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Full Length Research Paper

Cold-Active Alkaline Protease from Stenotrophomonas maltophilia: Optimization and Detergent Industry Applications

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Biodetergents are preferred over the conventional synthetic detergents in view of their better cleaning properties, low energy input and the alleviation of pollution. The biodetergents derived from mesophilic/thermophilic organisms and also peroxide-based synthetic detergents require high temperature for their optimum activity. Thus, cold-active enzymes are very useful as they work at lower temperatures and do not require the input of energy. The purpose of the present study was the production optimization and purification of cold-active alkaline protease from a novel psychro-tolerant *Stenotrophomonas maltophilia* MTCC 7528 and its application as a detergents additive for cold washing. Psychro-tolerant proteolytic bacterium *S. maltophilia* MTCC 7528 was isolated from soil of Gangotri glacier, Western Himalaya, India that produced maximum protease (56.2 U/ml) at 20°C and pH 9.0 after 120 h incubation in shaking condition (120 rev/min). The purified enzyme has molecular weight of 75 kDa with maximum activity and stability at pH 10 and 20°C temperature. It showed excellent compatibility with commercial detergents with improved cleansing power at low temperature. The enzyme completely removed blood and grass stains and increases the reflectance by 26 and 23%, respectively. Enzyme-based detergents find a wide range of applications in laundry and textile industries. Cold- active alkaline protease from psychro-tolerant *S. maltophilia* may be a potential component to be used as a detergent additive for cold washing that will be beneficial to save energy as they work at lower temperatures.

Key words: Alkaline protease, biodetergent, cold-active enzymes, wash performance.

INTRODUCTION

Biodetergents, also known as green chemicals, account for about 30% of the total worldwide enzyme production. Protease is used in detergents to remove protein-based stains by hydrolyzing them into small peptides which are readily dispersed in the washing liquor. Presently, it has been recognized that cold-adapted microorganisms provide a wide biotechnological potential and offering numerous economic and ecological advantages over mesophilic and thermophilic organisms and their enzymes (Gounot, 1991; Margesin and Schinner, 1994; Brenchley, 1996; Gerday et al., 2000; Soriano et al., 2000; Margesin et al., 2002; Soror et al., 2007). Cold-active enzymes had high catalytic efficiency at low temperatures and have been applied in biotechnology only quite recently as compared to their mesophilic and thermophilic counterparts (Margesin et al., 2005). The application of such enzymes enables lowering of the temperature and shortening of processing times without a loss of efficiency, which leads to saving time and energy consumption. Therefore, it is desirable to search new source of cold-active enzymes with novel properties from as many sources as possible. While there have been numerous reports on cold-active enzymes produced by

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microorganisms (Hamamoto et al., 1994; Hoshino et al., 1997; Kun-Hee et al., 1999; Hoffmann and Decho, 2000; Huston et al., 2000; Irwin et al., 2001; Zeng et al., 2003), very limited information has been published on microbes of Gangotri glacier regions of Western Himalaya that may be an ideal habitat for cold-adapted organisms and their enzymes. In this paper, we reported the production optimization, purification and applications of cold-active alkaline protease from *Stenotrophomonas maltophilia* MTCC 7528, a novel psychro-tolerant bacterium isolated from soil of Gangotri glacier.

MATERIALS AND METHODS

Microbial species and cultivation

A psychro-tolerant bacterium *S. maltophilia* MTCC 7528 was obtained from microbial type culture collection (MTCC), Chandigarh, India that produced an extracellular cold- active alkaline protease. The strain was grown on skim milk agar contained (g/l): skim milk powder, 100; peptone, 5 and agar, 15; and stored at 4°C (Baghel et al., 2005).

Protease production and enzyme assay

Production of protease was carried out in a medium containing (g/l): glucose, 10; peptone, 5; yeast extract, 5; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.2. The pH of autoclaved broth was adjusted (pH 7) by adding sterilized Na_2CO_3 solution (20% w/v). The media was inoculated at 1.0% (v/v) with 24 h old culture (OD 0.6) and incubated at 15±2°C in a refrigerated incubator shaker (100 rpm) for 48 h. The growth cultures were centrifuged at 4°C (10000×g, 15 min) and the supernatant was used for protease assay. Activity of protease with azocasein (Sigma) as a substrate was assayed by the modified method of Secades and Guijarro (1999). One unit of enzyme activity was defined as the amount which yielded an increase in A_{420} of 0.01 in 30 min at 20°C. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard protein.

