Microbiological Research **159** (2004) 135–140

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Microbiological Research
www.elsevier.de/micres

Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*

Rathindra Mohan Banik[a], Monika Prakash[b,*]

[a]*School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi 221005, India*
[b]*Institute für Biochemie, Johannes Gutenberg Universität, Becherweg-30, Mainz 55128, Germany*

Accepted 14 January 2004

KEYWORDS
Alkaline protease;
Thermostability;
Bacillus cereus;
Laundry detergents;
Detergent compatibility

Summary

The endogenous protease activity in various commercially available laundry detergents of international companies was studied. The maximum protease activity was found at 50°C in pH range 10.5–11.0 in all the tested laundry detergents. The endogenous protease activity in the tested detergents retained up to 70% on incubation at 40°C for 1 h, whereas less than 30% activity was only found on incubation at 50°C for 1 h. The alkaline protease from an alkalophilic strain of *Bacillus cereus* was studied for its compatibility in commercial detergents. The cell free fermented broth from shake flask culture of the organism showed maximum activity at pH 10.5 and 50°C. The protease from *B. cereus* showed much higher residual activity (more than 80%) on incubation with laundry detergents at 50°C for 1 h or longer. The protease enzyme from *B. cereus* was found to be superior over the endogenous proteases present in the tested commercial laundry detergents in comparison to the enzyme stability during the washing at higher temperature, e.g., 40–50°C.

Introduction

Proteases are one of the most important industrial enzymes produced by wide range of microorganisms such as bacteria, yeasts, molds, and are also found in plants and in various animal tissues (Walsh and Wilcox, 1970). Bacterial proteases are mostly extracellular, easily produced in larger amounts, thermostable and active at wider pH range. These properties make the bacterial proteases most suitable for wider industrial application. Alkaline proteases from the bacterial origin are the most important industrial enzymes, which contribute about 60% of the total world enzyme market (Ward, 1985; Kalisz, 1988; Outtrup, 1990). The economic importance of alkaline proteases came to light when bacterial alkaline proteases from *Bacillus* sp. were introduced in 1960s to the detergent industry, accounting for about 35% of the total microbial enzyme sales (Kalisz, 1988; Outtrup, 1990; Godfrey and West, 1985). Alkaline proteases from various *Bacillus* sp. such as *Bacillus brevis* (Banerjee et al., 1999), *Bacillus sp*. SSR1 (Singh et al., 2001), and *Bacillus stearothermophilus* (Dhandapani and Vijayaragvan, 1994) have been reported to use in

*Corresponding author.
E-mail address: monika1778@yahoo.co.in (M. Prakash).

0944-5013/$ - see front matter © 2004 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2004.01.002

laundry detergent formulations. However, there is limited information in open literature regarding the amount of active protease present in the detergent formulations and their thermal stability. Ideally, proteases used in detergent formulations should have considerably high level of activity in presence of soaps, alkali, and high temperature (Banerjee et al., 1999).

In this report, the endogenous protease activity in commercial laundry detergents such as Surf Excel Matic, Tide, Rin Shakti, Ariel Power Compact, Henko Stain Champion, Mr. White and Fena Super Power is described. The thermal stability of the endogenous proteases in these laundry detergents was also investigated. An alkaline and thermostable protease from *Bacillus cereus* was produced and its potential for use as an effective additive in laundry detergents was studied.

Material and methods

Chemicals

Casein, soybean meal and yeast extract were purchased from Hi-media (Mumbai, India). Trichloroacetic acid, glucose, beef extract and peptone were obtained from Qualigens (Mumbai, India). Other chemicals used in this study were of reagent grade and were commercially available. Various commercial detergents used in this study were purchased from the local market.

