RESEARCH ARTICLE

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Production and characterization of crude proteases from Halobacillus salinus strain DZ28 newly isolated from salt lake sediments in Algeria and their use as detergent bioadditives

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Abstract

New Algerian Gram-positive, rod-shaped, endospore-forming, salt-philic bacteria (DZ28 strain) that overproduce extracellular alkaline proteases have been isolated from salt lake deposits in Lake Oubeira, El Taref. Strain DZ28 was assigned as Halobacillus salinus DZ28 on the basis of phenotypic properties and 16S rDNA gene sequencing (ripotyping). The maximum protease activity registered after 36 hours of incubation in optimized medium at 30° C was 19,000 U / ml in a shaking bottle culture at 160 rpm. The crude extract protease showed optimal activity at 60 ° C temperature and pH 12. It is actively inhibited by PMSF and DIFP, indicating that it belongs to the serine protease family. Interestingly, the crude extract protease was not only very stable to nonionic surfactants and oxidants, but also showed high stability and compatibility with some commercial detergents. It retaining more than 100% of its initial activity after pre-incubation for 1 h at 40°C with ISIS, followed by Pril (98%), Tide (95%) and Dixan (90%). More curiously, the wash overall performance evaluation discovered that it may dispose of blood-stains remove at 40°C for 1 h with low supplementation (500 U/mL). This is the first report of a protease from Halobacillus salinus and has potential as a promising candidate for future applications as a bioadditive for detergent formulations.

Keywords: *Halobacillus salinus*, Protease, Process optimization, Laundry detergent, Wash performance

INTRODUCTION

Proteases denote an enzyme group with catalytic activity involving in various processes such as digestion, cell growth and apoptosis, blood coagulation, as well as protein catabolism and so others (Chew et al., 2019; Gurumallesh et al., 2019). Based on data from Allied Market Research, entitled, "Enzymes Market, by Type, Source, Reaction Type, and Application: Global Opportunity Analysis and Industry Forecast, 2018-2024", at the beginning of 2017 the global enzymes market was evaluated at $7,082 million. Hence, it recorded compound annual growth rate (CAGR) of 5.7% from 2018 to 2024 and it is estimated to reach $10,519 million in 2024. More than 60 % of commercially available enzymes worldwide are proteases (Gurumallesh et al., 2019). As the first commercial protease produced by Bacillus licheniformis in the 60s of the last century is an alkaline protease, researches focused more and more of such Bacillus derived-biomolecules with high catalytic activity and stability whilst keeping low production cost (dos Santos Aguilar and Sato, 2018; Raval et al., 2014).

Alkaline proteases enzymes are largely used by the industry (Maghsoodi et

al., 2013; Annamalai et al., 2014; Liu and Kokare, 2017;) in particular as additives in detergent formulations (Chew et al., 2019), registering 89% of the entire selling (Baweja et al., 2017). Of particular interest, proteases from extremophiles gained more attention over their classical strains counterparts regarding their promising performances and broad applicability. These enzymes are usually further benefits namely high activity in non-physiological conditions, such as extreme pH or temperature, intensive calcium chelating agents, and detergents solvent-tolerance, substrate selectivity and stability. Due to these unique properties, thermozymes are of tremendous importance for industrial applications and, accordingly, screening for novel biocatalysts from extremophiles represents a valuable alternative to elaborative engineering procedures for the optimization of available enzymes from mesophiles (Mechri et al., 2017a,b; Mechri et al., 2019a,b).

As a result of growing interest in proteolytic enzymes application by different industries like laundry detergents, wastewater treatment and tanning factories, researchers are looking into the diversity of salt lake deposits and salt resistant microorganism with prospetive biotechnology applications. Bacillus strains are often preferred as major sources for commercial alkaline proteases due to their exceptional ability to secrete large amounts of highly active enzymes, that are more stable at high temperature and pH (Nazari and Mehrabi, 2019). Bacillus licheniformis, Bacillus subtilis, Bacillus amyloliquefaciens, and Bacillus pumilus were traditionally the extra wide utilized species in industry operating with alkaline proteases (Baweja et al., 2016; Habicher et al., 2019). As far as we know, the protease capacity of Bacillus atrophaeus, which has been registered as a bioactive substance in surfactants, has not been described so far. Emphasizing the extremophile origins of Bacillus atrophaeus, its contribution to protease synthesis and improvement of detergent formulation and commercially available (Rahem et al., 2021). In this context, the present study reports, for the first time, on the optimization and biochemical characterization of the detergent-stable crude extract alkaline protease from Halobacillus salinus isolated from the saline Lake Oubeïra at the Province of El-Tarf (Algeria). It also provides basic information on the potential use of this crude enzyme as a prospective candidate for future applications in detergent formulations.

