Characterization of detergent compatible protease from halophilic *Virgibacillus* sp. CD6

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Abstract

A halophilic bacterium, *Virgibacillus* sp. strain CD6, was isolated from salted fish and its extracellular protease was characterized. Protease production was found to be highest when yeast extract was used as nitrogen source for growth. The protease exhibited stability at wide range of salt concentration (0–12.5%, w/v), temperatures (20–60 °C), and pH (4–10) with maximum activity at 10.0% (w/v) NaCl, 60 °C, pH 7 and 10, indicating its polyextremophilicity. The protease activity was enhanced in the presence of Mg²⁺, Mn²⁺, Cd²⁺, and Al³⁺ (107–122% relative activity), and with retention of activity > 80% for all of other metal ions examined (K⁺, Ca²⁺, Cu²⁺, Co²⁺, Ni²⁺, Zn²⁺, and Fe³⁺). Both PMSF and EDTA inhibited protease activity, denoting serine protease and metalloprotease properties, respectively. High stability (> 70%) was demonstrated in the presence of organic solvents and detergent constituents, and the extracellular protease from strain CD6 was also found to be compatible in commercial detergents. Proteinaceous stain removal efficacy revealed that crude protease of strain CD6 could significantly enhance the performance of commercial detergent. The protease from *Virgibacillus* sp. strain CD6 could serve as a promising alternative for various applications, especially in detergent industry.

Keywords Protease · Halophilic bacteria · *Virgibacillus* genus · Thermostable · Detergent compatible

Introduction

Halophilic microorganisms have received great interest as potential source of industrial enzymes that are equipped with exceptional properties ideal for industrial applications. The adaptations mechanisms and flexible metabolic pathway of halophilic microorganisms in the saline environments enable them to produce bioactive metabolites with applied value (DasSarma and DasSarma 2015; Edbeib et al. 2016). Research on halophilic enzymes especially hydrolases (cellulases, amylases, proteases and lipases) has recently increased dramatically (Dalmaso et al. 2015; Dammak et al. 2016; Yin et al. 2015), presumed to have optimal activities in extreme conditions, and could be a potential source for industrial applications.

Protease, a large group of noteworthy and versatile enzymes which hydrolyze peptide bonds of proteins, can be

categorized based on criteria such as reaction type which they catalyzed, chemical nature of catalytic site, and evolutionary protein structure relationship (Rao et al. 1998). Protease is extensively used in various industries such as food production (Tavano 2013), leather processing (Abraham et al. 2014; Karn and Kumar 2015), detergent formulation (Gupta et al. 2002; Kumar and Takagi 1999; Niyonzima and More 2015a, b; Singh et al. 2016), waste management (Cao et al. 2009; Karn and Kumar 2015), and pharmaceutical industry (Gupta et al. 2013). Protease accounted for more than total 60% production of enzymes for application in various industries (Homaei et al. 2016). In details, protease used in detergent industry contributed to approximately 30% of total enzymes production worldwide (Haddar et al. 2009).

In enzyme production industries, the major challenges that hinder the efficient and economic commercial scale application of proteases are their stability in broad range of pH, temperature, salinity, as well as their optimal activity in the presence of metal ions, organic solvents, and detergents. Moreover, the enzyme purification steps also contribute to the cost of production (Niyonzima and More 2015a). Therefore, characterization and production of crude protease with attractive

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properties from wild bacterial isolate could be an alternative as it is a more cost-effective way compared to production of protease that involves purification steps and protein engineering approach (Niyonzima and More 2015b). In this study, we reported a halophilic bacterium *Virgibacillus* sp. strain CD6 isolated from salted fish and its extracellular protease was characterized. The enzyme displayed polyextremophilic features and showed enzymatic compatibility in the presence of commercial detergents.

Materials and methods

Isolation and screening of protease-producing halophilic bacterium

Salted fish sample was obtained from groceries shop located in Terengganu, Malaysia. The sample was obtained aseptically from the fish and pounded in porcelain mortar. One gram of the sample was serially diluted and spread on Marine Agar 2216 (BD Difco™) medium for isolation of microbes. A strain designated as CD6 was isolated and maintained by routine culture on Marine Agar 2216 (BD Difco™) medium. The isolate was stored at -80°C with in marine medium supplemented with 20% (v/v) glycerol. The isolate was further screened for its proteolytic activity using marine agar supplemented with 10% (w/v) skimmed milk. The plates were incubated for 24 h at 37°C . The formation of halo zones (clear zones) around the colonies was taken as evidence of proteolytic activity.