Microorganism and culture conditions

B. cereus obtained from National Chemical Laboratory, India was maintained at 4°C on nutrient agar medium containing: beef extract 1.0%, NaCl 0.5%, peptone 1.0%, agar 2.0%. The organisms were grown in a medium having composition (%w/v): glucose 3.0%, soybean meal 1.0%, $CaCl_2$ 0.04% and $MgCl_2$ 0.2% (Lee and Chang, 1990). The pH of the medium was adjusted to 7.5 by dissolving the medium constituents in 0.1 M phosphate buffer. The growth medium was inoculated with the organism and incubated for 24 h at 30°C and 200 rpm in orbital shaker to develop inoculum. The liquid medium used for the production of alkaline protease had the following composition (%w/v): glucose 2.0%, soybean meal 2.0%, $CaCl_2$ 0.04% and $MgCl_2$ 0.02% (Lee and Chang, 1990). The pH of the medium was adjusted to 7.5 by dissolving the medium constituents in 0.1 M phosphate buffer. Fermentation was carried out using 50 ml of the production medium in 250 ml Erlenmeyer flask. The production medium was inoculated with 5% inoculum. The flasks were incubated for 72 h in a temperature-controlled (30°C) shaking incubator (200 rpm). The contents were then centrifuged (10,000*g*, 30°C, 20 min) and the cell free supernatant was used for determining extracellular protease activity.

Assay method

The alkaline protease activity was estimated by the procedure of modified Hagihara method using casein as substrate (Hagihara et al., 1958). The reaction mixture (11 ml) containing 5.0 ml of 1.2% casein solution in buffer solutions of appropriate pH, and suitably diluted enzyme (1.0 ml) was incubated at 50°C for 10 min. The reaction was terminated by addition of 5.0 ml of 0.3 M trichloroacetic acid, and after 30 min of incubation (30°C) the reaction mixture was centrifuged (10,000*g*, 30°C, 20 min). Absorbance of clear supernatant was measured at 275 nm using Elico spectrophotometer. Enzyme activity was expressed as protease unit, where, one unit of protease activity was defined as the quantity of the enzyme that liberated the digestion product not precipitated by protein precipitating reagent and gave absorbance at 275 nm equivalent to 1 μg/ml of tyrosine per min, under assay conditions.

Effect of pH and temperature on enzyme activity

To find out the optimum pH of the protease produced by *B. cereus* the activity of partially purified enzyme was measured at different pH. Protease enzyme activity was measured at different pH values (7.5, 8.5, 9.5, 10.5 and 11.5) using Phosphate buffer, Tris-HCl buffer and Na_2CO_3/NaOH buffer. Reaction mixtures of different pH were incubated at 50°C and the activity of enzyme was measured.

Effect of temperature on protease activity was studied by incubating the reaction mixture at different temperatures ranging from 30°C to 60°C. The reactions were carried out at the optimum pH 10.5 and the activity of the enzyme was measured.

Estimation of protease activity in laundry detergents

For estimation of protease activity in commercial laundry detergents most popular detergents of Indian market namely Surf Excel Matic (Hindustan Lever Limited), Tide (Procter and Gamble), Rin

Shakti (Hindustan Lever Limited), Ariel Power Compact (Procter and Gamble), Henko Stain Champion (Henkel Spic India Limited), Mr. White (Henkel Spic India Limited) and Fena Super Power (Saci Allied Product Limited) were studied. These detergent powders consist of different coloured granules. Three samples of each detergent were made. First type of sample was prepared by taking only coloured granules after separating them from whole detergents. Second type of sample was prepared by taking whole detergent powder. The coloured granules present in the detergents were crushed properly to make uniform distribution of the enzyme in detergent powder. Third sample was prepared by taking only detergent powder free from coloured granules. 2% solution (20 mg/ml) of each sample was prepared using distilled water and protease activity was assayed at pH 10.5 and absorbance was determined spectrophotometrically at 275 nm.

Protein assay of laundry detergents

Protein content in laundry detergents was estimated by the method of Lowry et al. Using bovine serum albumin as the standard (Lowry et al., 1951).

Compatibility of Bacillus cereus protease with laundry detergents

The compatibility of *B. cereus* protease with commercial laundry detergents was studied using Surf Excel Matic, Tide, Rin Shakti, Ariel Power Compact, Henko Stain Champion and Mr. White. For the study of compatibility these detergents were dissolved in distilled water in concentration of 7 mg/ml (Banerjee et al., 1999; Bhosale et al., 1995). Endogenous protease present in these detergents was inactivated by incubating the detergent solutions at 65°C for 1 h prior to the addition of the exogenous protease obtained from *B. cereus* (Samal et al., 1990). After addition of exogenous protease in detergent solution (1 ml enzyme in 4 ml detergent solution), the mixture was incubated at 50°C for 1 h (Samal et al., 1990; Bhosale et al., 1995; Banerjee et al., 1999; Singh et al., 2001) and the residual protease activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100%.

