Microbiol. Biotechnol. Lett. (2020), 48(3), 358–365
http://dx.doi.org/10.4014/mbl.1912.12015
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Biochemical Characterization of a Novel Alkaline and Detergent Stable Protease from *Aeromonas veronii* OB3

Laila Manni*, Asmae Misbah, Nouhaila Zouine, and Samir Ananou

Laboratory of Microbial Biotechnology, Faculty of Sciences and Techniques, Sidi Mohammed Ben Abdellah University, Moroco

Received: December 23, 2019 / Revised: June 5, 2020 / Accepted: June 8, 2020

An organic solvent- and bleach-stable protease-producing strain was isolated from a polluted river water sample and identified as *Aeromonas veronii* OB3 on the basis of biochemical properties (API 20E) and 16S rRNA sequence analysis. The strain was found to hyper-produce alkaline protease when cultivated on fish waste powder-based medium (HVSP, 4080 U/ml). The biochemical properties and compatibility of OB3 with several detergents and additives were studied. Maximum activity was observed at pH 9.0 and 60℃. The crude protease displayed outstanding stability to the investigated surfactants and oxidants, such as Tween 80, Triton X-100, and H_2O_2, and almost 36% residual activity when incubated with 1% SDS. Remarkably, the enzyme demonstrated considerable compatibility with commercial detergents, retaining more than 100% of its activity with Ariel and Tide (1 h, 40℃). Moreover, washing performance of Tide significantly improved by the supplementation of small amounts of OB3 crude protease. These properties suggest the potential use of this alkaline protease as a bio-additive in the detergent industry and other biotechnological processes such as peptide synthesis.

Keywords: **Protease, *Aeromonas veronii*, alkaline, detergent, fish waste medium, solvent-stable**

Introduction

Enzymes have fascinated the researchers due to their extensive power of catalysis [1]. Proteases are one of the major groups of industrial enzymes [2, 3]. Proteases cleave peptide links of proteins and peptides by hydrolysis reaction and are widely present in all living organisms such as plants, animals and microorganisms [4]. They are roughly classified into six major families based on their active sites and catalytic mechanisms, i.e., serine, aspartic, cysteine, threonine, asparagine and metalloproteases [5, 6]. The total enzyme sales around the world account for nearly 40–60% [7]. Bacterial proteases are the most studied enzymes due to their extensive potential in various industrial applications. The increased interest in microbially produced proteases is mainly due to their excellent properties which make them suitable for industrial application. They have in fact, distinct advantages such as easy production and the ability to operate under harsh pH and temperature conditions [2, 8, 9].

The increased industrial demand for detergent proteases over the years is due to their wide temperature and pH range, a broad specificity for substrates, and stability and compatibility with surfactants and oxidizing agents [6, 9, 10]. For these reasons, finding new protease producers and obtaining enzymes with improved properties is compulsory. Although different alkaline proteases have been isolated from several bacteria and fungi [2, 4, 9], few have the adequate properties that can be commercially exploited. Moreover, many bacteria to secrete new alkaline proteases have still remained uncharacterized.

Aeromonas genus was recently identified as an extracellular stable alkaline protease producer [11, 12]. However,

***Corresponding author**
Tel: +212 6 10186559, Fax: +212-535-60-82-14
E-mail: laila.manni@usmba.ac.ma

reports on optimization, characterization of *Aeromonas* alkaline proteases are still limited [13–15]. This work described the biochemical characterization of a novel surfactant and oxidant-stable alkaline protease produced by *A. veronii* OB3. The organic solvent stability was also investigated.

Material and Methods

Bacterial strain

Aeromonas veronii OB3 was isolated from valley in Fes, Morocco. The samples were preliminary screened for the production of extracellular proteases by using skimmed-milk agar plates and incubated from 24 to 48 h at 37℃. Protease-producing strains forming a clear hydrolysis halo were then purified. The isolate OB3 with the highest proteolytic activity was selected. The isolate was identified as *A. veronii* by combining Gram staining, oxidase test and by using the API 20E and 16S rRNA sequence analysis. The sequences were aligned using CLUSTALW software [16]. The accession number of the 16S rRNA sequence from this study is MN596812.

Preparation of fish wastes powder

Briefly, combined heads and viscera wastes from sardinella (*Sardinella aurita*) collected from a local market in Fez, Morocco were thoroughly washed with tap water. The wastes were then mixed with distilled water as described by Sellami-Kamoun *et al.* [17] and heated at 90℃ for 20 min to inactivate the endogenous enzymes. The resulting press cake was minced by mixer grinder, dried at 80℃ during 24–48 h and grinded into powder and then stored in clean and dry glass container at room temperature.

