RESEARCH PAPER | Biotechnology | Volume : 3 | Issue : 9 | Sept 2013 | ISSN - 2249-555X

Studies on Applicability of Alkaline Protease from Halophilic Bacteria in Detergent Formulations

KEYWORDS: Alkaline proteases, Bacillus, Bleaching agent, Detergents, Surfactants, Thermostable.

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ABSTRACT *Alkaline proteases are one of the major groups of industrial hydrolytic enzymes used in detergents formulations to clean various proteinaceous stains like blood, egg, milk, vegetable gravy as well as proteins from body secretions. The compatibility of crude alkaline proteases produced from halophilic Bacillus sp. have been evaluated in presence of different commercial detergents (Ariel, Tide, Wheel, Surf Excel and Ujala techno-bright etc.), surfactants, bleaching agents etc. at 50 oC and 60 oC respectively and it was found that the enzyme is stable in detergents (87-105% of residual activity), surfactants like Triton X-100 and Tween-80 (up to 1%), cetyltrimethylammonium bromide (5 mM) and bleaching agent sodium hypochlorite (0.2%), while 47% of residual activity was observed in presence of 0.5% sodium dodecyl sulfate. The maximum alkaline protease activity was observed at 50 oC and at a broad pH range of 8.0 to 10.0.*

1. Introduction

Alkaline proteases are one of the most important groups of hydrolytic enzymes that find varied uses in various industrial sectors such as leather, detergents, textile, food and feed etc. and constitute about 25% of the total enzyme market (Mrudula and Shyam, 2012; Singhal et al., 2012). Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases (Banerjee et al., 1999). This is because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Najafi et al., 2005). The Bacilli provides 70% of proteases hence the diverse sources has made these organisms the focus of attention in biotechnology. Till date, however, few thermophilic Bacillus sp. that produce proteases have been isolated, the earliest isolate being Bacillus stereothermophillus which is stable at 60 oC (Rajasekhar et al., 2011). Proteases can be classified according to their active pH range into neutral, acidic and alkaline proteases respectively. Alkaline proteases are those enzymes that are active at alkaline pH with optimum pH in between 9-11. Alkaline proteases are mainly used as cleansing additives in many ways (Tekin et al., 2012; Cheng et al., 2010). The major application of alkaline proteases is in detergent industry, because the pH in laundry detergent is generally in range of 9-12 (Kamoun et al., 2008; Kumar et al., 2008). Because of their eco-friendly nature and replacing phosphate detergent in laundry, they are termed as 'GREEN CHEMICALS' (Kumar et al., 1998). To meet the demand of rapidly growing detergent industry, there is constant search for better performing enzymes as well as good producer strains.

Since, halophilic proteases are adapted to extreme environments, they are usually stable and therefore they could serve as a suitable candidate for industrial process that are performed under harsh conditions (Sehar and Hameed, 2010). The thermostability, pH stability, activity and stability in presence of surfactants, oxidative and bleaching agents etc. are the major characteristics of proteolytic enzymes used in detergent applications (Anand et al., 2010). In this communication, the various characteristics of alkaline protease secreted from a halophilic Bacillus sp. has been studied for the applications in detergent industry.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Different halophilic alkaline protease producing bacteria were isolated from Sambhar Lake Rajasthan in presence of varied concentrations of sodium chloride using casein rich medium. The pure colonies were maintained at 4°C on 12% MGM-casein plate. The composition of alkaline protease production medium consisted of (g L^{-1}): $MgSO_4.7H_2O$, 14; $MgCl_2.6H_2O$, 12; NaCl, 120; KCl, 2.8; $CaCl_2$, 0.55; Yeast extract, 1; Casein, 5. The pH of the medium was adjusted to 8.0 before autoclaving. The different flasks were inoculated from a freshly grown plate and incubated at 37 °C for 72 h in an orbital shaker set at 150 rpm. The production broth was centrifuged at 10,000 rpm, 4 °C for 10 min to obtain the cell free supernatant which was used as enzyme source to measure the enzyme activity (Makhija et al., 2006).