The stability of *B. cereus* protease in presence of the best three laundry detergents namely Surf Excel Matic, Ariel Power Compact and Henko Stain Champion was also measured after incubation of the *B. cereus* protease with the laundry detergents at 40°C and 50°C for different periods upto 2 h. The activity of exogenous protease was compared with the residual endogenous proteases present in the laundry detergents after incubation at 40°C and 50°C upto 2 h.

Results and discussion

Characterization of the enzyme

The protease produced from *B. cereus* was found to be most active at pH 10.5. Protease activity increased progressively with increase in pH upto pH 10.5 and a further increase in pH showed decrease in protease activity (Fig. 1). At pH 7.5, only 18% of the maximum enzyme activity was obtained, increased to 81.7–86% at pH 8.5 and 9.5, respectively. Maximum enzyme activity was observed at pH 10.5, which is an essential characteristic for the use of alkaline protease as laundry

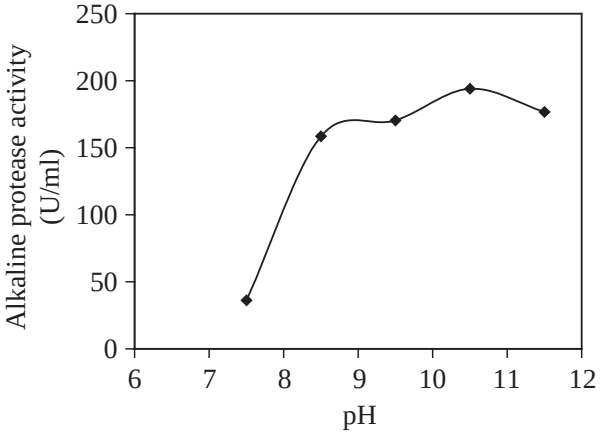

Figure 1. Effect of pH on alkaline protease activity by *B. cereus* at constant temperature 50°C.

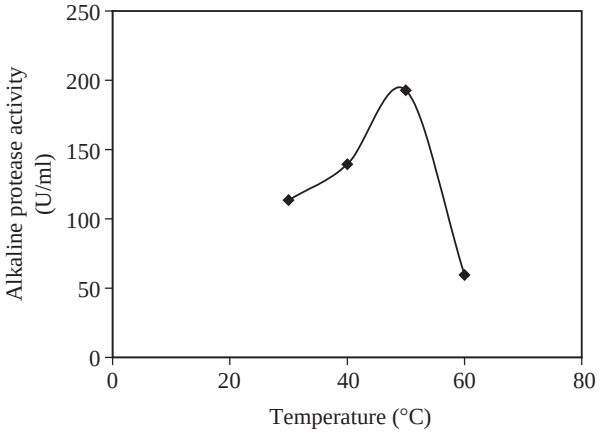

Figure 2. Effect of temperature on alkaline protease activity by *B. cereus* at constant initial pH 10.5.

detergent additives. Some earlier reports also showed pH optima of 10–10.5 for protease from *B. brevis* (Banerjee et al.), *Xanthomonas maltophila* (Debette, 1991) and *Vibrio metschnikovii* (Kwon et al., 1994).

The temperature profile (Fig. 2) of the *B. cereus* protease showed maximum protease activity at 50°C, however, at 30°C, 40°C and 60°C the enzyme showed 70%, 82% and 30% of maximum activity, respectively. The *B. cereus* protease possesses maximum activity at high pH and temperature range that indicates its usefulness in wide range of temperature wash programmes.