Enzyme production

Protease production by *A. veronii* OB3 was carried out in media composed solely of 10 g/l of Heads and Viscera Sardinella Powder (HVSP); pH 8.0. The pre-cultures were cultivated overnight in Luria-Bertani medium (LB) [18] and used for the inoculation of production medium. Cultures were maintained at 37℃ in rotatory shaker at 200 rpm for 24 h. The cell free supernatant collected by centrifugation of the culture broth was used to calculate protease activity.

Protease assay

The protease activity was carried out based on Kembhavi method [19] with slight modification. Briefly a 0.5 ml of suitably diluted enzyme solution was mixed with 0.5 ml of 1% (w/v) casein in the same buffer (pH 8.0) and the reaction mixture was kept at 60℃ for 15 min. After incubation, the reaction was stopped by adding 0.5 ml trichloroacetic acid (20%) and cooled at room temperature for 15 min. Finally, the absorbance was read in a spectrophotometer under 280 nm wavelength after centrifugation to remove the resulting precipitate.

Biochemical properties

Effect of pH on protease activity and stability. The optimum pH was determined with casein as substrate in various buffers from 3.0 to 12.0 at 60℃. For pH stability measurements, the enzyme was preincubated in the same buffers for 1 h at 30℃ and then the residual activity was assayed as described above. The non-pre-incubated enzyme is considered as a control for the calculation of residual activities.

Effect of temperature on protease activity and stability. The protease activity was assayed at the different temperature (20–80℃) and pH 9.0. The temperature stability was measured by preincubating the reaction mixtures at the same temperatures for 60 min. The residual protease activity was under optimum conditions.

Effects of protease inhibitors and metal ions. For determining the effect of various protease inhibitors, protease activity was assessed by adding to the enzyme solution, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol and benzamidin, at concentrations of 5 and 10 mM. The OB3 crude preparation was pre-incubated for 30 min at 30℃, and assayed under optimum conditions. To examine the effect of various metal ions (5 mM) on enzyme stability, monovalent and divalent metals were added to the reaction mixture. The ions included K^+, Na^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+}. The activities were compared with the control to which no inhibitors or metal ions were added.

Effect of organic solvent on stability. Stability of the crude preparation in organic solvents was assayed by monitoring the residual activity after 14 days incubation at 30℃ (150 rpm) in the presence of 50% (v/v) of different polar solvents (butanol, chloroform, DMSO, ethanol, formaldehyde, hexane and methanol). The residual proteolytic activity was assessed at pH 9.0 and 60℃ against a control.

Effects of some surfactants and commercial detergents on stability. The effect of various detergent additives was evaluated in the presence of varying concentrations of some surfactants, added in the standard assay reaction, such as Triton X-100, Tween 80, SDS and the oxidizing agent H_2O_2. The suitably diluted enzyme was pre-incubated for 60 min at 30℃ and the residual activity was estimated under optimum conditions and compared to a control in which no surfactants were added [20].

For testing the compatibility as detergent additive, 7 mg/ml of solid and 1% liquid commercial laundry detergents were added to the enzyme preparation (500 U) and preincubated at different temperatures (30, 40 and 50℃) for 1 h. To simulate washing conditions, detergents were diluted in tap water and incubated prior to use at 90℃ for 60 min to inactivate enzymes contained in these detergent formulations. The solid laundry detergents used were OMO (Unilever), Tide (Procter and Gamble, USA) and Ariel (Procter and Gamble, Suisse). The liquid laundry detergent used is El Kef (Lesieur Cristal, Morocco). A control test was carried out without any additives under the same incubation conditions.

Evaluation of washing performance using blood stain

To investigate the suitability of OB3 protease as an additive in detergent formulation, protein spot removal ability of enzymatic preparation was evaluated from blood soiled cotton cloth pieces. The soiled pieces were previously dried down naturally prior before wash test. The different flasks containing the stained-cloth pieces were incubated 1 h, at 30℃ and 140 rpm, with tap water, Tide and Tide supplemented with 500 UT of OB3 crude preparation. The washed cotton pieces were dried at room temperature for visual examination.

Statistical analysis

The results are expressed on average and the experiments were repeated at least 3 times. The data were analyzed for significant differences using the two-way analysis of variance using Graph Pad Prism software, version 8.0.2 for windows.

Results and Discussion

Isolation of OB3 protease

Among the isolates screened for protease production, one was retained as the highest alkaline protease producer. The isolate was Gram negative short rods, oxidase positive. The strain was identified as *A. veronii* and named OB3 on the basis of API and 16S rDNA sequence analysis.

Fish powders are rich in nutrients (carbon, nitrogen, and minerals) providing an excellent alternative inexpensive media for enzyme production. Therefore, protease production by OB3 was investigated on HVSP-based medium (10 g/l). A high level of protease activity of 4080 U/ml was obtained. Sellami-Kamoun *et al.* also reported similar level of protease production (5273 U/ml) in fish waste based medium supplemented by 1 g/l $CaCl_2$ [17].