MATERIALS AND METHODS

Strain isolation and culture conditions

Five sediments samples were collected from of the Oubeïra salt lake (Algeria, El Taref Province (GPS coordinates: latitude: 36 ° 50`38.246 "N, longitude: 8 ° 23 '14 .695" E)) using a sediment gripper. All of the samples were kept at 4°C until use. Once brought to the laboratory, samples were heat-treated (20 min at 90°C) to kill vegetative cells and used to inoculate skimmed milk agar plates (SMAP). The protease enzymatic potential for strain isolates was evaluated. These microbial strains were screened for the qualitative, quantitative protease activity and their ability to hydrolyze casein. Initial screening of protease activity was carried out using a plate assay in a SMAP medium. The solid medium contains in g/L: peptone 5, yeast extract 3, skimmed milk 250 mL, and agar, 20 at pH 7. The culture plates were incubated overnight at 30°C and colonies surrounded by clear halos, upon colonies were selected as putative protease producers. Among these isolates, a strain designed as DZ28, resulted in a large clear zone of hydrolysis and a high proteolytic potency (4,000 U/mL) in a casein-based initial liquid medium was selected for further experimental study.

A first growth medium for protease production at pH 7 comprised (in g/L): casein, 10; yeast extract, 1; CaCl2, 0.5; K2HPO4, 0.25; and KH2PO4, 0.25. In each time, one factor was altered to investigate individually the impacts of the different parameters on protease production. Various carbon sources (casein, gelatin, glucose, fructose, galactose, sucrose, and maltose) were individually selected then added to the production broth (100 ml) at a concentration of 5 g. Used / L. The impacts of the finest carbon source (casein) concentration on protease production were evaluated on adding up 5, 10, 15, 20, and 25 g / l casein to the production broth undergoing the overhead conditions. Different organic matter were investigated (yeast peptone, yeast extract, beef extract, and soya peptone) and inorganic compounds (ammonium chloride, ammonium sulphate, NaNO3, NH4Cl; (NH4)2SO4, and sodium nitrate) nitrogen sources, at a concentration of 2 g/L, were also studied in a medium containing casein at 10 g/L as the only carbon and energy source. The ideal source of organic nitrogen for protease synthesis was found to be yeast extract and their various levels were evaluated (2, 4, 6, 8, and 10 g / L) to recognize the ideal concentration needed for maximum protease activity. As reported by Jaouadi et al. (2009) an optimum growth medium for protease production at pH 7 composed of (g / l): casein, 10 g; yeast extract, 4 g; K2HPO4, 1.5 g; KH2PO4, 1.5 g; CaCl2, 1 g; 1% (v / v) and trace element. At 120 ° C for 20 minutes the medium was autoclaved and Incubations were accomplished in a 1000 ml Erlenmeyer flask with a functioning size of 100 ml at 30 ° C for 36 hours in a rotary shaker (160 rpm). To estimate bacterial growth, optical density was measured at 600 nm and transformed to cell dry weight (g / l) founded on the biomass vs. cell dry weight control. The cell-free supernatant was collected after centrifugation 8000 g, 40 minutes at 4 ° C and be as a source of protease in subsequent additional experiments.

