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Isolation and Characterization of a Broad Range pH and Temperature Active Protease from *Staphylococcus saprophyticus*

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Abstract

Increasing demand of protease with specific properties lead biotechnologists explore a new source of protease. Marine bacterium living in wide biological diversity and environmental change are one of the popular sources for biotechnological exploitation. This study aims to find a marine bacterium that shows ability to secrete high potent protease. *Staphylococcus saprophyticus* that isolated from marine sediments in Samaesan, Thailand secreted a broad range pH (pH 3.0-12.0) and temperature (10-90 °C) active protease. Metal ions (Na^+, Ba^{2+}, Ca^{2+}, Mg^{2+}, Hg^{2+}) did not affect the enzyme activity in contrast improve the activity. The enzyme was stable in surfactants (SDS, H_2O_2, Zeolite etc.) and hydrophobic solvents (benzene, hexane, hexadecane etc.). Broad rang pH and temperature stability and ability to work in metal ions, solvents and surfactants support the potential of this protease as a vigorous biocatalyst for industrial applications.

Keywords: metal-tolerant protease ; detergent-stable protease ; thermostable protease ; acidophilic protease

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1. Introduction

Worldwide demand of enzymes as industrial biocatalysts is growing increasingly. Among them, proteases share almost 60% contribution of the total industrial enzymes (Rao et al., 1998; Raval et al., 2014). Most of the proteases used in industrial applications are derived from plants, animals, or microorganisms (Rao et al., 1998). Microbial proteases are of major interest because of the diversity of catalytic activities, high yields, ease of genetic manipulation, rapid growth, and inexpensive culture (Kumar and Takagi, 1999; Bhunia et al., 2013). Driven by increasing industrial demands for proteases that can cope with industrial process conditions, considerable efforts have been devoted to the search for such enzymes (Bhunia et al., 2013; Iyer and Ananthanarayan, 2008). Marine environment is a source of unique microorganisms with great potential for biotechnological exploitation. Very few studies concerning the isolation and characterization of marine bacteria have been carried out and investigations in this field may lead to many new discoveries. This study attempts to find the marine bacteria that produce unique characteristic protease for application in industry.

2. Materials and Methods

2.1. Isolation of a protease-producing strain

Ten marine sediment samples were randomly collected from Koh Jan, Samaesan at different depth 9 m and 24 m. Ten grams of each of the samples were mixed with 100 ml of sterile water. The heavy particles were allowed to settle by sedimentation and the upper phase (100 µl) was directly used for screening by spreading onto Luria-Bertani agar (Sambrook et al., 1989). Cultures were maintained at 25°C for 24 hours. Colonies appeared on the plate were purified as a single colony by streaking repeatedly and the culture was maintained at the same conditions. Then, the bacteria capable of secreting protease were screened by a clear zone surrounding the bacterial colonies as shown on skim milk agar (1% (w/v) skim milk powder, 1.5% (w/v) bacto-tryptone, 0.25 % (w/v) yeast extract, 0.1% (w/v) glucose and 2.5% (w/v) NaCl, pH 7.2). Strains that showed a maximum ratio of clearzone: colony diameters were selected and assay for protease activity using azocasein as a substrate (see 2.8 Protease activity assay). Protein concentration was determined spectrophotometrically according to Bradford (Bradford, 1976) using Bio-Rad assay reagent and bovine serum albumin as the standard. The bacterial strain that showed the highest protease activity after 24 h cultivation was selected for further experiments.

2.2. Bacterial strain identification

Bacterial strain identification was based on “API Skills Bacterial Identification Method” and also by 16S rRNA gene sequence analysis (Weisburg et al., 1991). PCR amplification of 16S rRNA gene was performed using primers that were designed from the conserved regions at the base positions 22 to 41 and 1066 to 1085 located on *Escherichia coli* 16S rRNA gene (Precigou et al., 2004). Chromosomal DNA (100 ng) as purified by a GF-1 Nucleic acid extraction kit (Vivantis, Malaysia) was used as a DNA template. PCR was carried out by an initial denaturation step at 95°C for 10 min which was followed by 30 cycles at 95°C for 30 sec, 60°C for 45 sec and 72°C for 90 sec. Final extension was done at 72°C for 10 min.