Effect of pH on the activity and stability of OB3 protease

The OB3 protease was highly active in a range of pH 5.0 to 12.0 and exibited maximum activity at pH 9.0. Interestingly, as shown in Fig. 1A, more than 85% of its original activity was remained at pH 10.0 and about 78.78 and 78% were measured at pH 11.0 and 12.0. This is an important criterion for their successful incorporation in detergents since they exhibit highly alkaline environment [21]. The optimum pH of OB3 protease was slightly higher than other detergent stable proteases [22], and was similar to those described for *B. mojavensis* A21, *B. substilis* proteases [22, 23] and that of Thakur *et al.* [24] and Aryaei *et al.* [25]. However, Nakasone *et al.* [26] Karunakaran and Devi [27] and Divakar *et al.* [14] reported proteases from *Aeromonas* species, had an optimum pH around 7. Moreover, *A. veronii* PG01 protease described by Divakar *et al.* [14] had a residual activity of around 20% at pH 10.0. The pH stability revealed that the OB3 crude preparation exhibited broad pH stability profile. Between pH 7.0 and 9.0, there were still 100% of activity remaining (Fig. 1B) and around 78% of residual activity was recorded at pH 12.0. Because of its high activity and stability at alkaline con-

http://dx.doi.org/10.4014/mbl.1912.12015

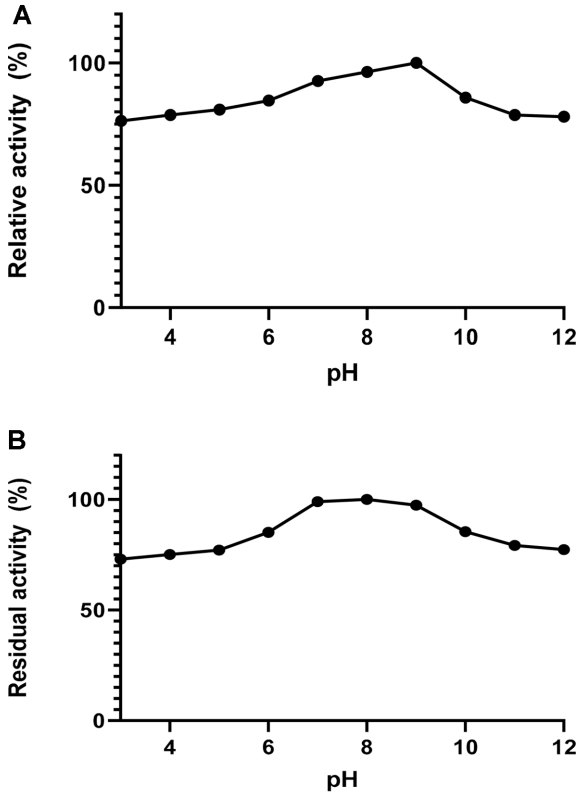

Fig. 1. Effects of pH on activity (A) and stability (B) of the OB3 protease. Protease activities were evaluated in the pH range of 3.0-12.0 using buffers of different pH values at 60℃. The maximum activity obtained at pH 9.0 was considered as 100%. The pH stability of enzyme was determined by incubating enzymes in different buffers for 1 h at 30℃ and the residual activity was measured at pH 9.0 and 60℃. The activity of enzyme before incubation was taken as 100%.