Phenotypic characterization

The colony shape, texture, and pigmentation of strain CD6 on marine agar were observed after 24 h of incubation at 37°C . Gram staining was carried out based on the method described by Madigan and co-workers (Madigan et al. 2009). The commercially available API kits including API 50 CHB/E, API 20E, API 20NE, and API ZYM were used to determine the biochemical properties of the strain according to the manufacturer's instructions (Bio-Mérieux, France). The oxidase reaction was tested using 1% (w/v) N,N,N,N,-tetramethyl-1,4-phenylenediamine reagent (Sigma Chemical Co., USA) and catalase activity was assessed using 3% (v/v) hydrogen peroxide. Antibiotic susceptibility was screened against strain CD6 using disc diffusion method on marine agar (Nokhal and Schlegel 1983). The antibiotics discs used in this study were ampicillin, kanamycin, and tetracycline.

DNA amplification, sequencing, and phylogenetic analysis

The genomic DNA of strain CD6 was extracted using Promega's Wizard® Genomic DNA Purification Kit according

to the manufacturer's instructions. The 16S rRNA gene was amplified using the universal primers 27F (5'-AGA GTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAG GTGWTCCARCC-3'), as described (Lane 1991). The PCR reaction mixture contained 12.5 μl of the GoTaq® Mastermix (Promega), 1 μl of each primer, and 2 μl of template in a final volume of 25 μl . PCR amplification included an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 1 min, 47°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 10 min. The 16S rRNA gene product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions and was sent to First BASE Laboratories, Seri Kembangan, Selangor, Malaysia for sequencing. The nearly full-length 16S rRNA sequence of strain CD6 was searched against sequences from National Center for Biotechnology Information (NCBI) database for identification. The closely related sequences were retrieved from GenBank and aligned using BioEdit software. The phylogenetic analysis was performed with MEGA 7 software program (Kumar et al. 2016) using Kimura 2-parameter model. The phylogenetic trees reconstructed using neighbor-joining (Saitou and Nei 1987) methods with bootstrap value of 1000.

Nucleotide sequence accession number

The 16S rRNA gene sequence of the strain CD6 has been deposited in the NCBI database under GenBank accession number KX907776.

Inoculum preparation and protease production in different nitrogen sources

For inoculum preparation, a loopful of fresh colonies of strain CD6 was inoculated in protease production medium containing K_2HPO_4 (7.0 g/l), KH_2PO_4 (2.0 g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (5.0 g/l), CaCl_2 (0.1 g/l), NaCl (20.0 g/l), $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ (5.0 g/l), and yeast extract (10.0 g/l), incubated at 37°C , 200 rpm for 24 h. Supernatant was removed and cells were re-suspended with equal volume of 0.1 M phosphate buffered saline. Then, 5% (v/v) of inoculum was transferred into new protease production medium (the same components as described above) and incubated at 37°C , 200 rpm for another 24 h. Culture was then centrifuged at 4°C , 10,000 rpm for 20 min to remove cells and insoluble materials. Cell-free supernatant was obtained as crude enzyme and was used in quantitative protease activity assays. Effect of nitrogen sources on protease production was determined by substituting yeast extract with soybean meal, peptone, tryptone, casein, gelatin, or casamino acid.

Protease assay

Protease activity was assayed by the modified method of Secades and Guijarro (1999) using azocasein (Sigma Chemical Co., USA) as a substrate. A 0.12 ml of crude enzyme solution was added into 0.48 ml of 50 mM Tris–HCl (pH 8.0) reaction buffer containing 1% (w/v) of azocasein and incubated at 37 °C for 30 min. Reaction was terminated by adding 0.6 ml of 10% (w/v) trichloroacetic acid (TCA) followed by incubation on ice for 30 min. Reaction mixture was centrifuged at 4 °C, 15,000 rcf for 10 min to remove precipitated protein. Next, 0.8 ml of supernatant was neutralized by adding 0.2 ml of 1.8 M NaOH. Absorbance at 420 nm (A_{420}) was measured using spectrophotometer. One unit of protease activity (U/ml) was defined as amount of enzyme which yields an increase in A_{420} of 0.01 under assay conditions. All enzyme assay experiments were conducted in biological triplicates. Control was prepared by adding enzyme solution after addition of 10% (w/v) TCA to the reaction mixture.