The expected PCR product (about 1.1 kb) was purified with a GF-1 Gel DNA recovery kit (Vivantis, Malaysia) and was then ligated into pTG19-T vector (Vivantis, Malaysia) according to the manufacturer’s instructions. After transformation into *E. coli* DH5α, plasmids were extracted and purified by a GF-1 Plasmid DNA extraction kit (Vivantis, Malaysia). A gene insert was verified by sequence analysis (Sanger et al., 1977). Similarity of nucleotide sequence was determined using BLAST (National Center for Biotechnology Information databases, Bethesda MD, USA) and subsequently analyzed at Ez-Taxon database (Chun et al.,

2007).

To generate a 16S rRNA gene-based phylogenetic tree, sequences were aligned using the SILVA aligner (Pruesse et al., 2007). Sequence divergence was calculated using the Kimura 2-parameter model (Kimura, 1980) in MEGA 6 software (Tamura et al., 2011). MEGA 6 software was also used to create the neighbor-joining tree and to perform bootstrap analysis (1000 replicates) (Saitou and Nei, 1987). The sequence data have been submitted to the GenBank databases under accession no. KM370127.

2.3. Effect of pH on protease activity and stability

Effects of pH on protease activity and stability were studied using crude enzyme (6 U/ml) at 37°C over a pH range of 3-12 and azocasein as substrate. Buffer systems were sodium acetate (pH 3.0-6.0), phosphate (pH 6.0-8.0), Tris-HCl (pH 7.0-9.0) and carbonate (pH 9.0-12.0). For optimum pH determination, reaction mixture was incubated at 37°C for 15 min. Effect of pH on protease stability was determined with 50mM buffer at the specific pH for different hours at 37°C and then the residual activity was determined at pH 7.0.

2.4. Effect of temperature on protease activity and stability

Effect of temperature on crude protease activity was analyzed by incubation of crude protease at various temperatures (10-90°C) for 15 min. Thermostability of the protease was time-course studied at different temperatures (range 10-80°C) in a water bath and subsequently measuring the residual activity using the activity at 50°C as control.

2.5. Effect of organic solvents on the stability of protease

Crude protease solutions were mixed with equal volumes of each of the selected organic solvent to prepare the 50% (v/v) organic solution, and the mixtures were consequently shaken and incubated at 37°C for 24 h at 150 rpm. The solvent contained in the mixture was partially eliminated by evaporation at 37°C for 5 min. Residual protease activity was compared to that of the control (no solvent).

2.6. Effects of metal ion and inhibitors on protease activity

Effects of metal ions and inhibitors on the enzyme were studied by incubating crude protease for 1 h at 37°C in 50 mM Tris-HCl buffer (pH 8.0) with the selected chemicals. The residual protease activity was compared to that of the control (no chemical added).

2.7. Effects of detergents and bleaching agents on protease activity

Crude proteases were mixed with detergents (SDS, Tween80 or Triton X-100) or bleaching agents (H_2O_2, sodium perborate, sodium carbonate, sodium carboxymethyl cellulose, sodium lauryl ether sulfate, tetra sodiumphosphate, sodium alkaline silicate, zeolite, sodium xylenesulfonate, sodium toluenesulfonate, sodium tripolyphosphte, alcoholethoxylate (lauryl alcohol ethoxylate), alkylphenol ethoxylate, alkylbenzene sulfonate sodium salt, sodium nitrotriacetate or sodium percarbonater) at 37°C for 1 h. The residual protease activity was compared with that of the control (without addition).

2.8. Protease activity assay

Protease activity was measured by the hydrolysis of azocasein (Haddar et al., 2009). The reaction mixture consisted of 1% (w/v) of azocasein (125 µl) and 125 µl of enzyme solution. The reaction was incubated at 37°C for 10 min. Then, the reaction was stopped by addition of 250 µl of 0.4 M trichloroacetic acid, and the sample was placed on ice for 15 min. The sample was centrifuged at 10,000 × g for 10 min to remove precipitate. After that, the obtained supernatant was mixed with 0.4 M sodium carbonate (625 µl) and Folin reagent (125 µl). Reaction was leave at room temperature for 10 min. Finally, the OD660 was spectrophotometically measured. In a reference tube, enzyme solution was added after trichloroacetic acid. One unit (U) of protease activity was defined as the amount of enzyme liberating 1µg of tyrosine per minute under assay conditions.