Optimization of fermentation conditions

The methods adopted to optimize growth parameters for cold-active protease production aimed to evaluate the effect of a single parameter at a time and later manifesting it as standard condition. Incubation time ranging from 24-168 h and effects of various inducers (BSA, casein, skim milk, egg albumin at the rate of 0.5-1% in the media) were evaluated in elation to enzyme yield. The culture conditions like temperature (4-45ºC), pH (5-10) and mode of incubation static and shake conditions (120 rpm) were optimized. To evaluate the impact of heavy metals, broth media was supplemented with maximal tolerance level of different metals and incubated under optimal condition for 48 h. The heavy metals used were Co^{2+} , Hg^{2+}, Cd^{2+}, Cu^{2+} , Zn^{2+} and Cr^{2+} at the final concentration of 50, 10, 25, 100, 50 and 150 g/ml, respectively. The enzymes produced by bacterial cells were represented in terms of units of protease activity in the culture supernatant and measured as per standard method (Secades and Guijarro, 1999). The experiments were conducted in triplicate and the results are average of three independent findings.

Extraction and purification of protease

The fermented broth, incubated at optimized conditions, was centrifuged at 4°C (10000×g, 15 min) and the extracellular protease in cell free culture supernatant was obtained by ammonium sulfate precipitation (Deutscher, 1990). The protein precipitate was dissolved in 0.05 M sodium acetate buffer (pH 5) and dialyzed against the same buffer for 24 h with 6-8 changes. The fraction with maximum enzyme activity was applied to DEAE-Cellulose column (3×12 cm, Bangalore Genei, India), pre-equilibrated with 0.02 M sodium acetate buffer (pH 5), and eluted with ten linear gradient of NaCl (0.1-1 M; 20 ml each) in the same buffer. Total 40 fractions, 5 ml each, were collected and assayed for protein and enzyme activity. All subsequent steps were carried out at 4°C. The fraction with high protease activity was concentrated by lyophilization (Lyophilizer MSW137, Macro Scientific Works, India).

Biochemical characterization of purified protease

Effect of pH and temperature on protease activity and stability

The optimum pH for enzyme activity was determined by using following buffers: citrate phosphate (pH 5-6), sodium phosphate (pH 7), Tris-HCl (pH 8) and glycine-NaOH (pH 9-11). Reaction mixtures were incubated at 20±2°C for 30 min and the respective activity was measured. The stability of protease was determined by pre-incubating the enzyme without substrate at different pH values (5-11) for 1 h at 20±2°C. The residual enzyme activity at each exposure was measured as per standard assay. The effect of temperature was determined by performing the standard assay procedure within a temperature range from 4-50°C. To determine the protease stability with changes in temperature, the enzyme was pre-incubated at different temperatures for 3 h and enzyme activity was assayed as per standard protocol.

SDS-PAGE and zymogram

The SDS-PAGE of lyophilized fraction was performed, using a mini slab gel apparatus (Bangalore Genei, India), by the method of Laemmli (1971). The protein bands were visualized by staining the gel with coomassie blue and the relative molecular mass of the protein was calculated using standard protein markers (Bangalore Genei, India) run simultaneously. The gel was analyzed with a Gel Documentation system (GeneLine, Spectronics Corporation, New York). Gelatin zymography was performed in polyacrylamide slab gel containing SDS and gelatin (0.1%) as a co-polymerized substrate. The activity band was observed as a clear colorless area depleted of gelatin in the gel against the blue background.