Bacterial strain identification

Analytical profiling index (API) strip examinations and 16S rDNA gene sequencing (ribotyping) were carried out to identify the genus that's the strain appertains. The API 50 CH strip (bioMérieux, SA, Marcyl`Etoile, France)

was utilized to investigate the phenotypic, biochemical and physiological properties of the DZ28 strain in accordance with the manufacturer's guidance. The 16S rDNA gene was amplified by PCR using forward primer 5'AGAGTTTGATCCTGGCTCAG3' and reverse primer 5' GGTTACCTTGTTACGACTT3'. Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used to purify Genomic DNA of strain DZ28 then utilized like a template for PCR amplifications (30 cycles, 94°C for 40 s denaturation, 64°C for 45 s primer annealing, and 72°C for 90 s extension). Amplified ~1.5 kb PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), conducting to pDZ28-16S plasmid (this study). The E. coli DH5α (Invitrogen, Carlsbad, CA, USA) was utilized like host strain. Every recombinant clones of E. coli were grown in Luria-Bertani (LB) medium with the addition of ampicillin (100 µg/mL), IPTG (0.4 mM), and X-gal (360 µg/mL) for screening. Every DNA purification, DNA electrophoresis, ligation, restriction, and transformation were carried out according to previously described method (Sambrook et al. 1989).

DNA sequencing and bioinformatics analysis

The nucleotide sequence of the cloned 16S rDNA gene was determined on both strands using the BigDye Terminator Cycle Sequence Ready Reaction Kit and the automated DNA sequencer ABIPRISM® 3100 AvantGeneticAnalyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequence was compared with sequences available in the public sequence databases and with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012), a web-based tool for the identification of prokaryotes based on 16S rDNA gene sequences from type strains. Phylogenetic and molecular evolutionary genetic analysis was performed using MEGA software v. 4.1 Implementation. Distances and clusters were calculated using the neighbor-joining method. Alignment of multiple nucleotide sequences was performed using the software program BioEdit version 7.0.2 and the program Clustal W2 available on the servers of the European Bioinformatics Institute. (http://www.ebi.ac.uk/Tools/msa/clustalw2/)(Rahem et al., 2021).

Protease activity assays

Protease activity was investigated using Hammerstein casein (Merck, Darmstadt, Germany) as a substrate, as well as elsewhere (Jaouadi et al. 2012). Unless otherwise stated, to an appropriately diluted enzyme solution (0.5 ml), add 2.5 ml of 100 mM disodium hydrogen phosphate-NaOH buffer at pH 12, add 1 mM CaCl2 (buffer A) containing 10 g / l of casein, and add. Mixed and incubated. 15 minutes at 60 ° C. The reaction was stopped by adding 2.5 ml of 20% trichloroacetic acid (TCA). The mixture was left at room temperature for 30 minutes and undigested protein was removed by centrifugation at 14,000 rpm for 20 minutes. Next, 0.5 ml of clear supernatant was mixed with 2.5 ml of 500 mM Na2CO3 and 0.5 ml of FolinCiocalteus phenolic reagent and incubated for 30 minutes at room temperature. The absorbance of the resulting supernatant was measured at 660 nm with respect to the blank. Protease activity was measured spectrophotometrically by detecting tyrosine released during protease hydrolysis. Protease activity present in detergent solutions has been described elsewhere using N,N-dimethylated casein (DMC) and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) as substrates. Measured at 450 nm by the method of (Jaouadi et al., 2009) and evaluated as a color indicator.

Biochemical characterization of the crude protease DZ28

Effects of some inhibitors, reducing agents, and metal ions on enzyme stability

Various inhibitors and divalent metal ions have been added to study the need for crude protease inhibitors, reducing agents, and metal ions. The experiment was carried out as described above using casein as a substrate after incubation at 40 ° C. for 1 hour.

Effects of different pH on enzyme activity and stability

Crude protease DZ28 activity was measured at a pH range of 2 to 13 at 60°C using casein as a substrate. The effect of pH on the crude protease DZ28 activity was calculated with measuring constantly the enzyme activity at 60°C over the pH range of 2–13 using the following buffers at 100 mM, supplemented with 1 mM CaCl2: glycine-HCl (pH 2-5), MES (pH 5-6), HEPES (pH 6-8), Tris-HCl (pH 8-9), glycine-NaOH (pH 9-11), bicarbonate-NaOH (pH 11-11.5), Na2HPO4-NaOH (pH 11.5-12), and sodium phosphate dibasic-NaOH (pH 12-13). Its pH stability was determined by preincubating at 40 ° C for 6 hours with different buffers at different pH values. Aliquots were removed and residual enzyme activity was measured at pH 12 and 60 ° C.