3. Results and Discussion

3.1. Isolation and identification of protease producing bacterium

Marine bacteria were isolated from Koh Jan, Sammesan using marine sediments as inoculums. Out of the 12 protease-producing isolates, one that showed high protease extracellular production (6.50 ± 0.03 U/ml) was selected as a candidate of protease producer. The strain was a cocci-shape gram-positive bacterium and reacted negatively with alkaline phosphatase. Positive results were recorded for the tests of catalase, urea and VP but negative for nitrate. Fermentations/oxidations of some substrates (D-glucose, D-fructose, D-maltose, D-trehalose, D-mannitol, D-saccharose) were detected but did not occur with others (D-mannose, D-lactose, Xylitol, D-melibiose, D-raffinose, D-xylose). In addition, the strain could produce L-arginine and gave negative results with methyl-α-D-glucopyranoside and N-acetyl-glucosamine. According to its biochemical characteristics, the strain was classified as *Staphylococcus warneri* with 97.4% probability.

The classified strain was then confirmed by 16S rRNA gene sequencing. The 16S rRNA gene sequence was 99% similar to the following *Staphylococcus saprophyticus* strains: *S. saprophyticus* ZK-3 (accession no. KM095954.1), *S. saprophyticus* SCD3-3 (accession no. KF476047.1), *S. saprophyticus* T86 (accession no. HQ407261.1), *S. saprophyticus* RW26 (accession no. EU419913.1), *S. saprophyticus* SCD2-6 (accession no. KF476046.1), *S. saprophyticus* ATCC15305 (accession no. NR115607.1), and *S. saprophyticus* SCD1-2 (accession no. KF476041.1). The same confidence level (99% identity) was also found in the same genus and subspecies including *Staphylococcus* sp. KJ1-5-94 (accession no. KJ623596.1), *Staphylococcus* sp. WW60 (accession no. JQ687115.1), *S. saprophyticus* subsp. *saprophyticus* ATCC15305 (accession no. AP008934.1), and *Staphylococcus* sp. An35 (accession no. AJ551173.1). Although biochemical characteristics suggested bacterial strain as *S. warneri*, a phylogenetic tree using neighbor-joining method (Fig. 1) suggested that the strain was close to *S. saprophyticus*, a common bacterium occurring in urine specimens (Hovelius and Mårdh, 1984). Hence, the strain was identified as a strain of *S. saprophyticus*.

3.2. Effect of pH and temperature on protease activity and stability

S. saprophyticus crude protease actives (>85% relative activity) in a wide-pH range between 3 and 12 and exhibited maximum hydrolytic activity towards azocasein at pH 7.0 (Fig. 2a). More than 80% residual activity still remained after 6 h incubation between 3.0 and 12.0 pH (Fig. 2b). It could be noted that the enzyme revealed a characteristic of broad-pH active protease different from other bacterial proteases.

Secreted protease of *S. saprophyticus* could hydrolyze azocasein at all temperature ranging of 10-90 °C (Fig. 3a). The optimum temperature of the enzyme was 50 °C and no significant change of the protease activity when the temperature increases up to 90 °C. The enzyme retained more than 80% of initial activity after 6 h incubation at 10–80 °C (Fig. 3b). Previously, lipase from *Staphylococcus* sp. was found to be stable

below 40 °C and bacterial proteases generally have temperature optima in the range 30–50 °C (Houmard and Drapeau, 1972; Akram et al., 2014; Shaw et al, 2005). Thus the ability to work in broad pH and temperature as well as the stability of this enzyme is therefore appropriate for industrial use (Bhunia et al., 2013; Iyer and Ananthanarayan, 2008).

