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Detergent Potential of a Spoilage Protease Enzyme Liberated by a Psychrotrophic Spore Former Isolated from Sterilized Skim Milk

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ABSTRACT

The organism isolated from sterilized skim milk was identified as *Bacillus cereus*. *B. cereus* was found to be capable of producing protease enzyme at an alkaline pH-10. To assess the possibility of exploiting this enzyme in cleaning formulations, temperature stability, pH stability and compatibility of crude enzyme with detergent components were looked into. The enzyme was found to be stable over a pH ranging from 7-12 with maximum activity at pH-11.0. The protease retained more than 60% activity after 90 min of exposure to all the selected temperatures of 20, 40, 60 and 80°C. Residual activity of enzyme after forty days of storage at 10 and -10°C was 77.4 and 69.5% of their initial activity, respectively. Enzyme remained stable to a very wide temperature range of -10 to 80°C. The anionic surfactant Sodium dodecyl sulphate enhanced the activity by 2.2 times under experimental conditions. Nonionic surfactant Tween 80 also improved the activity of protease enzyme. Hydrogen peroxide, a commonly used bleaching agent was found to have no significant adverse affect on the crude enzyme. Detergent compatibility of the enzyme was distinctly established by the wash test also. The results of this experiment confirmed the potential of enzyme to be used as a detergent additive.

Key words: Detergent potential, protease enzyme, *Bacillus cereus*, psychrotrophic spore former

INTRODUCTION

An important mandate in the performance of any food industry is the assurance of quality of its products. In food industry, as a part of quality assurance, always major emphasis has been on 'hygiene' which has eventually resulted in the indiscriminate use of chemicals for sanitation. This practice in the long run will result in residues that act as concealed threats in food chain. The milk stones and biofilms that are characterized by impervious protein matrix have been identified as major hurdles that interfere with the efficiency of cleaning regimes in milk processing units. Enzyme based sanitizers are better options in combating these problems (Kumar, 1997). The synthetic chemicals used presently for sanitation are to be replaced by eco-friendly alternatives to safeguard public health. Enzymes with its biological aura and green image are ideal for eco-friendly formulations.

For an enzyme to be used as a detergent additive it should be stable and active in the presence of typical detergent components like surfactants, builders, bleaching agents and other formulation aids at moderately high temperatures. Proteases exhibiting activity in high alkaline range are recognized as potential detergent additives (Anvar and Saleemuddin, 1998). Members of the genus Bacillus has been identified as a prolific source of alkaline proteases (Yang *et al*., 2000). The multitude of diverse habitats has challenged the nature to develop an equally numerous molecular adaptations in the microbial world. Hence, there is always a chance of finding microorganisms producing novel enzymes with better properties suitable for commercial exploitation (Kumar and Takagi, 1999). It is conceived that alkaline proteases of dairy microflora will be effective as cleansing additives in dairy industry. Identification of suitable enzymes and diligent use of these will be helpful in developing an eco-friendly sanitation methodology. In pursuit of an eco-efficient alternative for dairy plant sanitation, the present study was taken up to assess the detergent potential of the protease enzyme liberated by a psychrotrophic spore-former isolated from sterilized skim milk.

MATERIALS AND METHODS

Isolation and identification of microorganism: The organism used in this study was isolated from sterilized skim milk that showed signs of spoilage after two weeks of refrigeration. For isolation, the appropriately diluted samples were pour plated in Nutrient agar (Himedia) and incubated at 37°C for 48 h so as to get discreet colonies. After considering the colony characteristics, single colonies were selected and streaked to purity in nutrient agar plates. The isolates were subjected to biochemical characterization after activating the culture in nutrient broth at 37°C for 24 h (Barrow and Feltham, 1993). The isolate was maintained in nutrient agar slants, at 4°C. At regular intervals the purity of culture was confirmed by staining. Genetic identification (16SrRNA sequencing) was done by sending the isolate to Central Institute of Fisheries and Technology, Wellington Island, Cochin.