Proteolytic activity present in laundry detergents

Protease enzyme was present in all the commercial laundry detergents tested (Table 1). The maximum protease activity was found to be at 50°C, showed poor activity at 40°C and very low activity at 60°C. The protease activity was found both in coloured granules as well as in granule less part of the detergents. Surf Excel Matic consisted of three types of coloured granules in which protease activity was found in orange and blue granules, but no protease activity was found in green coloured granules of Surf Excel Matic. Two types of coloured granules were found in Mr. White in which blue granules were having protease activity but no protease activity was observed in green granules. Other detergents were having only blue granules possess protease activity (Table 2). Coloured granules were weighed separately and it was found that quantitatively these coloured granules differ in different detergents (Table 2). Coloured granules were not present in Fena but protease activity was found in the detergent powder. Protein estimation (Table 3) of these detergents was done to confirm that substrate degradation during estimation of protease is due to protease and not due to high temperature or high alkaline pH.

Table 2 indicates that the maximum protease enzyme is present in the coloured granules of Surf Excel Matic and Table 1 shows that the non-granular part of Surf Excel Matic has less activity in comparison to Henko Stain Champion and Tide. Table 1 indicates that the maximum protease activity is present in Henko Stain Champion. Good protease activity was found in Surf Excel Matic, Ariel Power Compact and Tide and relatively low protease activity was observed in Mr. White, Rin Shakti and Fena Super Power.

Compatibility of the B. cereus protease with laundry detergents

The suitability of an enzyme preparation for use in detergents depends on its compatibility with the detergents over a wide alkaline pH, and temperature range. An ideal detergent enzyme should be stable and active in the detergent solution for a longer period of time and should have adequate temperature stability to be effective in a wide range of washing temperatures. The *B. cereus* protease showed excellent stability and compatibility in the presence of the laundry detergents tested (Fig. 3). The enzyme produced from *B.cereus* retained more than 80% of its activity in all of the commercial detergents tested even after 1 h of incubation at 40°C and 50°C (Figs. 3–5). Singh et al. (2001) reported a serine alkaline protease from *Bacillus* sp. SSR1 showing nearly 70–80% of activity in most of the detergents at 40°C. They reported that above 40°C temperature for stability of enzyme in detergents addition of additive like $CaCl_2$ is required. Also Banerjee et al. (1999) and Bhosale et al. (1995) reported that protease enzyme retained high activity in commercial detergents after supplementation of additives $CaCl_2$ and glycine. Comparison of our results with that of others indicates that *B. cereus* protease is superior as it retains enzyme activity for longer period in presence of laundry detergents at higher

Table 1. Protease estimation in commercial laundry detergents at 50°C temperature and pH 10.5

Laundry detergents	Protease activity in coloured granules (U/ml)	Protease activity in granule less part of detergents (U/ml)	Protease activity in whole detergents (U/ml)
Henko stain champion	220.00	301.20	333.36
Tide	200.04	170.98	178.32
Ariel power compact	150.96	158.64	175.08
Surf excel matic	260.50	159.92	161.76
Rin shakti	72.36	40.24	48.48
Mr. White	92.64	14.04	16.32
Fena super power	—	—	32.54

Table 2. Quantitative analysis of coloured granules present in laundry detergents

Laundry detergents	Types of coloured granules present	Amount of coloured granules of detergents (mg/g)	Protease activity (U/ml)
Surf excel matic	Orange	9.5	165
	Green	3.6	95.2
	Blue	4.5	—
Henko stain champion	Blue	15	220
Tide	Blue	2.3	200.04
Ariel power compact	Blue	2.3	150.96
Mr. White	Blue	11.75	92.64
	Green	5.23	—
Rin shakti	Blue	20	72.36

Table 3. Protein content of commercial laundry detergents

Laundry detergents	Protein present in coloured granules (μg/ml)	Protein present in granule less part of detergents (μg/ml)	Protein present in whole detergents (μg/ml)
Henko stain champion	110.80	136.00	139.00
Tide	107.2	87.4	88.4
Ariel power compact	86.40	82.00	85.00
Surf excel matic	127.60	80.40	81.60
Rin shakti	23.80	56.2	71.40
Mr. White	30.60	48.30	52.40
Fena super power	—	—	68.20