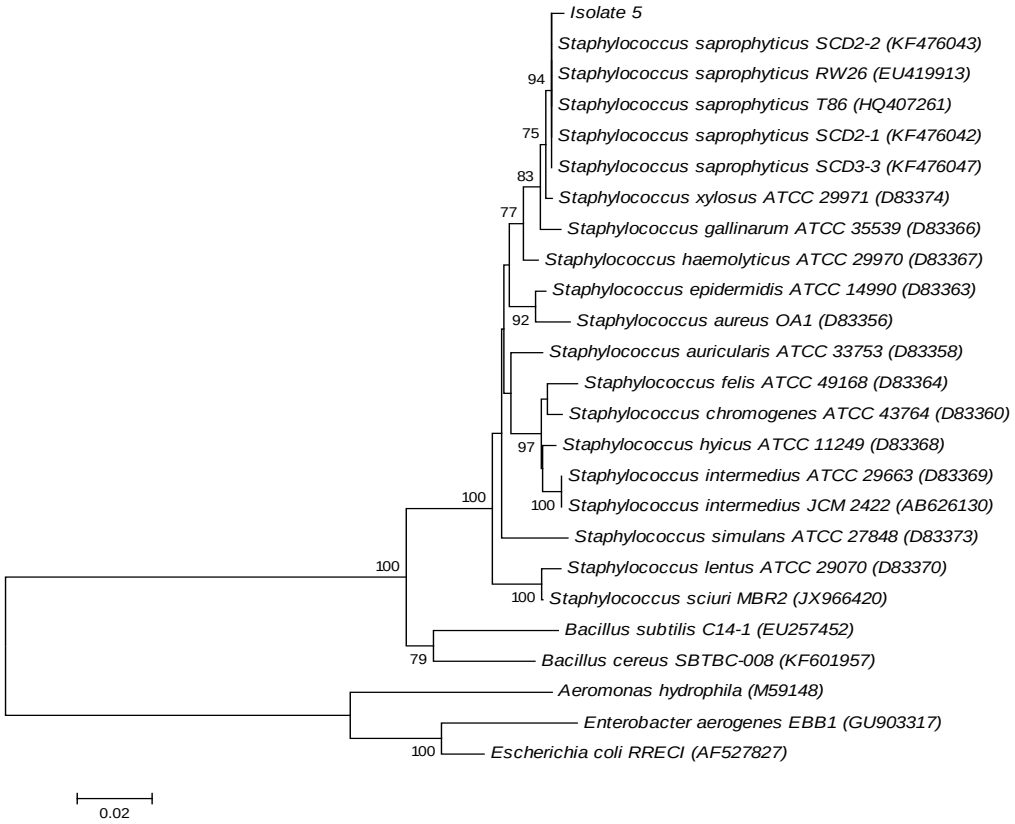

Fig. 1. Neighbor-joining phylogenetic tree of *Staphylococcus* sp. homologues to the isolate. The value next to the branch is the estimated confidence limit (expressed as a percentage, only more than 70 % is shown) for the position of the branch as determined by a bootstrap analysis (1000 replication). Evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

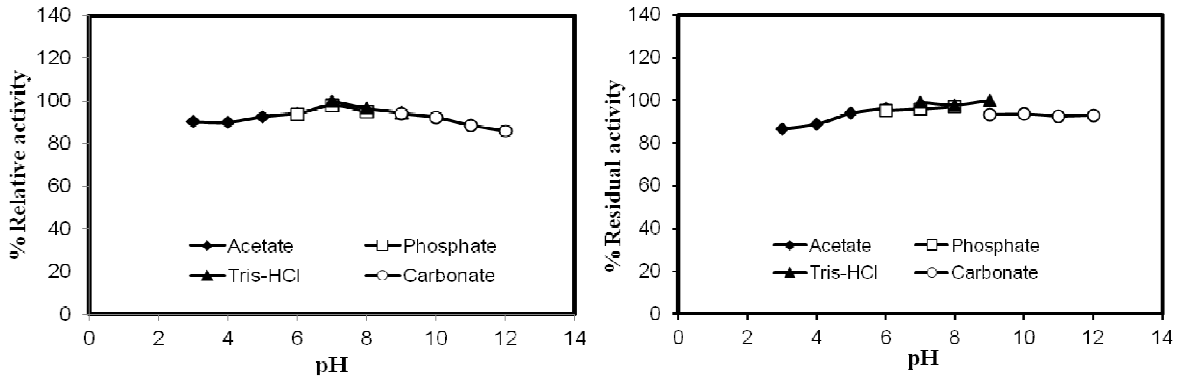

Fig. 2. Effect of pH on (a) protease activity and (b) stability. The activity was determined by incubating the reaction mixture in 50 mM buffer of a specific pH at 37 °C using azocasein as the substrate. Buffer systems used were acetate (pH 3.0–6.0; closed diamonds), phosphate (pH 6.0–8.0; open squares), Tris-HCl (pH 7.0–9.0; closed triangles) and carbonate (pH 9.0–12.0; open circles). The pH stability was studied by measuring the residual activities after 6 h incubation compares with control (100% residual activity) at pH 7.0.