2.2 Protease activity determination

1 ml of 10 g /L casein in 0.1 M Tris buffer (pH 9.0) was used as substrate to which 1 mL of suitably diluted enzyme supernatant was added and incubated at 50 °C for 30 min. The reaction was stopped by the addition of 0.5 mL of 10 % trichloro-acetic acid. The enzyme substrate reaction mixture was finally centrifuged at 10,000 rpm for 10 minute. The tyrosine released during the hydrolytic action of alkaline protease was measured by Lowry method (Lowry et al., 1951) against control where tricholoroacetic acid was added before the incubation. One unit of alkaline protease activity (U) is defined as the amount of the enzyme which produces 1 µg tyrosine / mL under specific conditions of assay.

2.3 Compatibility of alkaline proteases in presence of various commercial detergents

Compatibility of alkaline proteases produced from the bacteria in presence of various commercial detergents was evaluated at a final concentration of 7 mg mL^{-1}. The detergents used in the study were Tide, Ujala Techno Bright, Wheel, Arial, and Surf Excel respectively. The enzyme present in the detergents was deactivated by heating at 100 °C for 30 min before the detergents were added to the enzyme substrate reaction mixture. The protease activity was determined at 50 °C and compared with the control sample incubated at 50 °C without any detergent (Nascimento and Martins, 2006). In other studies, the detergents

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were directly (un- boiled) added to the enzyme substrate reaction mixture at a final concentration of 7 mg mL^{-1} and activity was performed at optimal conditions. All the assays were performed in triplicate and mean values were recorded.

2.4 Effect of calcium chloride on alkaline protease activity
The effect of calcium chloride ($CaCl_2$) on the enzyme activity was studied to observe increase or decrease in activity and stability of alkaline protease enzyme as the addition of calcium chloride has been observed to enhance the activity. 5.0 mM final concentration of $CaCl_2$ was added to the reaction mixture in presence of detergents and activity was calculated against control at optimal conditions where no $CaCl_2$ was added in the reaction mixture.

2.5 Effect of surfactants and oxidizing agents on alkaline protease activity
In addition to activity and stability in high pH range and at high temperatures, a good detergent protease must be compatible and stable with all commonly used detergent components such as surfactants, bleaches, oxidizing agents and other additives, which are present in the detergent formulations (Kamoun et al., 2008). The crude enzyme was evaluated in presence of Triton X-100 (0.5%, 1% and 2.5%), Tween-80 (0.5%, 1% and 2.5%), Cetyltrimethylammonium bromide (CTAB; 5mM and 10mM), Sodium dodecyl sulfate (SDS; 0.1%, 0.5, 1% and 5%), Sodium hypochlorite (0.02%, 0.1%, 0.2% and 0.4%) respectively and protease activity was measured under standard conditions. Similarly, in other experiment, same reaction mixtures were prepared and pre-incubated at room temperature for 30 minute and then activity was performed as stated above.

3. Results
During the preliminary investigations on production of alkaline proteases from some of the halophilic isolates, it was found that one of the isolates identified as Bacillus sp. by Institute of Microbial Technology, Chandigarh, India showed optimum keratinolytic activity at pH 9.0 and temperature of 50 oC with a half-life of more than 8 h (Nigam et al., 2013). The enzyme secreted from the isolate was further evaluated as detergent additives at various operating conditions. The morphological, physiological and various biochemical properties of the isolate are given in Table 1. The organism is gram positive, rod shaped, endospore producing bacteria with light yellowish pigments on surface. The isolate shows broad range of pH (5.0 to 11.0) for its growth and grows well at 10% of sodium chloride. It hydrolyses both casein and gelatin in the medium. The organism shows catalase positive reaction and hydrolyses most of the carbon sources. However, methyl red test and indole test was negative.

Table 1: Morphological, physiological and biochemical properties of Bacillus sp.