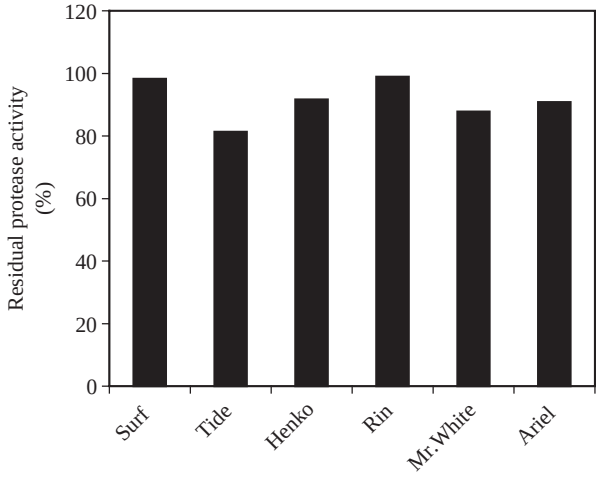

Figure 3. Compatibility of alkaline protease activity from *B. cereus* with laundry detergents at pH 10.5 and 50°C temperature.

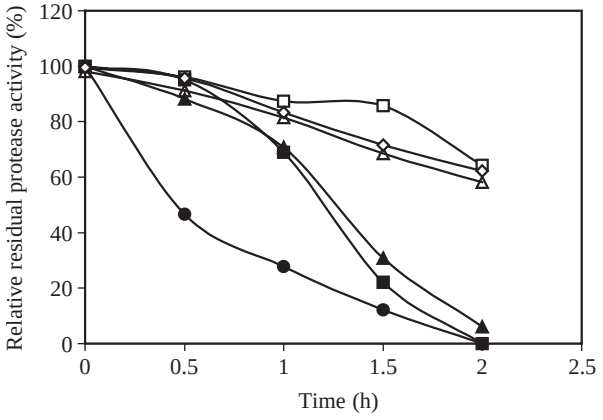

Figure 4. Comparison of protease activity present in detergents to *B. cereus* protease at 40°C (●, Surf; ▲, Ariel; ■, Henko; □, *B. cereus* protease in Surf; △ *B. cereus* protease in Ariel; ◇, *B. cereus* protease in Henko).

temperature and without supplementation of any additives.

In comparison to the enzyme present in commercial detergents tested, *B. cereus* protease showed better stability in these detergents at alkaline pH as well as at 40°C and 50°C temperatures. In presence of laundry detergents *B. cereus* protease retains nearly 95% activity after 30 min, more than 80% of activity after 1 h and even after 2 h of incubation more than 60% activity was remained (Figs. 4 and 5). The commercial detergents retained 80–90% of the maximum activity after 30 min and after 1 h negligible activity was observed (Figs. 4 and 5). Either hand washing or

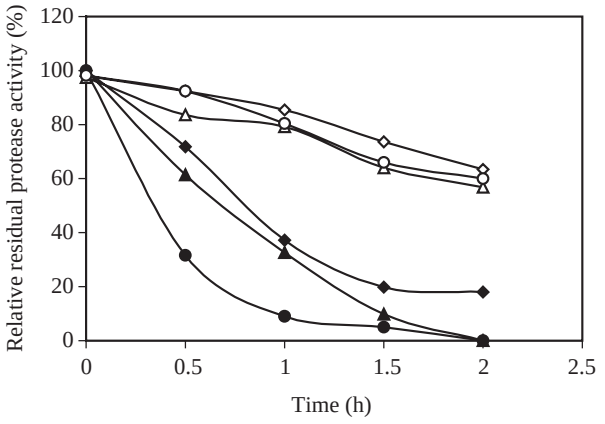

Figure 5. Comparison of protease activity present in detergents to *B. cereus* protease at 50°C (◆, Surf excel; ▲, Ariel; ●, Henko; ◇, *B. cereus* protease in Surf; △ *B. cereus* protease in Ariel; ○, *B. cereus* protease in Henko).

machine-washing of cloths normally takes 60–90 min, hence enzyme present in detergent should remain active during washing period.