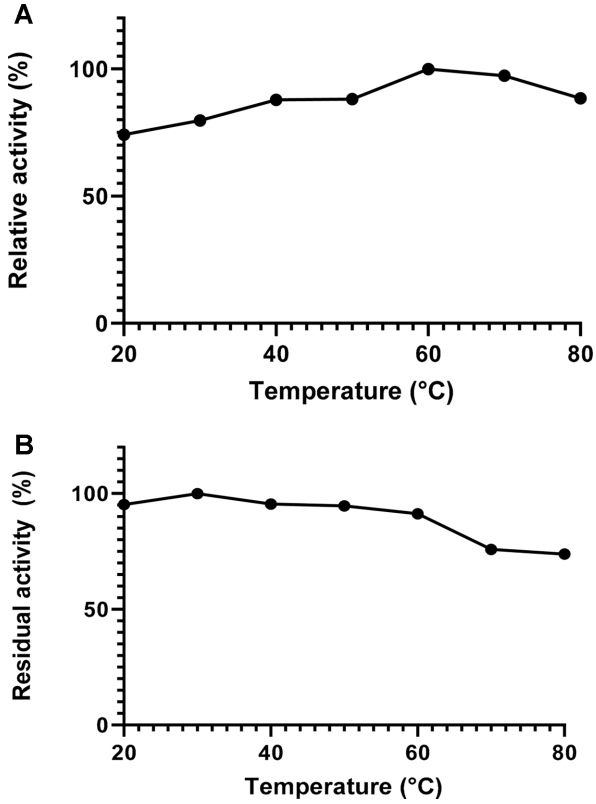

Fig. 2. Effect of temperature on activity (A) and stability (B) of the OB3 protease. The temperature profiles were determined by assaying protease activity at temperatures between 20 and 80℃. The activity of enzymes at 60℃ was taken as 100%. OB3 protease is pre-incubated at different temperatures for 1 h at pH 9.0, and then the residual enzyme activities were estimated under standard conditions. The non-heated enzymes were taken as 100%.

ditions, OB3 protease could be applicable in detergent formulation.

Effect of temperature on the activity and stability of OB3 protease

The OB3 enzyme was active in the temperature range tested with an optimum around 60℃ (Fig. 2A). About 74 and 88% of its original activity was obtained at 20 and 80℃, respectively. This optimum is in agreement with the data for the alkaline proteases from *A. veronii* PG01 [14], *A. caviae* [15] and the commercial detergent enzyme Subtilisin Carslberg and Subtilisin BPN' [28]. The OB3 crude enzyme (Fig. 2B) is very stable and maintains about 90 to 100% of its residual activity from 20℃ to 60℃ after 60 min incubation. The OB 3 crude preparation maintained at 70℃ and 80℃, 75.84 and 73.87% of its initial activity respectively. This activity and stability at lower and higher temperatures confirm its eventual use at various washing temperatures. Sareen and Mishra [29] also described a thermostable alkaline protease from *B. licheniformis*. However, proteases from *A. caviae* [15] and *A. caviae* AP34 [30] lost their activity after 30 and 15 min incubation at 80℃, respectively.

Effect of some metal ions and inhibitors on protease activity

Data presented in Table 1, showed stimulatory effect of the majority of the metal ions tested. Among the metals tested, the Ca^{2+} and Mg^{2+} stimulated protease activity by 142.66 and 139.11%, respectively. These results suggest that the enzyme requires metal ions as cofactors

Table 1. Effect of various enzyme inhibitors and metal ion son enzyme activity of OB3 protease.

Inhibitors/Ions	Concentrations (mM)	Activity (%)
EDTA	5	92.07 ± 0.30
	10	84.15 ± 0.59
β-mercaptoethanol	5	87.92 ± 1.35
	10	87.16 ± 0.11
PMSF	5	76.98 ± 1.4
	10	35.5 ± 0.03
Benzamidine	5	92.83 ± 0.57
	10	88.3 ± 0.49
Ca^{2+}	5	142.66 ± 0.46
Mg^{2+}	5	139.11 ± 0.15
Na^{+}	5	114.66 ± 0.09
K^{+}	5	104.44 ± 0.26
Zn^{2+}	5	119.11 ± 0.19
Cu^{2+}	5	108.88 ± 0.21
Fe^{2+}	5	99.55 ± 0.14

[31]. A similar effect of metal ions on the activity of alkaline proteases from *Bacillus subtilis* AU-2 and *Pseudomonasputida* was described by Patel *et al.* [32] and Iqbal *et al.* [33], respectively. Enhanced protease activity of *A. caviae* NRRL B-966 by Mg^{2+} and Ca^{2+}, has also been reported by Karunakaran and Devi [27]. Similar results were also reported by Zhang *et al.* [34], which revealed that when Ca^{2+} interacts with an alkaline protease, it causes a change into more active and stable conformation.

The effect of different protease inhibitors was studied (Table 1). The OB3 protease was inhibited by the serine proteases inhibitor PMSF. The activity was reduced in the presence of the metalloenzyme inhibitor EDTA with residual activities of 92.07% and 84.15% at 5 and 10 mM, respectively. According to Lee and Jang [35], the active structure of some serine proteases have two Ca^{2+} binding sites, and chelating agents such as EDTA reduced stability significantly at high temperatures. β-mercaptoethanol caused a slight inhibition (13%) of the crude enzyme suggesting the eventual cleavage of disulphide bridges in native conformation of this protease. Similar results were reported for other proteases from *A. hydrophila* [36] and *A. veronii* PG01 [14]. EDTA is used as a water softener in detergent formulations [37], thus OB3 protease may meet the requirements of additives in laundry detergents.