Colony morphology and Gram's reaction		Growth at temperature	Growth at pH	Growth on NaCl (%)	Biochemical Tests	
Gram's reaction	+ ve	15 °C +	pH 5.0 -	2.5 +	Maltose -	Casein and Gelatin hydrolysis +
Configuration	Round					
Margin	Wavy	25 °C +	pH 6.8 +	5.0 +	Fructose +	Catalase test +
Cell shape	Rods					
Surface	Rough	30 °C +	pH 8.0 +	7.0 +	Sucrose +	Indole test -
Endospore	Present (Central and oval)					
Density	Opaque	37 °C +	pH 9.0 +	8.5 +	Cellobiose+	Methyl red -
Size	Long and thick					
Pigments	Light yellowish	42 °C -	pH 11.0 +	10.0 +	Xylose +	Nitrate reduction +

+ = Positive; - = Negative

3.1 Compatibility with various commercial detergents
The suitability of an enzyme preparation for use in detergents depends on its compatibility with various detergents and certain additives used in laundry and house utensils cleaning. Alkaline protease from halophilic Bacillus sp. was studied for detergent compatibility at optimal conditions (50 °C and pH of 9.0) and the results are presented in Figure 1. The observations revealed that 96%, 104%, 105%, 102% and 107% of its original activity was retained in the presence of boiled-detergents like Tide, Ujala, Wheel, Ariel and Surf Excel respectively. The results of addition of un-boiled detergents (non-denatured enzyme) to the enzyme reaction mixture showed that the activity is comparatively higher in the reaction where the boiled detergents were added (denatured enzyme).The increase in enzyme activity is probably due to presence of protease in the un-boiled detergents which was denatured in boiled detergents at 100 oC for 30 min (Figure 2).

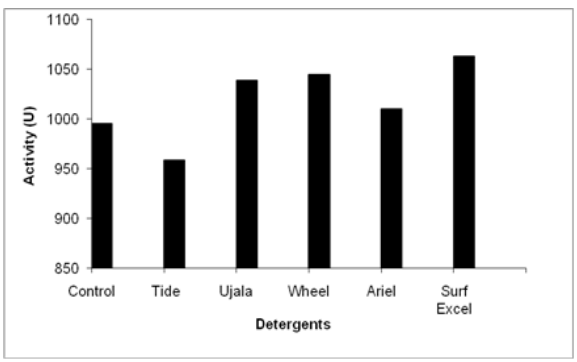

Figure 1: Compatibility of alkaline protease of Bacillus sp. in presence of various detergents.

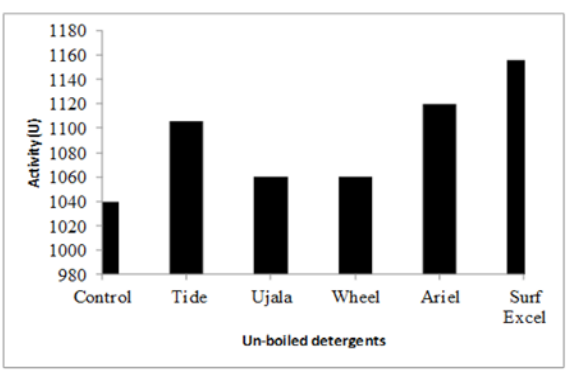

Figure 2: Compatibility of alkaline protease of Bacillus sp. in presence of un-boiled detergents.

3.2 Effect of calcium chloride on alkaline protease activity
The effect of calcium chloride ($CaCl_2$) on alkaline protease activity was evaluated by adding $CaCl_2$ in enzyme catalyzed reaction mixture at a final concentration of 5 mM in presence of boiled detergents (7mg/mL) and the activity calculated is shown in Figure 3. It is evident from the figure that no appreciable effect of calcium chloride was observed on alkaline protease activity. Very slight increase in activity (105%) was observed against control (100%).

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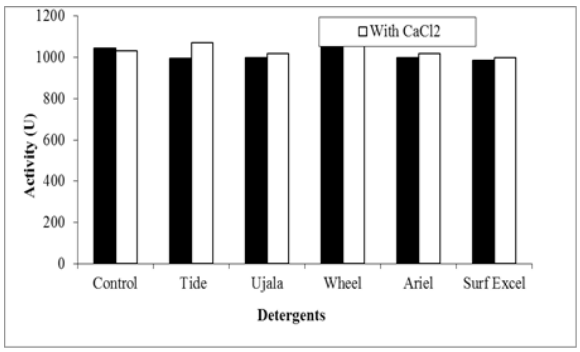

Figure 3: Effect of CaCl2 on alkaline protease activity in presence of detergents.