Enzyme compatibility and wash performance analysis of alkaline protease for cold washing

Cold-active protease (crude) as a detergent additive was studied by using method of Beg and Gupta (2003). The enzyme compatibility with commercially available laundry detergents was studied. The detergents solution (1% w/v) was boiled for 10 min to destroy any protease already present (enzyme assay was performed to check the activity). The detergents solution was incubated with fixed protease concentration for different time intervals (0.5-3 h) at 20ºC and the residual activity was determined in comparison to control (without any detergent). The used detergents were Ariel and Tide (Procter and Gamble, India), Surf Excel and Wheel (Hindustan Lever Ltd., India) and Nirma (Nirma Chemical, India). Also, the wash performance analysis of crude enzyme was studied on white cotton cloth pieces (5 x 5 cm) soiled with chicken blood and grass

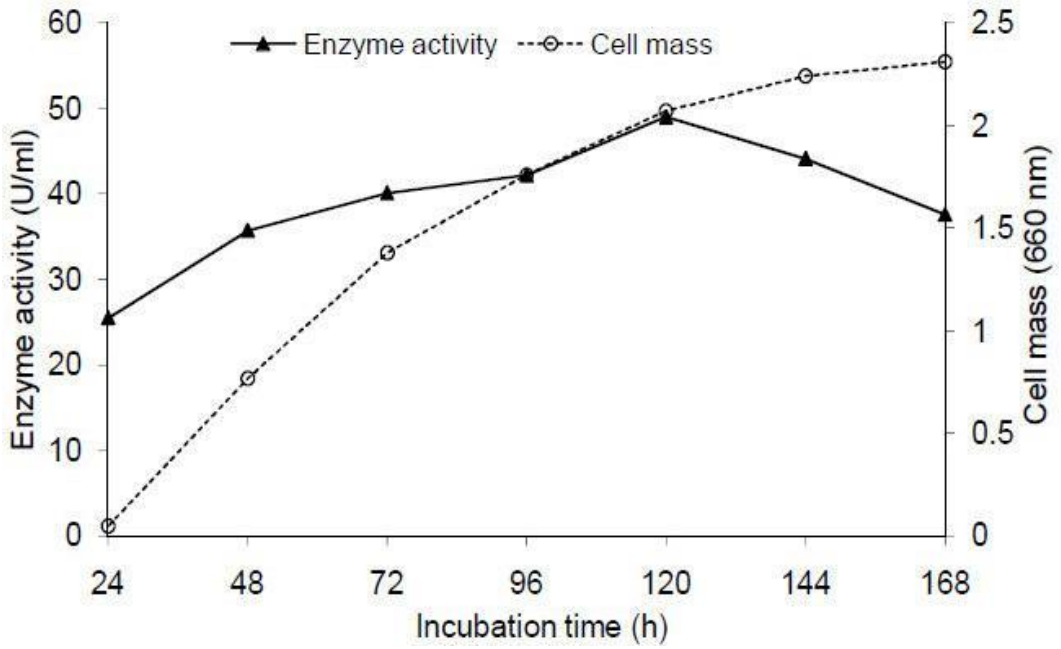

Figure 1. Effect of incubation time on growth and protease production (15±2°C).

sap. Concentrated grass sap was obtained by macerating fresh grass (100 g in 100 ml distilled water) in a mortar and pestle and filtered by using cheesecloth. The soiled cloth pieces were allowed to sit overnight and taken in separate flasks. The following sets were prepared:

a) Flask containing distilled water (100 ml) + soiled cloth (with blood and grass sap, independently).
b) Flask containing distilled water (98 ml) + soiled cloth + 2 ml wheel detergent (1% w/v).
c) Flask containing distilled water (96 ml) + soiled cloth + 2 ml wheel detergent (1% w/v) + 2 ml crude enzyme.

The flasks were incubated for 30 min (20ºC) and rinsed with cold water then air dried. The wash performance was analyzed by using digital reflectance meter (Photo Electric Instruments, India). The relative reflectance of cloth pieces were examined to test enzyme efficiency of stains removal. Untreated cloth pieces soiled with blood and grass sap were taken as control (Beg and Gupta, 2003; Adinarayana et al., 2003).

RESULTS AND DISCUSSION

Optimization of enzyme production

Effect of incubation time

The growth pattern and enzyme production was observed for 168 h in protease production media at 15±2ºC (pH 7). The protease production increases gradually and it was maximum (49 U/ml) at 120 h incubation. The enzyme production was growth independent (Figure 1). Similar to many proteolytic enzymes, it is also secreted largely at the late exponential growth phase (Dube et al., 2001; Kuddus and Ramteke, 2008).