Effects of different temperature on enzyme activity and stability

To measure the temperature effect of the crude protease DZ28, enzyme activity was measured at various temperatures in the range of 40-100 ° C. The thermal stability of the crude protease DZ28 i was determined after enzyme incubation at different temperatures (50, 60, and 70°C) and pH 12 for 6 h with and without 1 mM CaCl2 and measuring the residual enzyme activity at specific time. The unheated crude protease was 100% and was considered as a control.

Effect(s) of some polyols and/or calcium on enzyme thermal stability

The effect of the addition of several polyols [PEG 1000, PEG 1500, PEG 6000, glycerin, sorbitol, mannitol, and xylitol] with a final concentration of 100 g / L on the thermal

stability of the crude protease DZ28 was investigated after preincubation at 70°C for 6 hours. Residual activity was determined at pH 12 and 60 ° C compared to two controls performed in the absence of polyol (I) and unincubated (NI). The effect of calcium (1 mM) alone and / or mannitol (100 g / L) on the thermal stability of the crude protease DZ28 after 6 hours of preincubation at 70 ° C was also investigated. The residual activity of the enzyme without additives was assumed to be 100% and was considered as a control.

Substrate specificity determination

The substrate specificity profile of the crude protease DZ28 was measured on natural (gelatin, casein, albumin, ovalbumin and keratin) and modified (albumin azure, azocasein and keratin azure) protein substrates. Enzyme activity was previously determined elsewhere in each substrate according to standard conditions (Jaouadi et al., 2013).

Performance evaluation of the crude protease DZ28

Effect of detergent additives and compatibility of enzyme with laundry detergents

Crude protease DZ28 was pre-incubated for 6 h at 40°C to study the effect of bleaches [hydrogen peroxide (H2O2), sodium perborate], surfactants [SDS, linear alkylbenzenesulfonate (LAS), sulfobetaine], non-ionic surfactants (Tween 20, Tween 40, Tween 80, and Triton X-100), anti-redeposition agents (Na2CO3, STPP, TAED, and Na2CMC) and other detergent additives on the enzyme stability. Residual activity was performed at 60 ° C and pH 12. The effect of H2O2 on the activity and stability of each enzyme was also monitored with 100 mM NaOH borate buffer under the above conditions. Crude enzyme activity (without any additive) was set to 100%.

Stability and compatibility of enzyme with laundry detergents

The stability and compatibility of the crude protease DZ28 with some currently commercially available liquid and solid detergents was investigated. Detergents liquid list included Dipex and Ecovax (Klin Productions, Sfax, Tunisia), Class (EJM, Sfax, Tunisia), Omino Bianco, Fairy, ISIS, and Pril (Henkel, Algeria), and Skip (Unilever, France). The solid detergents used were Ariel, Persil, and Tide (Procter & Gamble, Switzerland), OMO (Unilever, France), Dixan, Axion, and Nadhif (Henkel, Tunisia), and Det (Sodet, Sfax, Tunisia). In order to research their balance and compatibility, the above referred to industrial detergents had been diluted in tap water to acquire a final concentration of 7 mg/mL (to simulate washing conditions) (Vojcic et al. 2015). The endogenous proteases found in those laundry detergents had been inactivated with the aid of heating the diluted detergents for 1 h at 65°C according to Banik et al., (2004). A 500 U/mL of every crude protease DZ28 become shake-incubated with every laundry detergent for 1 h at 40°C, and residual activity become decided at finest pH and temperature of every used enzyme using DMC as a substrate. The enzyme activity of a control (with none detergent), incubated under similar conditions, was taken as 100%.

Removal of protein stains from cotton fabrics

A new cotton cloth (8 cm x 10 cm) was stained with blood and used to simulate wash conditions and measure the efficiency of the crude protease DZ28 as a detergent bio-additive compared to the commercial protease Alcalase ™. The stained pieces of cloth were shaken (200 rpm) with tap water and a commercially available isis detergent (7 mg / ml), and varied in a 1 liter beaker with a total volume of 100 ml at 40 ° C for 30 minutes. After the treatment, a piece of cloth was taken out, rinsed with water, dried, and visually observed to examine the effect of removing the enzyme stain. An untreated blood stain cloth was used as a control.

Statistical analysis

All measurements were performed on three independent replicas and enzyme-free control experiments were performed under the same conditions. Experimental results were expressed as repeat mean and standard deviation (mean ± SD).