Protein content determination

Protein content was determined by Lowry assay (Lowry et al. 1951) using bovine serum albumin (BSA) as standard.

Effect of temperature, pH, and salt concentration on protease activity and stability

The effect of temperature, pH, and salt concentration on protease activity was investigated by incubating reaction mixture at different temperatures (20–70 °C), pH (4–11), or various NaCl concentration (0–15%, w/v). Protease stability was studied by pre-incubating the protease for 1 h at different temperatures (20–70 °C), different pH buffers (4–11), or various NaCl concentration (0–15%, w/v) prior to residual activity (%) determination by standard protease assay. Buffer system used was: 50 mM citrate phosphate (pH 4–6), 50 mM sodium phosphate (pH 6–8), and 50 mM glycine–NaOH (pH 9–11).

Effect of metal ions and inhibitors on protease activity

Effect of various metal ions including K^+ , Ca^{2+} , Cd^{2+} , Cu^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , Al^{3+} , and Fe^{3+} (final concentration 5 mM) on protease activity was examined. Meanwhile, effect of potential inhibitors on protease activity was investigated using various substrates including phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid (IAA), urea, dithiothreitol (DTT), and β -mercaptoethanol. Protease was pre-incubated with aforementioned metal ions or inhibitors at 50 °C for 1 h

and then assayed by standard protease assay. Relative activity (%) was expressed by taking enzyme activity without any metal ions and inhibitors (control) as 100%.

Effect of organic solvents on protease activity

Protease was pre-incubated at 50 °C for 4 h in the presence of various organic solvents [acetone, chloroform, dimethyl sulfoxide (DMSO), ethanol, isopropanol, or methanol in 25% (v/v)], and the protease activity was measured by standard protease assay. Relative activity (%) was determined by taking enzyme activity without any organic solvent (control) as 100%.

Effect of detergent constituents on protease activity

Influence of 1% final concentration of surfactants (ionic and non-ionic) and oxidizing agent [sodium dodecyl sulfate (SDS) in w/v, sodium deoxycholate, Tween 20, Tween 40, Tween 60, Tween 80, Triton X-100, and hydrogen peroxide (H_2O_2) in v/v] on protease activity were determined. Protease was pre-incubated with detergent constituents at 50 °C for 1 h and then assayed by standard protease assay. Relative activity (%) was expressed by taking enzyme activity without any detergent constituent (control) as 100%.

Compatibility of protease with commercial detergents

Compatibility of protease with commercial detergents (Axion®, Surf Excel®, Ariel®, Dixan®, Sunlight® and Top®) were assessed. Solid detergents were diluted in distilled water (0.7%, w/v), whereas liquid detergents were diluted 100-fold to stimulate washing condition (Niyonzima and More 2015b). Diluted detergent solutions were boiled for 15 min to denature the potential enzymes originally presented, if any, and confirmed by protease assay. Detergent solutions were incubated with crude protease from strain CD6 at 50 °C for 1 h and then assayed by standard protease assay. Relative activity (%) was expressed by taking enzyme activity without any commercial detergent (control) as 100%.

Proteinaceous stain removal activity of protease

The efficacy of the crude protease in stain removal was assessed using white cotton cloth (10 × 10 cm) stained separately with various proteinaceous stains (chicken blood, egg yolk and soy sauce) and dried at 100 °C for 10 min. The efficacy of crude protease in enzymatic commercial detergent (Surf Excel®) and non-enzymatic commercial detergents were examined. Non-enzymatic detergent was prepared by boiling the detergent for 15 min to denature the enzymes that originally presented in commercial detergent.

The stained cloth was placed in separate tray and the set-up was as follows:

Set 1 (control): Tray with 100 ml distilled water + proteinaceous stained cloth.