Effect of incubation temperature and pH of media

Temperature has profound influence on production of microbial enzymes. The maximum protease production (56.2 U/ml) was obtained at 20ºC and totally inhibited at 45°C. The results indicated that high quantity of enzyme can be produced in between the temperature range of 15 to 25°C (Figure 2a). Therefore, it may be applicable for industrial purposes having small temperature variation in process. The pH of culture strongly affects enzymatic processes and transport of compounds across the cell membrane. The maximum enzyme production was achieved at pH 9 (62.2 U/ml) at 120 h incubation and 20°C (Figure 2b). Thangam and Rajkumar (2002) also reported protease production in alkaline medium by *Alcaligenes faecalis* but it was within a narrow pH range.

Effect of agitation and different substrates

The enzyme production was increased two fold from 18.2 to 45.7 Um/l in shaking condition as compared to unruffled condition. The use of cheap carbon and nitrogen sources are important as these can significantly reduce the cost of enzyme production. The isolate utilized casein for maximum enzyme production (52.4 U/ml) followed by skim milk (46.6 U/ml), BSA (37.6 U/ml) and egg albumin (26.3 U/ml). However, skim milk is the best substrate for cold-adapted enzyme production from

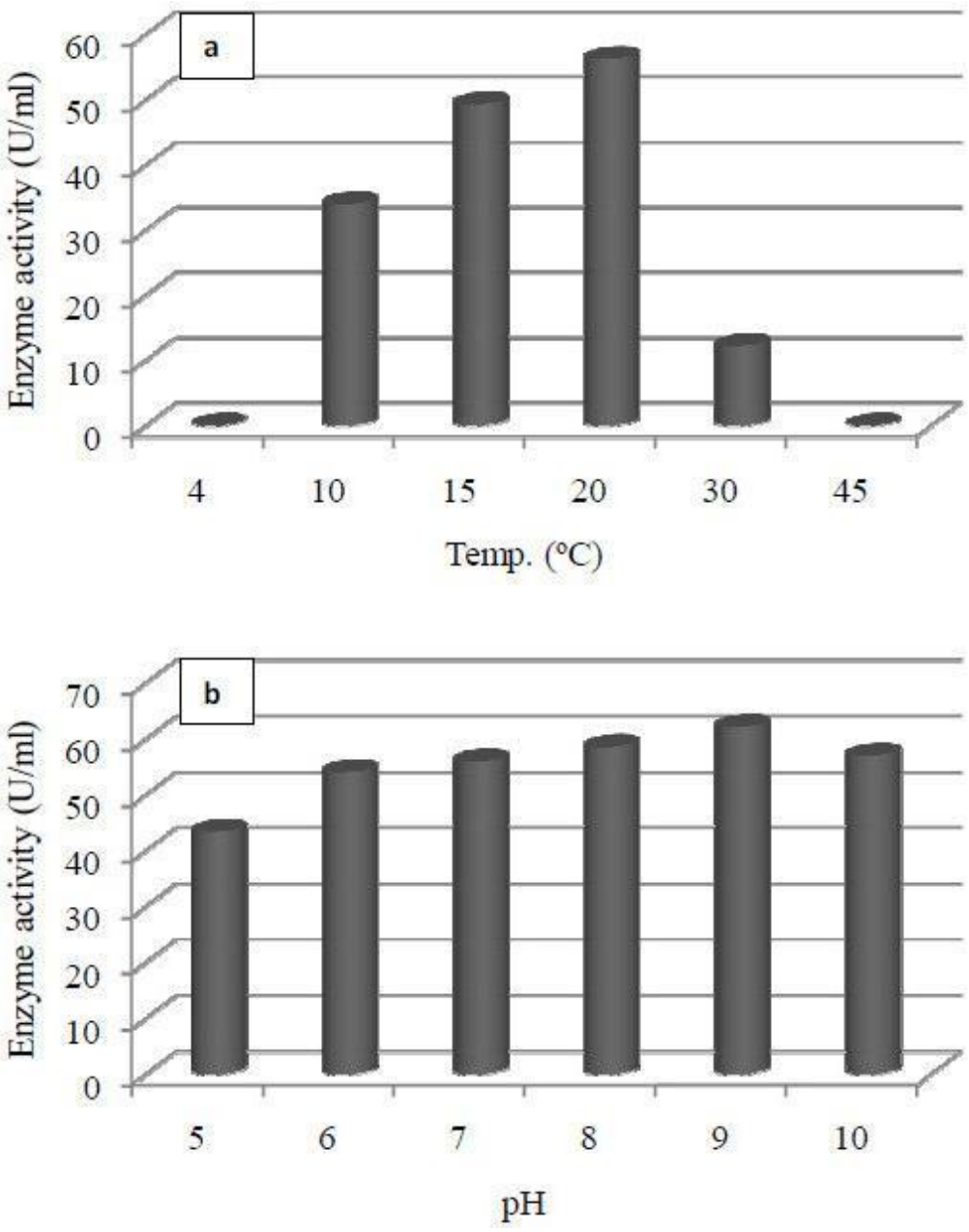