Nucleotide sequences accession number

The nucleotide sequence data of 16S rDNA (1501 bp) gene from Halobacillus salinus DZ28 strain reported in this paper has been submitted to the GenBank/ENA/EMBL databases under accession number: MZ156960.

RESULTS AND DISCUSSION

Screening of protease-producing strains

In the current study, seven salt-tolerant bacterial strains are protease producers based on the pattern of clear zone formation in protein-containing media from 45 bacteria isolated from the deposits of Lake Oubeira (El Taref, Algeria). Using the ratio of clear zone diameter (on skim milk agar plate) to colony diameter served as an indicator for selecting strains with high protease production capacity. Seven isolates (DZ19, DZ25, DZ28, DZ33, DZ35, and DZ44) with the highest proportions (> 3.5 mm) were tested for protease production in liquid cultures. Of the seven strains, DZ28 showed the highest ratio of 4.3 mm and the highest extracellular protease activity (about 4,000 U / mL) after culturing in non-optimized medium for 36 hours, so all subsequent strains retained in the study.

Identification of the strain DZ28

A new isolated bacterium (termed DZ28) was recognized utilizing some molecular and catabolic methods. According to the Bergey's Manual of Systematic Bacteriology methods described in (Yabuuchi 2001), morphological, biochemical and physiological properties

happen in the DZ28 isolate in the form of bacilli, aerobic, gram-positive and rod-shaped. It was shown to be endospore formation oxidase, catalase positive, motile, colonies were round to slightly irregular and smooth. Carbohydrate degradation profiles of the isolate were additionally investigated using the API 50CH gallery test. Our results indicate that besides to monosaccharides, this strain is citrate, malate, glucose, glycerol, D-trehalose, ribose, D-xylose, D-galactose, D-fructose, D-mannitol, D-maltose, D-sucrose, D-turanose, D-tagatose, gluconate and lacate. It was shown to have metabolized L-aspartate and L-glutamate. Lactose, sorbitol, glycogen, L-xylose, xylitol, drixose, darabinose, starch, adonitol, sorbose, erythritol, inulin, D-arabitol, L-arabitol, capric acid, adipic acid, phenylacetic acid, propionic acid and glycine are not metabolized like energy origins. For that reason, the whole results acquired concerning the biochemical and physiological properties and phenotype of this isolate completely established that DZ28 strain belongs to the genus Halobacillus (Table 1).

A phylogenetic tree based on the 16S rDNA gene Fig. 1 reveal that the novel isolates clustered with members of the genus related Bacillus, the nearest neighbor being Halobacillus salinus HSL-3T with an average similarity of 98.60% (accession no.: AF500003). Based on the results obtained in the course of the present study, we suggest the assignment of this isolate (accession no. MZ156960) as Halobacillus salinus strain DZ28.

Table 1. Phenotypic, physiologic, and biochemical characteristics of the DZ28 isolate.

Characterization features of the strain DZ28		
Phenotypic characteristics	Colony density	Translucent
	Colony morphology	Smooth circular to slightly irregular
	Cell shape	Ellipsoidal
	Cell arrangement	Single/pairs
	Motile	+
	Gram	+
Physiological characteristics	Temperature range (°C)	10–45 (30)
	pH range	6–9 (8)
	NaCl range (%)	2–20 (10)
Biochemical characteristics	Catalase	+
	Oxidase	+
	Nitrate reduction	-
	Sporulation	+
	ß-galactosidase	+
	Arginine Dihydrolase	-
	Lysine Decarboxylase	-
	Ornithine Decarboxylase	-
	Citrate	-
	H_2S	-
	Urease	-
	Tryptophane Desaminase	-
	Indol	-
	Voges-Proskauer	+
	Aesculin	+
	Casein	+
	ONPG	+
	Gelatin	-
	D-Glucose	+
	D-Mannitol	+
	D-Fructose	+
	D-Maltose	+
	D-Raffinose	+
	D-Sucrose	+
	D-Melibiose	+
	D-Galactose	+
	D-Trehalose	+