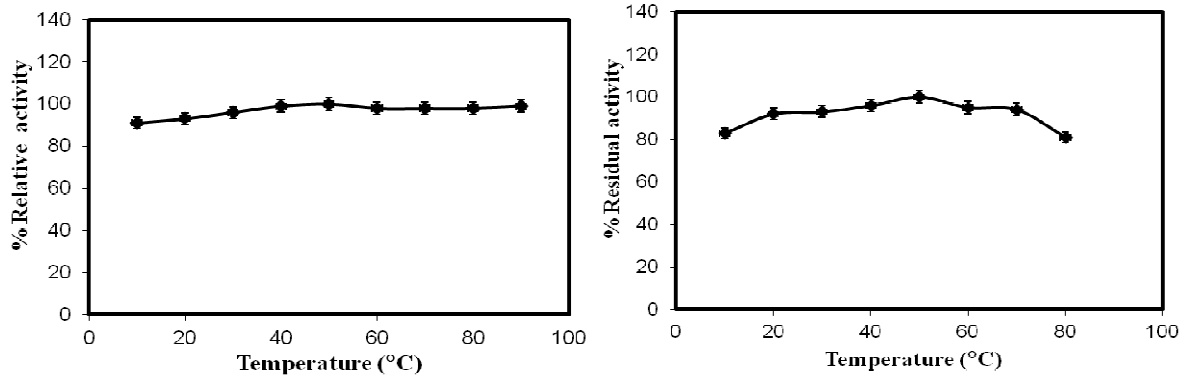

Fig. 3 Effect of temperature on (a) protease activity and (b) stability. The activity was measured by incubating the crude enzyme in the temperature range of 10–90 °C in a thermostatically controlled water bath. The thermal stability of protease was measured by pre-incubating protease solution at different temperature for 6 h, after which the residual activity was calculated comparing with control (100% of residual activity) at 50 °C.

3.3. Effect of organic solvents on the stability of protease

The enzyme retained almost 60% of its original activity in the presence of short-chain alcohols: methanol (log $P_{o/w}$ = -0.8), ethanol (log $P_{o/w}$ = -0.24), and propanol (log $P_{o/w}$ = 0.05) while isoamyl alcohol (log $P_{o/w}$ = 1.3) slightly reduced the activity (Table 1). Hydrophobic solvents: benzene (log $P_{o/w}$ = 2.0), hexane (log $P_{o/w}$ = 3.5), heptanes (log $P_{o/w}$ = 4.0), decane (log $P_{o/w}$ = 5.6), and hexadecane (log $P_{o/w}$ = 8.8) did not affect the protease activity. Perhaps these solvents hinder efficient interaction between enzymes and substrates (Laane et al., 1987). It is possible that hydrophilic solvents are able to dissolve enzyme more efficiently thus resulting in high degree of inactivation (Sugihara et al., 1991) and short-chain alcohols have low solubility in azocasein forming a new liquid phase at moderate concentrations thus leading to enzyme inactivation (Shimada et al., 1999). Recently, proteases are increasingly being used for peptide synthesis and pharmaceutical application (Rao et al., 1998; Raval et al., 2014). These require the reactions containing water-immiscible organic solvents. Thus the stability of this protease in hydrophobic solvents at wide ranges of temperatures makes the enzyme suitable for such applications as well.

Table 1. Effect of organic solvents on protease activity

Organic solvents (50%)	log $P_{o/w}$	Relative activity (%)[a]
Control	-	100
Methanol	-0.8	58
Ethanol	-0.24	60
Propanol	0.05	56
Isoamylalcohol	1.3	85
Benzene	2.0	94
Hexane	3.5	93
Heptane	4.0	95
Decane	5.6	93
Hexadecane	8.8	97

[a] The crude protease was incubated at 37°C with shaking at 150 rpm in the presence of 50% organic solvent for 24 h. The remaining protease activity was measured and expressed as the mean of three determinations comparing to control.