Figure 2. Effect of temperature (pH 7) (a), and pH (at 20ºC) (b) on the production of protease (120 h incubation).

different isolates of Antarctica is reported by Dube and coworkers (2001).

Effect of metal ions

Heavy metals present in surroundings play an important role in the growth of bacteria. The enzyme production was enhanced by Cu^{2+} (126.8%) and Cr^{2+} (134.6%) while Co^{2+} (43.5%) worse production. The other heavy metals such as Hg^{2+}, Cd^{2+} and Zn^{2+} have no significant effect but maintain more than 50% of enzyme production.

Purification and characterization of protease

The enzyme was partially purified by single step ion-exchange chromatography. The protein pellet obtained after 80% saturation of ammonium sulphate was dissolved in sodium acetate buffer and loaded onto a DEAE-cellulose column pre-equilibrated with same buffer. The elution profile of DEAE-cellulose column

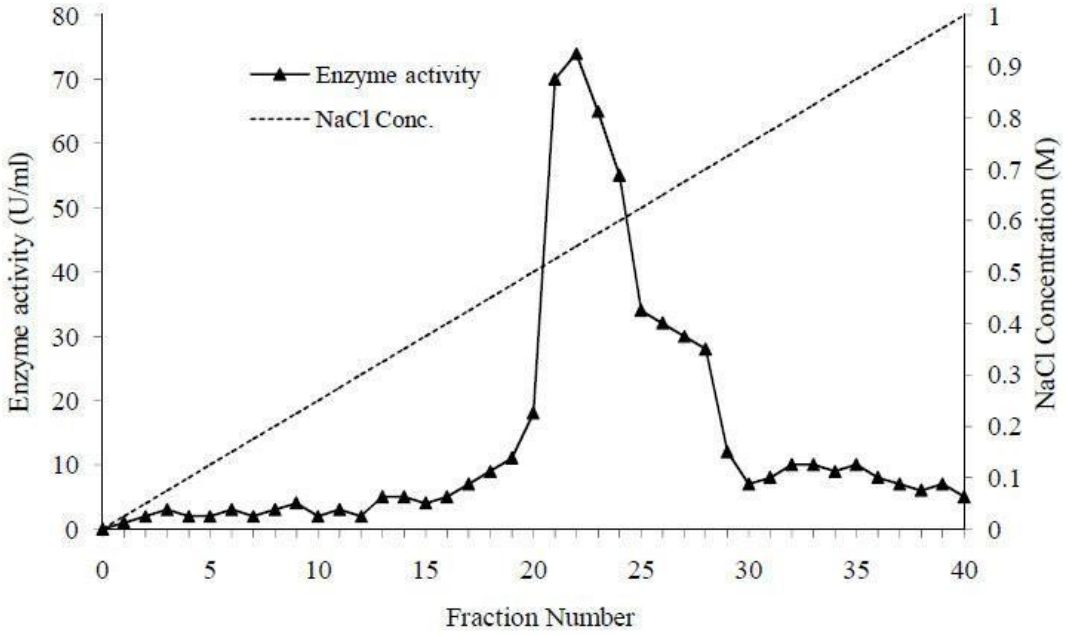

Figure 3. DEAE-Cellulose ion-exchange chromatography of ammonium sulfate-precipitated *S. maltophilia* culture supernatant. Dialyzed precipitate was applied to a column (3x12 cm) equilibrated in sodium acetate buffer (0.02 M, pH 5). Elution was accomplished with 0.1 to 1 M NaCl gradient. The material removed from the column (0.6 M fraction) was concentrated for further application.