Set 2: Tray with 100 ml distilled water + 1% non-enzymatic commercial detergent + proteinaceous stained cloth.

Set 3: Tray with 100 ml distilled water + 1% enzymatic commercial detergent + proteinaceous stained cloth.

Set 4: Tray with 100 ml distilled water + crude protease + proteinaceous stained cloth.

Set 5: Tray with 100 ml distilled water + 1% non-enzymatic commercial detergent + crude protease + proteinaceous stained cloth.

Set 6: Tray with 100 ml distilled water + 1% enzymatic commercial detergent + crude protease + proteinaceous stained cloth.

All experimental sets were incubated at 50 °C for 30 min. During the incubation, the clothes were rinsed by with water every 5 min. The stain removal activity was visually examined and compared after the cotton cloth was dried.

Results and discussion

Isolation, phenotypic characterization, and identification of protease-producing halophilic bacterium

A halophilic bacterium designated as CD6 was isolated from salted fish. The qualitative proteolytic screening showed the

formation of clear halo zone around colonies on skim milk agar, confirming its extracellular protease activity. This strain was identified as *Virgibacillus* sp. based on 16S rRNA gene analysis, demonstrating closest similarity (99%) to *Virgibacillus massiliensis* Vm-5^T (accession no NR144700) (Khelaifia et al. 2015). In neighbor-joining phylogenetic tree, strain CD6 clustered within the clade comprising species of the genus *Virgibacillus* (Fig. 1). The phenotypic characteristics of strain CD6 in comparison with the closest *Virgibacillus* species are listed in Table 1.

The *Virgibacillus* sp. strain CD6 is Gram-positive bacterium with rod shape, catalase, and oxidase positive, able to produce acid from D-melibiose but not D-fructose, corresponded to description of this genus (Heyrman et al. 2003). When compared strain CD6 to its closely related species (Table 1), some distinctions in biochemical characteristics were observed, suggesting the uniqueness of this strain. For example, urease, alkaline phosphatase, and N-acetyl-β-glucosaminidase were produced by strain CD6 but not in case of *Virgibacillus massiliensis* Vm-5^T (Khelaifia et al. 2015). On the other hand, unlike *Virgibacillus salexigens* C-20Mo^T (Garabito et al. 1997; Heyrman et al. 2003), nitrate was reduced by *Virgibacillus* sp. strain CD6.

Protease production in different nitrogen sources

Effect of various nitrogen sources on protease production of *Virgibacillus* sp. strain CD6 was determined. Results

Fig. 1 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationship between strain CD6 with other members of genus *Virgibacillus* and other closely related bacteria. Percentages at nodes are levels of bootstrap support based on 1000 resampled data set

Table 1 Phenotypic characteristics of *Virgibacillus* sp. strain CD6 and other two closely related *Virgibacillus* species

Characteristics	1	2	3
Gram stain	+	+	+
Salt requirement	+	+	+
Production of			
Catalase	+	+	+
Oxidase	+	+	+
Urease	+	–	ND
Alkaline phosphatase	+	–	ND
<i>N</i> -Acetyl- β -glucosaminidase	+	–	ND
Hydrolysis of			
Gelatin	+	ND	+
Casein	+	ND	+
Aesculin	+	ND	+
Reduction of nitrate	+	+	–
Acid production from			
D-Galactose	–	–	w
D-Glucose	–	+	w
D-Fructose	–	+	w
D-Mannose	–	+	w
D-Mannitol	–	+	–
Susceptibility			
Ampicillin	+	+	+
Habitat	Salted fish	Human gut	Salterns

Strains: 1, *Virgibacillus* sp. CD6; 2, *V. massiliensis* Vm-5^T (Khelaifia et al. 2015); 3, *V. salexigens* C-20Mo^T (Garabito et al. 1997; Heyrman et al. 2003)

+, positive; –, negative; w weakly positive reaction; ND not determined

revealed that this bacterium could utilize variety of organic nitrogen sources for growth and for protease production. Protease production was seen highest when cells were grown in medium containing yeast extract as a sole nitrogen source (29.02 ± 0.35 U/mg), followed by casein (25.23 ± 2.40 U/mg), casamino acid (24.19 ± 1.78 U/mg), soybean meal (23.69 ± 0.48 U/mg), tryptone (22.85 ± 2.05 U/mg), and peptone (17.30 ± 0.94 U/mg), and lowest protease production was observed when gelatin was used as a nitrogen source (10.82 ± 0.65 U/mg). Therefore, yeast extract was selected as a nitrogen source for crude protease production in subsequent experiments.