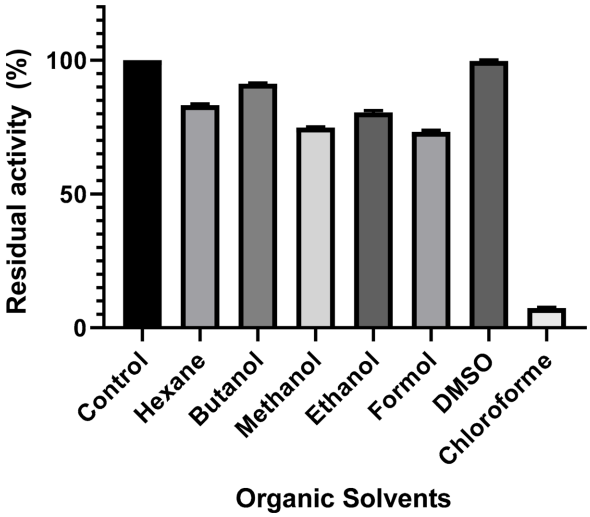

Fig. 3. Effect of different organic solvents on OB3 protease from *A. veronii* OB3.

Effect of various organic solvents on OB3 protease activity

Hydrolytic enzymes have gained increasing interest due to their wide industrial applications such as catalysts in the peptide synthesis in the presence of organic solvents [38]. In the present study, the effect of different water miscible organic solvents on the protease stability is shown in Fig. 3. There was no effect on protease activity in presence of DMSO, while it was slightly affected in the presence of hexane (83.85%), butanol (91.45%), methanol (64.62%), ethanol (80%) and formol (73.66%). However, OB3 crude enzyme was considerably affected by chloroform. Similar stability has been reported for some bacterial alkaline proteases [39, 40]. The stability in some solvents, known to be used for most synthetic reactions, such as methanol and DMSO, indicate that the OB3 protease will be an ideal candidate in comparison with the described *Aeromonas* proteases.

Compatibility of OB3 protease with surfactants and oxidizing agents

In order to be incorporated as bioadditive in laundry detergent, the alkaline proteases and other detergent enzymes must have specific characteristics such as stability under harsh conditions [41]. Commercial detergent proteases such as Alcalase and Subtilisin are compatible with detergent ingredients, but the majority show poor stability towards oxidizers [42].

Table 2. Stability of the alkaline protease OB3 in the presence of various surfactants and bleaches.

	Concentrations (%)	Residual activity (%)
SDS	0.5	75.08 ± 0.76
	1	35.15 ± 0.95
Tween 80	5	198.29 ± 0.50
Triton x-100	5	147.43 ± 0.56
H_2O_2	1	111.53 ± 1.03
	5	109.82 ± 0.57

As summarized in Table 2, an improvement in activity of 98.29 and 47.43% was recorded in with Tween 80 and Triton X-100, respectively. Similar activating effect of Tween 80 was reported by Rekik *et al.* [43]. While the *B. invictae* protease described by Hammami *et al.* [44] was less stable than OB 3 in the presence of the same surfactants. Furthermore, the OB3 protease retained about 75.08 and 35.15% of its initial activity in the presence of 0.5 and 1% SDS, respectively. The stability of OB3 protease greatly exceeds the protease from *B. invictae* with only 19.48% residual activity when incubated 1 h with 1% SDS [44]. Interestingly, the protease activity was improved by H_2O_2, the activity was 111.53 and 109.82% in the presence of respectively, 1 and 5%. A slight stimulating effect by low concentrations of the oxidizing agent was reported for other alkaline serine proteases [39, 45]. Similarly, proteases from *B. mojavensis* A2 retained less than 76% of their activity after 1 h at 30℃ [46]. Interestingly, when compared to other proteases from *A. veronii* PG01 (58% (2%)) [14] and other detergent stable alkaline proteases [47–49], OB3 protease exhibit significant stability in the presence of H_2O_2.

Compatibility with commercial detergents

As it can be seen in Fig. 3, OB3 protease exhibited high stability with several commercial detergents. Furthermore, the activity of alkaline protease is improved in Tide (113%), Ariel (110%), El Kef (109%) and OMO (107%). At 40℃, remarkable stability was also recorded in the presence of Ariel, Tide and El kef retaining its maximal activity and negligible loss was observed with OMO. However, OB3 activity was slightly decreased at 50℃ in all the detergents tested. In contrast, proteases from *Bacillus safensis* RH12 [43] and *Bacillus* sp. SSR1 [49] retained at 40℃ with Ariel, 75 and 37% of their initial activity, respectively.

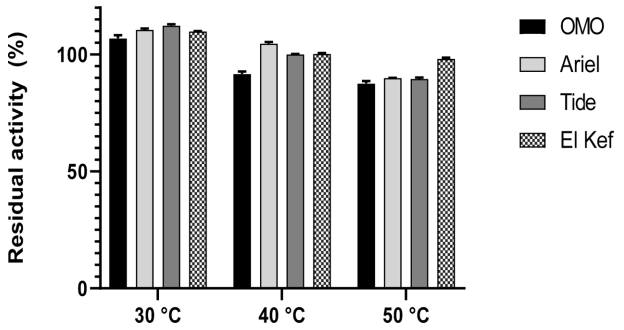

Fig. 4. Activity of the alkaline protease OB3 in commercial solid and liquid laundry detergents.