3.4. Effects of metal ions and inhibitors on protease activity

The protease activity of *S. saprophyticus* was two-fold enhanced by all metal ions (1mM). Around 40% increased activities were still detected when the ion concentrations reached 5 mM (Table 2). These suggest that *S. saprophyticus* protease is a metal-tolerant protease. The stabilities in the presence of Ca^{2+}, Mg^{2+} as well as Hg^{2+} recommend this protease suitable to be detergent supplements. Interaction between the enzyme and ions probably stabilize the structure preventing heat denaturation and protease attack (Brockerhoff and Jensen, 1974). EDTA did not affect the enzyme activity but improve. This implies lack of metal ion at the enzyme active site. An enhancement in protease activity after the addition of PMSF may be a result of nature of the active site serine imparts resistance to serine hydrolase inhibitor (Dharmsthiti et al., 1998). A large increase on protease activity in the presence of DTT suggested that the *S. saprophyticus* protease has a characteristic of cysteine protease in which a disulphide bond could be resided in the enzyme active site (Brockerhoff and Jensen, 1974; Dharmsthiti et al., 1998).

Table 2. Effects of metal ion and inhibitors on protease activity

Metal ions or inhibitors	Relative activity (%)[a]	
	1 mM	5 mM
Control	100	100
Na^{+}	239	146
Ba^{2+}	221	197
Ca^{2+}	251	140
Mg^{2+}	238	141
Hg^{2+}	245	133
PMSF	212	162
DTT	321	689
EDTA	232	150

[a] The crude protease was incubated at 37°C with shaking at 150 rpm in the presence of metal ion or inhibitors for 1 h. The remaining protease activity was measured and expressed as the mean of three determinations comparing to control.

3.5. Effects of detergents and bleaching agents on protease activity

The effects of detergents and bleaching agents on protease activity were tested with the concentration up to 10% ((w)v/v). As summarized in Table 3, SDS had little effect on enzyme activity by changing the conformation of protease or the interfacial properties. Addition Triton X-100 did not affect the enzyme

activity while Tween80 promotes the hydrolytic activity. Traces of sodium carbonate and sodium carboxymethyl cellulose slightly reduced the protease activity to 93% and 60%, respectively. The enzyme retained half of its original activity in the presence of sodium carboxymethyl cellulose at 5-10% concentration. A little antagonistic effect on protease activity was found by the action of 10% bleaching agents: sodium carbonate (92%), sodium perborate (91%), zeolite (87%), sodium silicate (84%), sodium pyrophospho tetrabasic (83%), sodium dihydrogen orthophosphate (80%), sodium tripolyphosphate (80%), sodium xylenesulfonate (77%), sodium p-toluene sulfonate (75%), and sodium percarbonate (71%) while H_2O_2 and nitriloria acetic acid did not inhibit the enzyme activity. The surfactant-stability makes *S. saprophyticus* protease is a good candidate biocatalyst for laundry application (Raval et al., 2014; Iyer and Ananthanarayan, 2008).

Although proteases from *Staphylococcus* sp. are well-known applicable protease, activity at broad pH and temperature as well as stability on solvents, surfactants and metal ions could not be reported. Thus, to the best of our knowledge, this is the first report on the production of versatile protease by *Staphylococcus* sp. This unique property makes the enzyme attractive for various biotechnological applications.

Table 3 Effects of detergents and bleaching agents on protease activity

Detergents or bleaching agents	Relative activity (%)[a]		
	1% (w/v, v/v)	5% (w/v, v/v)	10% (w/v, v/v)
Control	100	100	100
SDS	84	76	70
Tween80	386	342	165
Triton X-100	124	112	103
H_2O_2	137	116	106
Nitriloria acetic acid	103	107	100
Sodium dihydrogen orthophosphate	100	94	80
Sodium carbonate	93	90	92
Sodium carboxymethyl cellulose	60	55	52
Sodium perborate	106	105	91
Sodium percarbonate	100	95	71
Sodium pyrophosphate tetrabasic	109	110	83
Sodium p-toluene sulfonate	107	102	75
Sodium silicate	103	92	84
Sodium tripolyphosphate	103	94	80
Sodium xylenesulfonate	98	96	77
Zeolite	108	99	87

[a] The crude protease was incubated at 37°C with shaking at 150 rpm in the presence of detergents or bleaching agents for 1 h. The remaining protease activity was measured and expressed as the mean of three determinations comparing to control.

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