Optimum pH, salinity, and temperature of protease

The extracellular protease from strain CD6 was found to have maximum activity at pH 10 (Fig. 2a), in accordance to protease from *Virgibacillus pantothenicus* (Gupta et al. 2008) and *Virgibacillus marismortui* NB2-1 (Chamroensaksri et al. 2008). Nevertheless, when compared to

Fig. 2 Effect of different pH (a), salt concentration (b), and temperatures (c) on activity and stability of protease from *Virgibacillus* sp. CD6. Relative activity (%) and stability (%) were calculated by relative to the case of reaction at which maximum activity was taken as 100%. Mean values ($n = 3$) are expressed and standard deviations are indicated as error bars

proteases of *Virgibacillus halodenitrificans* RSK CAS1 and *Virgibacillus* sp. SK37 which exhibited maximal activity at pH 9 and 8–8.5, respectively (Sathishkumar et al. 2015; Sinsuwan et al. 2007), protease from strain CD6 disclosed maximum activity at higher pH. Protease active at such alkaline conditions was demonstrated by strain CD6 indicating its suitability to be incorporated into detergent formulations (Kumar and Takagi 1999; Niyonzima and More 2015b) and applications in sliver

recovery process (Debette 1991; Karn and Kumar 2015). Furthermore, protease of *Virgibacillus* sp. strain CD6 was also stable over wide range of pH (4–10) (Fig. 2a), showing a slightly broader range of pH stability than proteases from other members under the same genus (pH 5–10) (Gupta et al. 2008; Sathishkumar et al. 2015; Sinsuwan et al. 2009), displaying a better versatility in different pH conditions.

Besides that, optimal activity of protease of strain CD6 was observed at 10% (w/v) NaCl (Fig. 2b). This result indicated that it is a moderately halophilic enzyme. Optimal activity of protease from strain CD6 at such salt concentration did not share exact similarity with other species within the same genus. Proteases of *V. halodenitrificans* RSK CAS1 and *Virgibacillus* sp. SK37 exhibited optimal activity up to 20–25% (w/v) of salt concentration (Sathishkumar et al. 2015; Sinsuwan et al. 2007), indicating higher salt tolerance compared to this study. Nonetheless, protease in this case manifested more exclusive activity in terms of halophilicity when compared to protease from *V. halodenitrificans* SK1-3-7, *Virgibacillus marismortui* NB2-1, and *Virgibacillus dokdonensis* VITP14 where they have protease with maximum activity at 5–8% (w/v) NaCl (Chamroensaksri et al. 2008; Montriwong et al. 2015; Rajeswari et al. 2012). It is also notable that protease of strain CD6 was stable from 0 to 12.5% (w/v) NaCl (Fig. 2b), perceived considerably high stability in both non-halophilic and halophilic conditions. These properties allowed them to be potentially applied in food fermentation and soy sauce production (Setyorini et al. 2006).

Moreover, the protease of strain CD6 exhibited optimal activity at 60 °C, with > 75% relative activity at 70 °C (Fig. 2c). Its optimal activity at this temperature is a distinguishing feature as is higher than optimal temperatures of most proteases reported previously from *Virgibacillus* spp. (40–50 °C) (Chamroensaksri et al. 2008; Gupta et al. 2008; Rajeswari et al. 2012; Sathishkumar et al. 2015; Sinsuwan et al. 2008, 2009). The protease of strain CD6 displayed a better thermal stability up to 60 °C (Fig. 2c) when compared to other strains from *Virgibacillus* (< 60 °C) (Gupta et al. 2008; Montriwong et al. 2015; Sinsuwan et al. 2009). This protease was also found to be stable at lower temperatures (20–30 °C) which are well suited for applications in sustainable washing using minimal energy. Multifaceted potential was clearly demonstrated by this protease in terms of temperature.