Qualitative assessment of production of protease in alkaline pH: The potential of the isolate to grow and produce protease enzyme in an alkaline medium was assessed qualitatively by streaking an appropriately diluted sample of the active culture on Glucose Peptone Yeast extract Carbonate medium (Glucose-10.0 g, Peptone-5.0 g, Yeast extract-1.0 g, K_2HPO_4-1.0 g, $MgSO_4$-0.2 g, Agar-18 g, Distilled water 1000 mL) fortified with 10% skim milk and having a pH-10. (Kumar *et al*., 1999). Sodium carbonate (2%) was sterilized separately and added aseptically to adjust the pH to 10.0. The proteolytic potential of the isolate was qualitatively assessed by the zone of clearance obtained after incubation at 37°C for 48 h.

Protease assay: To obtain the crude enzyme, active culture with an optical density of 0.6 at 600 nm was inoculated at a level of two per cent to basal fermentation medium having 0.5% skim milk and 0.5% whey powder (pH-11.0). The Cell Free Supernatant (CFS) collected by refrigerated centrifugation (4°C/8000 rpm) after incubation at 37°C for 48 h served as the crude enzyme. Protease activity was measured using 0.5% casein as substrate (pH-11.0). Enzyme activity was expressed in terms of micromoles of tyrosine released/ml/min under standard assay conditions (Joo *et al*., 2003). The amount of enzyme which liberates one micromole of tyrosine/mL/minute was taken as one unit of activity.

pH stability of protease: This was determined by exposing the crude protease solutions to varying pH of 7.0, 8.0, 9.0, 10.0 11.0 and 12.0) by mixing the enzyme with Tris-HCl/glycine-NaOH buffer in the ratio 1:1. The enzyme solutions at different pH values were incubated at 30°C for 24 h. The residual protease activity was subsequently determined in terms of tyrosine value, using 0.5% casein as substrate. The original enzymatic activity before incubation at various pH values was taken as 100% (Nascimento and Martins, 2004).

Temperature stability of protease: The crude enzyme solution was exposed to different temperatures of 20, 40, 60 and 80°C for a total duration of 90 min. Aliquots of enzyme solution were drawn after 30, 60 and 90 min of exposure to different temperatures. The heat treated enzyme samples were immediately cooled and the residual enzyme activity was determined using 0.5% casein as substrate under standard assay conditions. Storage stability of crude preparation at 10 and -10°C was evaluated by storing the enzyme preparation at the corresponding temperatures for forty days and assessing the residual activity at ten days interval. The enzyme activity at '0' time was taken as 100% (Nilegaonkar *et al*., 2007).

Compatibility with detergent components: The crude protease enzyme solution was pre-incubated with Sodium dodecyl sulphate, Tween-80 and Hydrogen peroxide each at 10 mM concentration in the ratio 1:1 at 60°C for 30 min. The residual protease activity was determined on 0.5% casein substrate under standard assay conditions. Activity of enzyme without the addition of inhibitor was taken as 100%.

The efficiency of the crude protease enzyme (cell free supernatant with an activity of 25 U mL^{-1}) in combination with a locally available detergent (wheel) in removing blood stain was also assessed by a wash test. The possibility of using this enzyme as a detergent additive was looked into by washing blood stained cloth pieces with water alone, with detergent solution (0.1 g detergent, water-50 mL) and with the test solution containing detergent and crude enzyme (0.1 g detergent, CFS-10 mL, Water-50 mL). Untreated cloth piece stained with blood was taken as control. Efficiency to remove blood stain was assessed by visual examination of dried cloth pieces (Adinarayana *et al*., 2003).