chromatography exposed that the protease was eluted as a well-resolved single peak of enzyme activity at 0.6 M NaCl concentration (Figure 3). Approximately 55- fold purification of the crude enzyme was achieved with the specific activity of 20964 U/mg and migrated as a single band in SDS-PAGE suggesting that the purified protease was homogenous.

Effect of pH and temperature on protease activity and stability

The enzyme was active over pH range of 7 to 11 with maximum activity at pH 10 towards azocasein. The activity increased almost linearly from pH 6 to 10, and 84% of the total enzyme activity was manifested between pH 8 and 11 (Figure 4a). The results recommended that the enzyme is alkaline protease. It was relatively stable between pH ranges of 8 to 10 and retained more than 93% of its original activity (Figure 4a). Similar result is also obtained by Margesin et al. (2005) for purified protease from *Pedobacter cryoconitis*. The effect of the temperature on protease activity is shown in Figure 4b. The optimum temperature was 20ºC retaining 100% of enzyme activity and thus can be classified as cold-tolerant protease (Morita, 1975). Protease activity was increased from 4 to 20ºC after that it was declined. The activity of enzyme was not affected after repeated freezing and thawing. The enzyme was stable at 20ºC along with 85% activity between 4 to 20°C (Figure 4b).

Proteases with these characteristics and their producing organisms may have interesting applications in biotechnological processes.

Molecular mass of the protease

The molecular mass of the enzyme was determined on the basis of its mobility relative to the protein standards on SDS-PAGE by using gel documentation system. By interpolation, the molecular mass of single polypeptide chain was 75 kDa. The proteolytic activity of the protein band was confirmed by zymogram analysis. Similar molecular masses have also been reported for other alkaline proteases previously characterized (Thangam and Rajkumar, 2002).

Stability of protease with detergent and wash performance analysis at low temperature

In addition to pH, a good detergent protease is expected to be stable in the presence of commercial detergents at low temperature for cold washing. Purified protease from *S. maltophilia* exposed tremendous stability and compatibility with a wide range of locally available commercial detergents at 20°C (Figure 5a). Similar results are also obtained by other workers from different strains of *Bacillus* sp. but at 60°C (Adinarayana et al., 2003; Beg and Gupta, 2003). It was most stable with