Effect of metal ions and inhibitors on protease activity

In terms of effect of metal ions, protease of strain CD6 disclosed considerably stability (> 80%) in the presence of all of the tested metal ions (Table 2), with enrichment

in activity by metal ions such as Mg^{2+} , Mn^{2+} , Cd^{2+} , and Al^{3+} (107–122% relative activity). It has been recognized that Mg^{2+} and Mn^{2+} play a vital role in stabilizing enzyme to prevent thermal degradation (Kumar and Takagi 1999), protease activity increased by Cd^{2+} also observed in similar strains (Sinsuwan et al. 2007, 2008); however, this is the first time reported that Al^{3+} can promote activity of protease. In addition, the protease of strain CD6 also remained high activity in the presence of Ni^{2+} which was not seen in other *Virgibacillus* spp. formerly (Gupta et al. 2008; Montriwong et al. 2015; Sathishkumar et al. 2015; Sinsuwan et al. 2008, 2009). The ability of protease from strain CD6 which remained high activity in the presence of metal ions makes it useful for removal of proteinaceous waste co-contaminated by metal ions.

Protease can be classified by assessing its sensitivity to inhibitors (North 1982) and was examined in this study (Table 2). Protease activity was decreased sharply to 69.4% when pre-incubated with 5 mM of PMSF and significantly inhibited protease activity indicating the presence of serine residue in enzyme catalytic site. Similarly, protease activity was moderately inhibited by EDTA, showing metalloprotease property. Meanwhile, reducing agent such as DTT also

Table 2 Effect of various metal ions and inhibitors on activity of protease from *Virgibacillus* sp. strain CD6 after pre-incubation at 50 °C for 1 h

Additives	Concentration (mM)	Relative activity (%)
Control	–	100
Metal ions		
Ca^{2+}	5	85.4
Cu^{2+}	5	85.8
Co^{2+}	5	88.1
Cd^{2+}	5	107.3
Mg^{2+}	5	111.9
Mn^{2+}	5	113.5
Al^{3+}	5	122.3
Ni^{2+}	5	93.5
Zn^{2+}	5	90.4
Fe^{3+}	5	95.8
K^{+}	5	99.2
Inhibitors		
PMSF	5	69.4
EDTA	5	70.3
IAA	5	96.3
Urea	10	80.6
DTT	10	71.4
β -Mercaptoethanol	10	89.4

Each value represents mean of three determinations (SD < 5% of the mean)

causes inhibitory effect to protease activity with decrease in activity around 30%, while protease was slightly hindered by β -mechaproethanol and hydrogen breaker urea. Nevertheless, IAA causes no adverse effect on protease activity, indicating no sign of cysteine protease.

Effect of organic solvents on protease activity

Various organic solvents exhibited different effects on protease activity, as summarized in Table 3. Enhancement in protease activity of strain CD6 in the presence of methanol (polar solvent) was noticed during stability testing of organic solvents, make this protease a promising candidate in water-organic solvent systems. In addition, this is first seen that chloroform could promote the protease activity by 14%, while depleted activity (59% relative activity) was spotted for previously reported protease from *Virgibacillus* sp. SK33 (Sinsuwan et al. 2009) and no stability assessment was reported in the presence of this solvent for protease of other members from *Virgibacillus* genus (Montriwong et al. 2015; Sathishkumar et al. 2015; Sinsuwan et al. 2009).

Table 3 Effect of organic solvents and detergent constituents on activity of protease from *Virgibacillus* sp. strain CD6 after pre-incubation at 50 °C

Additives	Relative activity (%)
Control	100
Organic solvent (25%, v/v)	
Acetone	71.7
Ethanol	75.6
DMSO	85
Isopropanol	91.7
Chloroform	114.4
Methanol	144.4
Detergent constituents (1%)	
SDS	77.8
Sodium deoxycholate	69.6
Triton X-100	85
Tween 20	106.2
Tween 40	84
Tween 60	100
Tween 80	86.6
H ₂ O ₂	97.4

Each value represents mean of three determinations (SD < 5% of the mean)