RESULTS AND DISCUSSION

The results of morphological and biochemical characterization were suggestive of the isolate to be *B. cereus*. The 16SrRNA sequencing confirmed the identity of isolate. The fact that the organism was isolated from sterilized milk kept under refrigeration is indicative of the psychrotrophic potential of the isolate. Incidence of such psychrotolerant strains of *Bacillus* in milk had been reported earlier (Meer *et al*., 1991; Pacova *et al*., 2003). Murphy *et al*. (1999) opined that *Bacillus* species with adverse proteolytic activities are so widely distributed in the environment that they could be introduced into milk and milk products at any stage of production and processing. Isolation of *Bacillus* species from sterilized milk is in agreement with Bellow *et al*. (2007) who have reported occasional occurrence of *Bacillus* originating from raw milk in high heat treated milks. *Bacillus* species with potential to produce alkaline proteases have been isolated from a wide variety of sources like soil (Nascimento and Martins, 2004) and waste water near milk processing plant (Chu, 2007).

Alkaline proteases are the major industrial work horses in different process applications. This underscores the need to assess the potential of enzyme to withstand a wide range of pH. As per the

Table 1: Stability of enzyme at different pH

pH of the medium	Protease activity (μ moles of tyrosine mL^{-1})	Residual activity (%)
7	314.34±3.53	92.6[a]
8	325.41±3.69	110.8[b]
9	336.97±6.54	114.3[b]
10	355.15±2.06	118.7[bc]
11	385.53±3.13	125.1[d]
12	262.74±2.59	135.8[e]

The mean difference is significant ($p \leq 0.05$). Values bearing same superscript do not differ significantly. Activity is expressed as μ moles of tyrosine released under standard assay conditions

results (Table 1), the crude enzyme used in this trial preferred an alkaline medium. With increasing pH, enzyme showed a gradual increase in activity. A similar gradual increase in protease activity above pH 7.0 till pH 10 had been reported by Son and Kim (2002). Takami *et al.* (1990) reported that pH was important in deciding the binding function between enzyme and substrate. Better activity at alkaline pH might be due to the better binding of enzyme to the substrate at this pH. The present observation of maximum activity at pH 11 is in agreement with the reports of Joo *et al.* (2003). In this trial, enzyme retained 92.6% of the original activity after 24 h of exposure to pH 12. Stability of protease enzyme from *Bacillus* species over a pH ranging from 7-12 had been reported earlier by Aftab *et al.* (2006). Such good stability at pH values up to 12 had been reported by Hogueira *et al.* (2006). This distinctive feature of high pH optimum is a common feature of all alkaline proteases. The *Bacillus* isolate from sterilized skim milk also showed the ability to produce extra cellular alkaline protease with possible industrial applications.

Assessment of thermostability of enzyme showed that protease retained more than 60% activity after 90 min of exposure to all the selected temperatures of 20, 40, 60 and 80°C (Table 2). Comparable thermostability and high optimum temperature for *Bacillus* protease had been reported by Sookkheo *et al.* (2000) and Johnvesly *et al.* (2002). Thermostability of enzyme will vary significantly with strain, growth medium and incubation period (Koka and Weimer, 2001). At 20, 40 and 60°C, protease activity tended to decrease with increase in time of exposure. But the activity profile at 80°C showed a different pattern. After 30 min of exposure to 80°C, there was a sharp decline in activity to 46.4%. This could be because enzyme was not capable of getting adapted to high temperature within 30 min. As the exposure period advanced, enzyme would have got adapted to the high temperature environment such that activity increased to 79.4% at 60 min. Such high thermostability for *Bacillus* protease has been reported by Chopra and Mathur (1985). Inactivation of protease inhibitors at high temperature, also would have contributed to the enhanced enzyme activity (Poffe and Mertens, 1988). Reduction in protease activity on further exposure to temperature could be due to the thermal denaturation of enzyme resulting from prolonged exposure to high temperature.

