retain activity up to 14 d but there after its efficiency of washing decreased by 21 d. Alkaline proteases have been incorporated in the detergents to hydrolyze and remove proteinaceous stains on clothes (Kobayashi *et al.*, 1995; Ito *et al.*, 1998).

The suitability of any protease for inclusion in detergent formulation is dependent on its stability and compatibility with detergent components (Kumar and Takagi, 1999; Venugopal and Saramma, 2006). Besides, the enzyme should be alkaline in nature and thermostable. However, the stability and compatibility of any component should not be the only prerequisite for its inclusion in detergent formulation. Krik *et al.* (2002) emphasized that the detergent protease must be active at room temperature in order to save energy required in heating of water. The protease secreted by *Aspergillus versicolor* PF/F/107 found to retain its stability at high temperature and at alkaline pH range, thus the enzyme can be used as additive in formulation of laundry detergents.

Fig. 5. Effect of alkaline protease on stain removal.



A: 7 d old blood stain; B: 14 d old blood stain; C: 21 d old blood stain  
 Row: 1. Cotton cloth; 2. Silk mixed cloth; 3. Polyester fabric  
 Column c: Control (without detergent and enzyme); x: Treated with detergent solution (7 mg/mL);  
 y: Treated with detergent solution (7 mg/mL) + 1 mL of crude enzyme sample.

The efficiency of alkaline proteases in removing stains has been proved with different washing conditions and detergent compositions (Greene *et al.*, 1996; Kalisz, 1998; Banerjee *et al.*, 1999).

## Conclusion

The alkaline protease isolated from *Aspergillus versicolor* PF/F/107 is thermostable and it is stable at alkaline pH, at high temperature and in the presence of commercial detergents and is compatible with commercial and local detergents. These properties indicate the possibilities for use of the enzyme in detergent and can be exploited commercially in near future.

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