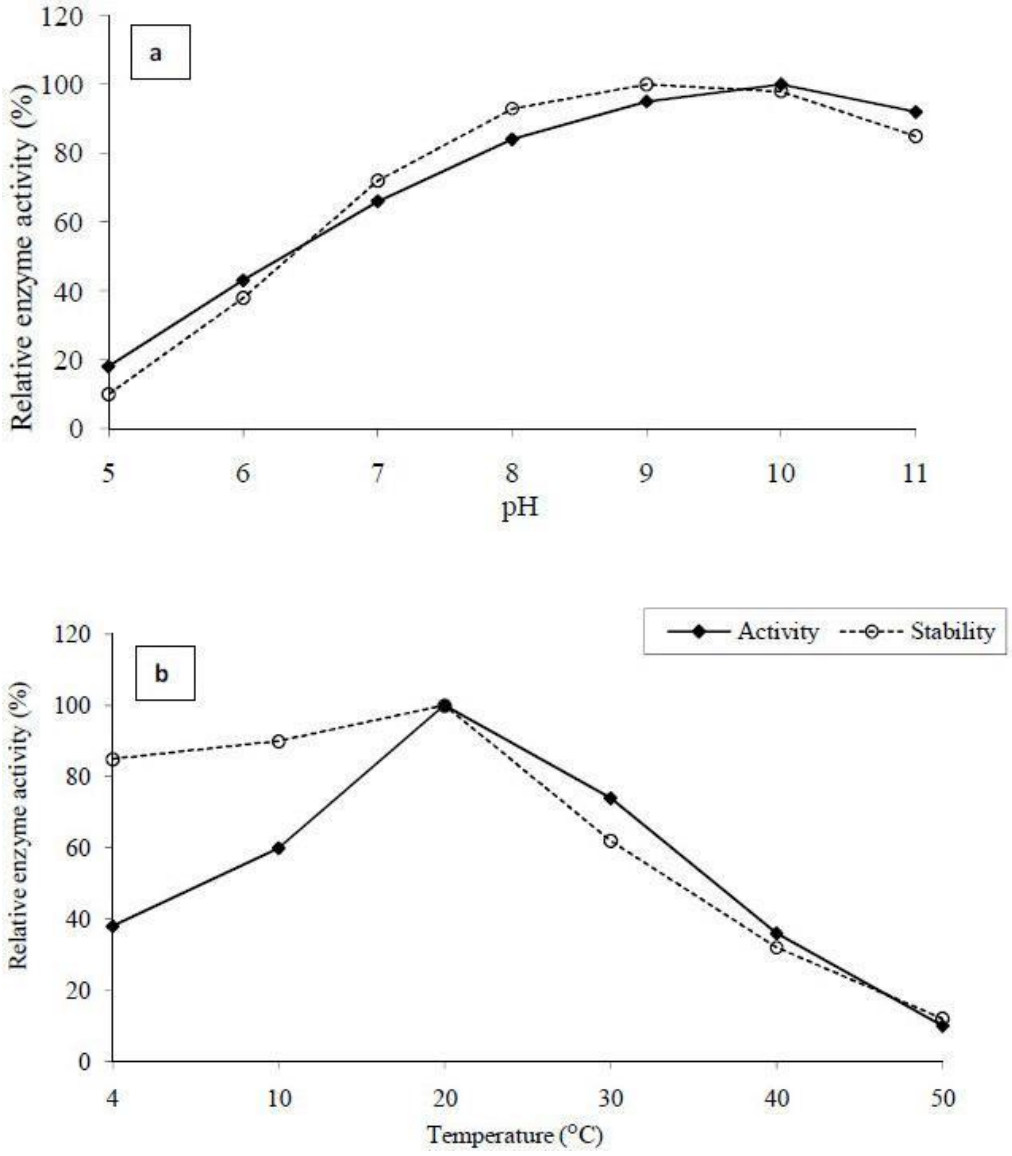

Figure 4. Influence of pH (a), and temperature (b) on the activity ( ) and stability ( ) of the purified protease. The relative activity was defined as the percentage of activity detected with respect to the maximum protease activity.

'Wheel detergent' retaining 92 and 87% activity after 0.5 and 1.0 h incubation (Figure 5a). However; more than 65% activity was maintained with all the tested detergents even after 3 h. In comparison to preceding results, it is evident from Figure 5b that alkaline protease from *S. maltophilia* exhibited high efficiency for the removal of blood and grass stains at 20ºC and increases the reflectance by 25.7 and 23.2% for blood and grass stains, respectively (Figure 5b). Therefore, it may be recommended that the supplementation of the cold-active alkaline protease isolated from *S. maltophilia* MTCC 7528 could significantly perk up the cleansing of the proteinaceous stains resulting in complete stains removal at low temperature.

Conclusion

The high activity of enzymes at low and moderate temperatures offers potential economic benefits (Gerday et al., 2000) . The ability of microorganisms to grow over wide range of temperature makes them attractive for industrial and biotechnological applications (Kuddus and Ramteke, 2009). The present microbial analysis concluded that the alkaline protease isolated from *S. maltophilia* MTCC 7528 is a cold-active protease that is stable at alkaline pH and with commercial detergents. These properties indicate the possibilities of this protease as a detergent additive for cold washing to improve the cleansing of the proteinaceous stains. Washing with