Remarkably, at 40 and 50℃, *A. veronii* OB3 protease was more stable than the commercial proteases present in some detergent formulations. As compared to *A. veronii* OB3 protease (80%), the activity of the commercial detergent Ariel was severely affected when incubated 1 h at 50℃ [51]. These findings strongly advocated the use of OB3 protease as a bioadditive in detergent industry.

Wash performance analysis

As seen in Fig. 4, the use of the Tide alone is not effective in removing blood stain. The best washing performance was given by the detergent supplemented with the enzyme preparation (500 U). Addition of OB3 protease improved the stain removal efficiency and can enhance the ability of detergents to clean clothes. It is noticed that the crude enzyme from *A. veronii* OB3 is more effective than other reported detergent-stable alkaline proteases [16, 31]. Moreover, low level of supplementation of OB3 protease (500 U) in comparison with previously reported levels described by other papers (845, 1690, 5070 U) [46, 52].

In this study, an alkaline protease from *A. veronii* was isolated. Biochemical characteristics, along with compatibility and stability towards surfactants, oxidizers, organic solvents and commercial detergents were demonstrated. Such impressive properties make OB3 alkaline protease promising for its use in organic peptide synthesis and detergent industry applications. In addition, the use of fish wastes powder constitutes an economic alternative to synthetic medium during large-scale production of commercial proteases.

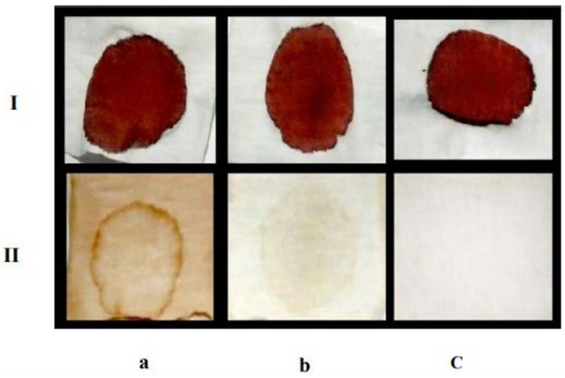

Fig. 5. Washing performance analysis of the *A. veronii* OB3 protease in the presence of the commercial detergent Tide (7 mg /ml). (a) Cloth coton stained with blood washed with tap water; (b) bloodstained cloth coton washed with Tide; (c) bloodstained cloth washed with Tide supplemented with crude preparation of OB3 (500 U). I: untreated cloths (control) and II: treated cloths.

Conflicts of Interest

The authors have no financial conflicts of interest to declare.