It was also found that these laundry detergents showed very poor protease activity at 40°C in comparison to 50°C, in contrast *B. cereus* protease showed good activity at wider temperature range (40–50°C). The *B. cereus* protease is, therefore, superior to the enzymes used in laundry detergents as most of the reputed garment industries recommend normal temperature washing.

In conclusion, protease activity was found to exist in all the commercial detergents tested. Most of these laundry detergents contained coloured granules, protease activity was found both in coloured granules as well as in granule less part of the detergents. The enzyme present in these laundry detergents showed maximum activity at 50°C and pH 10.5 (detergent's pH). At 40°C endogenous enzyme of laundry detergents showed very poor activity in comparison to 50°C and at 60°C became inactivated. At 40°C and 50°C, protease enzyme present in these detergents retained its 80–90% activity only upto 30 min, after 1 h showed very low activity and after that negligible activity. In contrast, the extracellular protease isolated from *B. cereus* was stable over a wide range of alkaline pH and temperature. It also showed excellent compatibility with various laundry detergents tested and the stability of the enzyme in detergents was much better for longer time period than the endogenous enzymes of laundry detergents. This study indicates that the *B. cereus* protease is superior to the conventional enzymes used in manufacture of laundry detergents.

References

Banerjee, U.C., Sani, R.K., Azmi, W., Sani, R., 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterisation as a laundry detergent additive. Process Biochem. 35, 213–219.

Bhosale, S.H., Rao, M.B., Deshpande, V.V., Srinivasan, M.C., 1995. Thermostability of high-activity alkaline protease from *Conidiobolus coronatus* (*NCL 86.8.20*). Enzyme Microbial Technol. 17, 136–139.

Debette, J., 1991. Isolation and characterisation of an extracellular protease produced by a soil strain of *Xanthomonas maltophila*. Curr. Microbiol. 22, 85–90.

Dhandapani, R., Vijayaragvan, R., 1994. Production of thermophilic, extracellular alkaline protease by *B. stearothermophilus* AP-4. World J. Microbiol. Biotechnol. 10, 33–35.

Godfrey, T., West, S., 1985. The application of enzymes in Industry. In: Industrial Enzymology. The Nature Press, London.

Hagihara, B., Matsubara, H., Nakai, M., Okumuki, K., 1958. Crystalline bacterial proteinase I. Preparation of crystalline proteinase of *Bacillus subtilis*. J. Biochem. 45, 185–194.

Kalisz, H.M., 1988. Microbial proteinases. In: Fiechter, A. (Ed.), Advances in Biochemical Engineering/Biotechnology, Vol. 36. Springer, Berlin, pp. 1–65.

Kwon, Y.T., Kim, J.O., Moon, S.Y., Lee, H.H., Rho, H.M., 1994. Extracellular alkaline protease from alkalophilic *Vibrio metschnikovi* strain RH 530. Biotechnol. Lett. 16, 413–418.

Lee, Y.H., Chang, H.N., 1990. Production of alkaline protease by *Bacillus licheniformis* in an aqueous two-phase system. J. Ferment. Bioeng. 69, 89–92.

Lowry, O.H., Rosebrough, N., Farr, A.L., Rondall, R.L., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–273.

Outtrup, H., 1990. Boyee Col. Microbial proteases and biotechnology. In: Fogarty, W.M., Kelly, C.T. (Eds.), Microbial Enzymes and Biotechnology. Elsevier Science, New York, pp. 227–254.

Samal, B.B., Karan, B., Stabinsky, Y., 1990. Stability of two novel serine proteinases in commercial laundry detergent formulations. Biotechnol. Bioeng. 35, 650–652.

Singh, J., Batra, N., Sobti, R.C., 2001. Serine alkaline protease from a newly isolated *Bacillus sp. SSR1*. Process Biochem. 36, 781–785.

Walsh, K.A., Wilcox, P.E., 1970. In: Perlmann, G.E., Lorand, L. (Eds.), Methods in Enzymology. Vol. 19, Academic Press, New York, pp. 31–226.

Ward, O.P., 1985. Proteolytic enzymes. In: Moo-Young, M. (Ed.), Comprehensive Biotechnology, Vol. 3. Pergamon, Oxford, pp. 789–818.