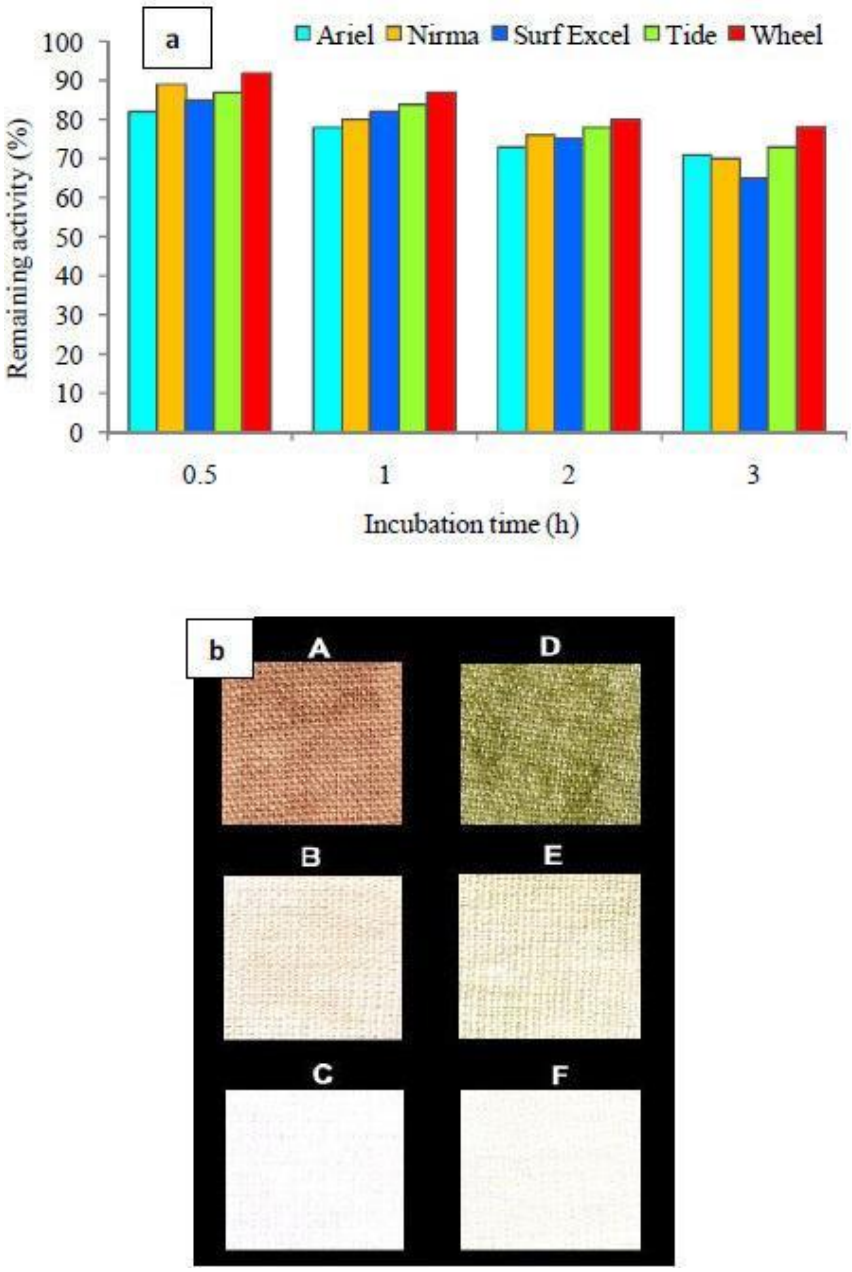

Figure 5. (a) Stability and compatibility of alkaline protease of *S. maltophilia* in the presence of commercial detergents at pH 10 and 20ºC temperature. (b) Washing performance of alkaline protease from *S. maltophilia* in combination with commercial detergent (Wheel) at 20ºC. Where, (A) Cloth stained with blood, (B) Blood-stained cloth washed with detergent only, (C) Blood- stained cloth washed with detergent and enzyme, (D) Cloth stained with grass sap, (E) Grass sap stained cloth washed with detergent only, (F) Grass sap stained cloth washed with detergent and enzyme.

detergents also requires a lot of energy particularly when done at high temperature because peroxide-based bleaches need higher temperature to work properly. Thus, lowering the wash temperature by using cold-active protease can save lots of energy. The organism can also find applications in environmental bioremediation of proteinaceous waste in cold regions.

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