Storage stability of the enzyme at 10 and -10°C were assessed at 10 days interval for forty days (Table 3). Stability of *Bacillus* protease at temperatures below 20°C had been reported by Son and Kim (2002). For 20 days protease activity increased gradually to 145.4 and 129.3% at 10 and -10°C, respectively. This could be probably because of the inhibition of protease inhibitors at low temperature. On all tested days, frozen samples exhibited lesser activity than the corresponding samples at 10°C. Ice crystal formation at -10°C might have affected the active conformation of enzyme thereby initiating enzyme denaturation and loss of activity. Even after 40 days of storage, samples at 10 and -10°C exhibited a residual activity of 77.4 and 69.5%,

Table 2: Stability of enzyme at high temperature

	Duration of exposure (min)							
	0 h		30 min		60 min		90 min	
Temperature (°C)	Activity (μ moles of tyrosine mL^{-1})	Residual activity (%)	Activity (μ moles of tyrosine mL^{-1})	Residual activity (%)	Activity (μ moles of tyrosine mL^{-1})	Residual activity (%)	Activity (μ moles of tyrosine mL^{-1})	Residual activity (%)
20	300.98±3.00	100	240.78±0.47	80.0	212.49±1.50	70.6	210.69±1.86	70.0
40	300.98±3.00	100	235.97±3.60	78.4	206.17±1.11	68.5	184.80±0.74	61.4
60	300.98±3.00	100	238.98±0.96	79.4	183.00±0.85	60.8	180.08±1.11	60.1
80	300.98±3.00	100	139.65±1.18	46.4	224.80±2.18	74.7	224.83±1.36	67.2

Table 3: Stability of enzyme at low temperature

	Temperature			
	10°C		-10°C	
Days of storage	(Activity μ moles of tyrosine mL^{-1})	Residual activity	(Activity μ mole of tyrosine mL^{-1})	Residual activity
0	300.98±3.00	100.0	300.98±3.00	100.0
10	378.62±2.43	125.8	370.83±2.18	123.2
20	437.75±1.90	145.4	389.01±2.78	129.3
30	384.30±1.86	127.7	360.00±1.77	119.6
40	233.04±2.37	77.4	209.16±1.73	69.5

Table 4: Effect of detergent components on the activity of protease enzyme

Compounds	Activity (μ moles of tyrosine mL^{-1})	Residual activity (%)
Control	273.20±5.18[a]	100.0
Hydrogen peroxide	272.17±2.99[a]	99.6
Tween-80	303.67±3.80[e]	111.2
Sodium dodecyl sulphate	597.97±8.86[f]	218.9

The mean difference is significant ($p \leq 0.05$). Values bearing same superscript do not differ significantly

respectively. Adaptation of enzyme to low temperatures could be because enzyme was produced by a psychrotolerant strain of *Bacillus*. Results of this trial conclusively suggest the stability of enzyme to a very wide temperature range of -10 to 80°C.

Compatibility with surfactants and oxidizing agents are advantageous for enzymes to be used as detergent additives. In the present trial, protease activity was found to enhance significantly in the presence of surfactants (Table 4). The anionic surfactant, SDS acted as an activator such that activity increased by 2.2 times for the enzyme tested, under experimental conditions. This is in accordance with the findings of Hogueira *et al.* (2006) who found that SDS enhanced the protease activity of *Teredinobacter turnirae* by 3.7 times. The protease enzyme exhibited a better performance in the presence of nonionic surfactant Tween 80 also. Enzyme activity increased to 111.2% when compared to control. Similar effect of nonionic surfactant has been reported by Joo *et al.* (2003) for the alkaline protease of *B. clausii*. Stability with oxidizing agent is beneficial as it facilitates better performance in bleach based formulations. Hydrogen peroxide, a commonly used bleaching agent was found to have no significant adverse affect on the crude enzyme. Stability of *Bacillus* protease to H_2O_2 is in corroboration with the findings of Joo *et al.* (2003) and Nilegaonkar *et al.* (2007).

CONCLUSION

The results suggest that protease preparation is an ideal candidate to be used as a detergent additive. The wash test conclusively proved that incorporation of enzyme improved the performance of the detergent solution. Stability of enzyme at wide temperature range indicate its potential to be used in hot as well as cold wash cycles. Detergent compatibility of the enzyme is distinctly established by the wash test. Stability in alkaline pH, thermostability of enzymes and stability in the presence of oxidizing agent and surfactants suggest that the enzyme used in this trial is an ideal candidate to be used a detergent additive in cleaning formulations.

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