References

1. Singh S, Bajaj BK. 2017. Potential application spectrum of microbial proteases for clean and green industrial production. *Energy Ecol. Env.* **2**: 370-386.
2. Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A, *et al.* 2019. Microbial proteases applications. *Front. Bioeng. Biotechnol.* **7**: 110.
3. Sarrouh B, Santos TM, Miyoshi A, Dias R, Azevedo V. 2012. Up-To-Date insight on industrial enzymes applications and global market. *J. Bioproces. Biotech.* **4**: 002. doi:10.4172/2155-9821.S4-002
4. Niyonzima FN, More S. 2014. Detergent-compatible proteases: Microbial production, properties, and stain removal analysis. *Prep. Biochem. Biotechnol.* **45**: 233-258.
5. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* **62**: 597-635.
6. Gupta R, Beg QK, Lorenz P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.* **59**: 15-32.
7. Sharma KM, Kumar R, Panwar S, Kumar A. 2017. Microbial alkaline proteases: Optimization of production parameters and their properties. *J. Gen. Eng. Biotechnol.* **15**: 115-126.
8. Verma J, Pandey S. 2019. Characterization of partially purified alkaline protease secreted by halophilic bacterium *Citricoccus* sp. isolated from agricultural soil of northern India. *Biocat. Agric. Biotechnol.* **17**: 605-612.
9. Salwan R, Sharma V. 2019. Trends in extracellular serine proteases of bacteria as detergent bioadditive: alternate and environmental friendly tool for detergent industry. *Arch. Microbiol.* **201**: 863-877.
10. Lakshmi BK, Muni Kumar D, Hemalatha KP. 2018. Purification and characterization of alkaline protease with novel properties from *Bacillus cereus strain S8*. *J. Gen. Eng. Biotechnol.* **16**: 295-304.
11. Zacaria J, Delamare APL, Costa SOP, Echeverrigaray S. 2010. Diversity of extracellular proteases among *Aeromonas* determined by zymogram analysis. *J. Appl. Microbiol.* **109**: 212-219.
12. Laishram S, Pennathur G. 2015. Purification and characterization of a membrane-unbound highly thermostable metalloprotease from *Aeromonas Caviae. Arab. J. Sci. Eng.* **41**: 2107-2116.
13. Cho SJ, Park JH, Park SJ, Lim JS, Kim EH, Cho YJ, *et al.* 2003. Purification and characterization of extracellular temperature-stable serine protease from *Aeromonas hydrophila*. *J. Microbiol.* **41**: 207-211.
14. Divakar K, Deepa Arul Priya J, Gautam P. 2010. Purification and characterization of thermostable organic solvent-stable protease from *Aeromonas veronii PG01*. *J. Mol. Catal. B: Enz.* **66**: 311-318.
15. Datta S, Menon G, Varughese B. 2016. Production, characterization, and immobilization of partially purified surfactant-detergent and alkali-thermostable protease from newly isolated *Aeromonas caviae. Prep. Biochem. Biotechnol.* **47**: 349-356.
16. Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**: 4673-4680.
17. Sellami-Kamoun A, Ghorbel-Frikha B, Haddar A, Nasri M. 2011. Enhanced *Bacillus cereus BG1* protease production by the use of sardinelle *(Sardinella aurita)* powder. *Ann. Microbiol.* **61**: 273-280.
18. Miller JH. 1972. Experiments in moleculer genetics. pp. 431-435. Cold Spring Harbor, NY: Cold483 Spring Harbor Laboratory Press.
19. Kembhavi AA, Kulkarni A, Pant A. 1993. Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis NCIM* No. 64. *Appl. Biochem. Biotechnol.* **38**: 83-92.
20. Manni L, Jellouli K, Ghorbel-Bellaaj O, Agrebi R, Haddar A, Sellami-Kamoun A, *et al.* 2009. An oxidant- and solvent-stable protease produced by *Bacillus cereus* SV1: Application in the deproteinization of shrimp wastes and as a laundry detergent additive. *Appl. Biochem. Biotechnol*. **160**: 2308-2321.
21. Maurer KH. 2004. Detergent proteases. *Curr. Opi. Biotechnol.* **15**: 330-334.
22. Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M. 2009. A novel surfactant-stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. *Proc. Biochem.* **44**: 29-35.
23. Uttatree S, Charoenpanich J. 2016. Isolation and characterization of a broad pH- and temperature-active, solvent and surfactant stable protease from a new strain of *Bacillus subtilis. Biocat. Agricul. Biotechnol.* **8**: 32- 38.
24. Thakur N, Kumar A, Sharma A, Bhalla TC, Kumar D. 2018. Purification and characterization of alkaline, thermostable and organic solvent stable protease from a mutant of *Bacillus* sp. *Biocat. Agricult. Biotechnol.* **16**: 217-224.