3.3 Effect of surfactants and oxidizing agents on protease activity

Alkaline proteases used in detergent formulations should be stable and active in presence of different surfactants, oxidative and bleaching agents etc. respectively. Even though a number of proteases have been described and used as detergent additives, they have some limitations with respect to their stability and activity in oxidants and surfactants, which are common ingredients of the detergent formulations. The effect of various surfactants on residual activity of alkaline proteases produced by Bacillus sp. at two different temperatures is presented in Table 2. In the subsequent studies, the reaction mixture in presence of surfactants was pre-incubated at room temperature for 30 minute and assayed against control (activity as 100%). It is clear from the table 2 that the enzyme secreted from the halophilic bacteria can tolerate triton X-100 up to 1% at 60 °C of reaction incubation. Similarly, the residual activity of 84% was recorded when tween-80 at a concentration of 1% was added in the enzyme substrate reaction mixture at 60 °C of incubation. The residual activity of 80% and about 50% respectively were observed when CTAB (10 mM) and SDS (0.5%) were added in the reaction at 50 °C of incubation. At higher concentrations, the alkaline protease activity decreased because of the denaturation of enzyme protein.

Table 2: Residual alkaline protease activity of Bacillus sp. in presence of various surfactants.

Surfactants (Concentrations)	Residual activity (%)			
	50 °C		60 °C	
	Direct	Pre-incubation	Direct	Pre-incubation
Triton X-100 (0.5%)	158	147	119	108
Triton X-100 (1.0%)	157	135	104	95
Triton X-100 (2.5%)	33	27	33	25
Tween-80 (0.5%)	106	102	98	96
Tween-80 (1.0%)	92	81	84	78
Tween-80 (2.5%)	59	50	55	47
CTAB (5 mM)	100	80	81	83
CTAB (10 mM)	81	66	58	65
SDS (0.1%)	57	54	46	32
SDS (0.5%)	47	40	44	30
SDS (1.0%)	29	25	39	17
SDS (5.0%)	18	12	17	6

The use of enzymes in laundry products has become limited because enzymes get deactivated by chlorine bleach and hence bleach stable enzymes are required in detergent industry. The effect of bleaching agent sodium hypochlorite was also studied in a concentration range of 0.02% to 0.4% keeping other conditions constant and the results showed that 63% of residual activity was retained both at 50 °C and 60 °C of incubation at 0.2% concentration of hypochlorite (Figure 4).

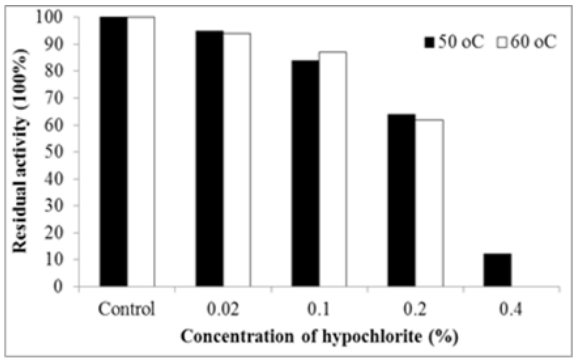

Figure 4: Effect of hypochlorite on alkaline protease activity.