Effect of surfactants and oxidizing agent on protease activity

The effect of detergent constituents including (ionic and non-ionic) surfactants and oxidizing agent was also examined (Table 3). The order of protease stability in the presence of different detergent constituents is as: Tween 20 > Tween 60 > H₂O₂ > Tween 80 > Triton X-100 > Tween 40 > SDS > sodium deoxycholate. Reduced protease activity was observed in the presence of SDS (77.8%) and sodium deoxycholate (69.6%), which could be due to conformation of enzymes changed in the presence of these ionic surfactants (Niyonzima and More 2015a). Nevertheless, substantial activity still maintained (> 80%) in the presence of non-ionic surfactants (Triton X-100, Tween 20, Tween 40, Tween 60, and Tween 80) and oxidizing agent (H₂O₂). This could be due to non-ionic surfactants create suitable environment for catalytic activity of protease by lessen surface tension of medium and protease can resist oxidizing reaction (Niyonzima and More 2015a).

Compatibility of protease with commercial detergents

Interestingly, protease activity of strain CD6 was highly enhanced in the presence of commercial detergents Axion®, Surf Excel®, Ariel® and Top® (118–165% relative activity) and remained high protease activity was seen in the presence of Dixan® (99.3%), as shown in Table 4. While protease activity in Top® and Dixan® has not been reported before in other *Virgibacillus* spp., our results showed that the protease from strain CD6 remained stable in the presence of both of these commercial detergents. Furthermore, protease from strain CD6 also displayed a better compatibility in Surf Excel® and Ariel® (enhanced activity of 138.8 and 146.2%,

Table 4 Compatibility of protease from *Virgibacillus* sp. strain CD6 with various commercial detergents after 1 h of pre-incubation at 50 °C

Commercial detergents	Relative activity (%)
Control	100
Axion ®	164.7
Surf Excel ®	138.8
Ariel ®	146.2
Dixan ®	99.3
Sunlight ®	63.8
Top ®	118.4

Enzyme activity without any commercial detergents (control) was taken as 100%. Each value represents mean of three determinations (SD < 5% of the mean)

Fig. 3 Proteinaceous stain removal activity of crude protease from *Virgibacillus* sp. CD6. **a** Blood stained cloth; **b** egg yolk stained cloth; **c** soy sauce stained cloth; i, stained cloth washed with distilled water (control); ii, stained cloth washed with non-enzymatic deter-

gent alone; iii, stained cloth washed with enzymatic detergent alone; iv, stained cloth washed with protease alone; v, stained cloth washed with non-enzymatic detergent and protease; vi, stained cloth washed with enzymatic detergent and protease

respectively) compared to proteases from other *Virgibacillus* spp. (ranging from 0 to 76.4% relative activity) (Gupta et al. 2008; Sathishkumar et al. 2015) and *Bacillus* spp. (ranging from 52 to 97% relative activity) (Niyonzima and More 2015a). These consequences demonstrated that the protease of strain CD6 manifested impressive suitability to most of commercial detergents investigated.

Proteinaceous stain removal efficacy of protease

Various proteinaceous stains' (chicken blood, egg yolk, and soy sauce) removal efficacy of protease from strain CD6 was visualized as shown in Fig. 3. When non-enzymatic detergent, enzymatic detergent, and crude protease were individually applied onto blood [Fig. 3a(ii)–(iv)], egg yolk [Fig. 3b(ii)–(iv)], and soy sauce stained cloth [Fig. 3c(ii)–(iv)], respectively, removal activities were observed in comparison to control [Fig. 3a(i), b(i), c(i)]. Similarly, a certain degree of removal activities was also shown in non-enzymatic detergent added with crude protease on different proteinaceous stained clothes, as displayed in Fig. 3a(v), b(v), c(v) for blood, egg yolk and soy sauce, respectively. Interestingly, a nearly complete stain removal was observed for all proteinaceous stains

investigated when both crude protease and enzymatic commercial detergent were applied together [Fig. 3a(vi), b(vi), c(vi)]. This finding demonstrated that protease from strain CD6 could significantly enhance the performance of commercial detergent, which, therefore, could serve as a potential candidate in detergent industry.

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Compliance with ethical standards

Conflict of interest All authors declared that there are no conflicts of interest.

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