25. Aryaei A, Farhadi A, Moradian F, Mianji GR. 2019. Cloning, expression and characterization of a novel alkaline serine protease gene from native Iranian *Bacillus* sp.; a producer of protease for use in livestock. *Gene* **693**: 10-15.
26. Nakasone N, Toma C, Song T, Iwanaga M. 2004. Purification and characterization of a novel metalloprotease isolated from *Aeromonas caviae*. *FEMS Microbiol. Lett.* **237**: 127-132.
27. Karunakaran T, Devi BG. 1995. Proteolytic activity of *Aeromonas caviae*. *J. Basic Microbiol.* **35**: 241-247.
28. Horikoshi K. 1990. Enzymes of alkalophiles. In: Louis, M.O., St. pp. 275-294. (Ed.), Microbial Enzymes and Biotechnology, second ed. Elsevier Applied Science, Amsterdam.
29. Sareen R, Mishra P. 2008. Purification and characterization of organic solvent stable protease from *Bacillus licheniformis* RSP-09-37. *Appl.Microbiol. Biotechnol.* **79**: 399-405.
30. Toma C, Ichinose Y, Iwanaga M. 1999. Purification and characterization of an *Aeromonas caviae* metalloprotease that is related to the *Vibrio cholera* hemagglutinin/protease. *FEMS Microbiol. Lett.* **170**: 237-242.
31. Hadjidj R, Badis A, Mechri S, Eddouaouda K, Khelouia L, Annane R, *et al*. 2018. Purification, biochemical, and molecular characterization of novel protease from *Bacillus licheniformis* strain K7A. *Int. J. Biol. Macromol.* **114**: 1033-1048.
32. Patel AR, Mokashe NU, Chaudhari DS, Jadhav AG, Patil UK. 2019. Production optimisation and characterisation of extracellular protease secreted by newly isolated *Bacillus subtilis AU-2* strain obtained from *Tribolium castaneum gut*. *Biocatal. Agric. Biotechnol.* **19**: 101-122.
33. Iqbal A, Hakim A, Hossain MS, Rahman MR, Islam K, Azim MF, *et al*. 2018. Partial purification and characterization of serine protease produced through fermentation of organic municipal solid wastes by *Serratia marcescens A3* and *Pseudomonas putida A2*. *J. Gen. Eng. Biotechnol.* **16**: 29-37.
34. Zhang J, Wang J, Zhao Y, Li J, Liu Y. 2019. Study on the interaction between calcium ions and alkaline protease of *Bacillus*. *Int. J. Biol. Macromol.* **124**: 121-130.
35. Lee S, Jang DJ. 2001. Progressive rearrangement of subtilisin Carlsberg into orderly and inflexible conformation with Ca^{2+} binding. *Biophys. J.* **81**: 2972-2988.
36. Rivero O, Anguita J, Paniagua C, Naharro G. 1990. Molecular cloning and characterization of an extracellular protease gene from *Aeromonas hydrophila*. *J. Bacteriol.* **172**: 3905-3908.
37. Beg QK, Gupta R. 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enz. Microb. Technol.* **32**: 294-304.
38. Vulfson EN, Halling PJ, Holland HL. 2001. Methods in Biotechnology: Enzymes in Nonaqueous Solvents. Part II, 532, pp. 241-422.
39. Manni L, Ghorbel-Bellaaj O, Jellouli K, Younes I, Nasri M. 2009. Extraction and characterization of chitin, chitosan, and protein hydrolysates prepared from shrimp waste by treatment with crude protease from *Bacillus cereus SV1*. *Appl. Biochem. Biotechnol.* **162**: 345-357.
40. Sundus H, Mukhtar H, Nawaz A. 2016. Industrial applications and production sources of serine alkaline proteases: a review. *J. Bacteriol. Myc.* **3**: 191-194.
41. Kumar CG, Takagi H. 1999. Microbial alkaline proteases from a bioindustrial viewpoint. *Biotechnol. Adv.* **17**: 561-594.
42. Gupta R, Gupta K, Saxena RK, Khan S. 1999. Bleach-stable, alkaline protease from *Bacillus* sp. *Biotechnol. Lett.* **21**: 135-138.
43. Rekik H, Jaouadi NZ, Gargouri F, Bejar W, Frikha F, Jmal, N, *et al*. 2019. Production, purification and biochemical characterization of a novel detergent-stable serine alkaline protease from *Bacillus safensis strain RH12*. *Int. J. Biol. Macromol.* **121**: 1227-1239.
44. Hammami A, Hamdi M, Abdelhedi O, Jridi M, Nasri M, Bayoudh A. 2017. Surfactant- and oxidant-stable alkaline proteases from *Bacillus invictae*?: Characterization and potential applications in chitin extraction and as a detergent additive. *Int. J. Biol. Macromol.* **96**: 272-281.
45. Biver S, Portetelle D, Vandenbol M. 2013. Characterization of a new oxidant-stable serine protease isolated by functional metagenomics. *Springerplus* **2**: 410.
46. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M. 2009. Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: purification, characterization and potential application as a laundry detergent additive. *Bioresour. Technol.* **100**: 3366-3373.
47. Sellami-Kamoun A, Haddar A, Ali NEH, Ghorbel-Frikha B, Kanoun S, Nasri M. 2008. Stability of thermostable alkaline protease from *Bacillus licheniformis RP1* in commercial solid laundry detergent formulations. *Microbiol. Res.* **163**: 299-306.
48. Yu P, Huang X, Ren Q, Wang X. 2019. Purification and characterization of a H_2O_2-tolerant alkaline protease from *Bacillus* sp. *ZJ1502*, a newly isolated strain from fermented bean curd. *Food Chem.* **274**: 510-517.
49. David A, Singh Chauhan P, Kumar A, Angural S, Kumar D, Puri N, Gupta N. 2018. Coproduction of protease and mannanase from *Bacillus nealsonii* PN-11 in solid state fermentation and their combined application as detergent additives. *Int. J. Biol. Macromol.* **108**: 1176-1184.
50. Singh J, Batra N, Sobti R. 2001. Serine alkaline protease from a newly isolated *Bacillus sp.* SSR1. *Proc. Biochem.* **36**: 781-785.
51. Banik RM, Prakash M. 2004. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. *Microbiol. Res.* **159**: 135-140.
52. Jain D, Pancha I, Mishra SK, Shrivastav A, Mishra S. 2012. Purification and characterization of haloalkaline thermoactive, solvent stable and SDS induced protease from *Bacillus* sp.: a potential additive for laundry detergents. *Bioresour. Technol.* **115**: 228-236.