4. Discussion

The suitability of an enzyme preparation for use in detergents depends on its compatibility and stability with the detergents as well as with the various additives used in detergent formulations. The compatibility of alkaline protease from halophilic Bacillus sp. revealed that approximately 92%, 104%, 105%, 91%, 87% of its original activity was recorded at 50 °C in the presence of the detergents Tide, Ujala Techno Bright, Wheel, Ariel and Surf Excel respectively (Figure 1). The compatibility of enzyme produced from Bacillus sp. against studied detergents is greater than Bacillus circulans BM15 which showed 67-78 % compatibility (Venugopal and Saramma, 2007) and Bacillus species Y with 70-75 % compatibility (Mala and Srividya, 2010). It is further observed that the compatibility of enzyme from this strain against detergents is higher with other alkaline protease producing bacteria such as Bacillus cereus SIU1 (Singh et al., 2011), Bacillus licheniformis BWU-1 (Wakte and Bhusare, 2011), Bacillus RV.B2.90 (Vijayalakshmi et al., 2011), Stenotrophomonas maltophilia MTCC 7528 (Kuddus and Ramteke, 2011), Bacillus subtilis VSG-4 (Giri et al., 2011), Bacillus licheniformis KBDL4 (Pathak and Deshmukh, 2012) and Bacillus licheniformis (Mani et el., 2012). The activity of enzyme produced by the isolate is comparable with Bacillus brevis having activity in the range of 85-92% (Banerjee et al., 1999), Bacillus mojavensis A21(BM1) 79.4-100 %, Bacillus mojavensis A21 (BM2) in the range of 87.5-100 % (Haddar et al., 2009) and Bacillus alcalophilus TCCC11004 (Cheng et al., 2010). It has been reported that alkaline proteases require divalent cations like Ca2+, Mg+2, Mn+2 or combination of two or more for maximum activity. These cations not only protect enzyme from thermal denaturation but also help in maintaining the active conformation of the enzyme at high temperature (Kumar and Takagi, 1999; Akel et al., 2009; Cheng et al., 2010). In this study the residual activity in presence of CaCl2 was found to be in range of 94 -104% in presence of different detergents (Figure 3) which is higher compared to activity of proteases from Bacillus licheniformis RP1 (Kamoun et al., 2008) and Bacillus licheniformis (Mani et el., 2012)

The enzymes used as detergent additives should be stable and active in presence of surfactants, oxidative and bleaching agents etc., which are common ingredients of detergent formulations (Anand et al., 2010; Venugopal and Saramma, 2007; Singh et al., 2011; Nascimento and Martins, 2006). The protease from halophilic Bacillus sp. showed higher activity at 1% triton X-100 i.e., 158% of residual activity (Table 1) compared to other alkaline proteases where residual activity of 47%, 100%, 72%, 100% and 78% was observed with Bacillus sp. (Nascimento and Martins, 2006), Bacillus licheniformis RP1 (Kamoun et al., 2008), Bacillus circulans BM15 (Venugopal and Saramma, 2007), Bacillus RV B2.90 (Vijayalakshmi et al., 2011) and Bacillus licheniformis KBDL4 (Pathak and Deshmukh, 2012) respectively. The alkaline protease from the isolate is also quite stable in presence of tween-80 showing 92% of its residual activity at 1% concentration of tween-80 compared to proteases from Bacillus cohnii APT5 (Tekin

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et al., 2012) and Bacillus licheniformis KBDL4 (Pathak and Deshmukh, 2012) where 50 and 40% residual activity was obtained at the same conditions. Similarly, in presence of 5 mM cationic surfactant CTAB, 19 % of residual activity was lost with Bacillus used in present work at 60 0C (Table 1) whereas 70% of residual activity was lost with alkaline protease from Bacillus subtilis VSG-4 (Giri et al., 2011). Approximately 50% of activity of enzyme was noticed in presence of 0.5% of SDS at 50 oC in the present investigation which is much higher than alkaline protease (26% of residual activity) with Bacillus sp. (Nascimento et al., 2006). Complete loss of enzyme activity was reported at 0.1% of SDS with Chromohalobacter sp. TVSP101 (Vidyasagar et al., 2007). The use of enzymes in laundry products has become limited because enzymes get deactivated by chlorine bleach hence bleach stable enzymes are required in detergent industry. The alkaline protease from halophilic Bacillus sp. was very stable in presence of sodium hypochlorite and retained 67% of its residual activity at 0.2% of sodium hypochlorite (Figure 4). In other study, 71% of residual activity of enzyme was reported with Bacillus sp. APR-4 at 0.05% concentration of sodium hypochlorite (Kumar and Bhalla, 2004).

5. Conclusions

The studies on the applicability of alkaline protease from halophilic Bacillus sp. as detergent formulation showed that the enzyme is stable in presence of both boiled and un-boiled detergents at high temperature and pH. The results also showed that it is able to tolerate high concentrations of Triton X-100 (1%), Tween-80 (1%), CTAB (5mM), SDS and bleaching agent respectively and hence, could be a potential source for using as detergent additives.

6. Acknowledgments

Authors duly acknowledge the financial assistance received from University Grants Commission, Government of India [Ref No: 41-504/2012 (SR)] to carry out this study. Authors are grateful to Central Instrumentation Facility (Teqip), Birla Institute of Technology, Mesra, Ranchi for the infrastructure support.

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