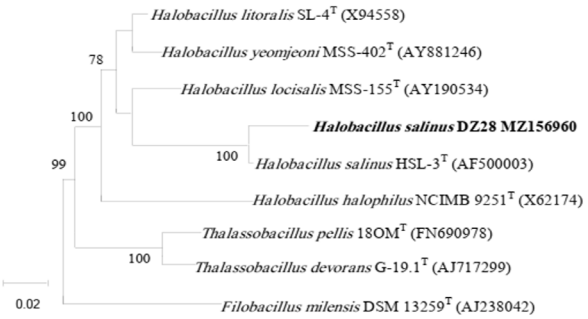

Figure 1. A phylogenetic tree derived from 16S rDNA gene sequence specifying the position of the DZ28 strain (accession number MZ156960) within the radiation of the genus Halobacillus. Bar, 0.02 nt substitutions per base. The node number (> 50%) designates support for internal branches in the tree acquired by bootstrap analysis (percentage of 1000 bootstraps). The NCBI accession number is shown in parentheses.

Protease production by strain DZ28

No defined medium has been established for the best production of proteases from different microbial sources. Each strain has its own special conditions for maximum enzyme production (Zhu and Zhang, 2019). Protease production was tested in the initial medium, containing 5 g/L of different carbohydrates. Results conclude that, the best carbon source for protease production was casein (4,000 U/mL) followed by gelatin (2,500 U/mL). However, enzyme production was significantly low (570 and 500 U/mL) when strain DZ28 was grown on glucose and sucrose, respectively, and was nearly the same as that of the control without carbon source. Since casein was the best carbon source, the effect of its various concentrations on protease production was studied. The optimum concentration of casein for protease production was 10 g/L (10,300 U/mL). Different workers have used different organic nitrogen sources (simple or complex), inorganic nitrogen sources and amino acids for enhancing protease production. The best nitrogen source for protease production was yeast extract, 12,500 U/mL; followed by beef extract, 11,230 U/mL and (NH4)2SO4, 10,250 U/mL. Protease production was significantly lower with NH4Cl, 855 U/mL and NaNO3, 1,060 U/mL as nitrogen sources. Based on these observations, yeast extract was selected and its various concentrations were tested for the protease production. Maximum protease activity was achieved at a concentration of 4 g/L (14,900 U/mL), giving about 19.22-fold enzyme activity, compared to the medium without nitrogen sources (775 U/mL). In the medium containing (in g/L): casein, 10; meat extract, 4; CaCl2, 1; K2HPO4, 1.5; and KH2PO4, 1.5; the addition of trace elements at 2% (v/v) significantly improved protease production by 1.27-folds, reaching 19,000 U/mL. Considering the overall modifications, this last optimized medium was retained for all further studies. Under this particular condition, the enzyme production started after a 4 h lag phase and then increased exponentially and concomitantly with the increase on cellular growth and reached the maximum within 36 h of cultivation Fig. 2.

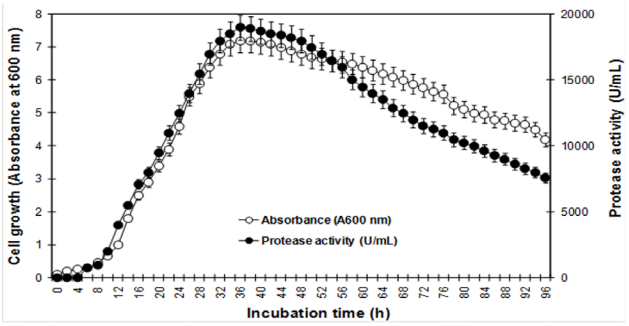

Figure 2. Proteases production kinetics from the DZ28 strain. Switches gradually in cell proliferation (○) of the DZ28 strain and production (•) of the crude protease DZ28. Cell generation was managed after calculating absorbance at 600 nm then converted to dry weight (g / l) of cells. The results shown as mean (n = 3) ± standard deviation.

Biochemical characterization of the crude protease DZ28

Effects of some inhibitors, reducing agents, and metal ions on enzyme stability

As may be seen in Table 2 two well-known inhibitors of serine proteases; PMSF and DIFF actively inhibited crude enzyme activity. However, further inhibitors such as TPCK, TLCK, benzamidine and SBTI tested in this experiment resulted in no inhibitory effect against enzyme activity. Moreover, thiol reagents (NEM, iodoacetamid and DTNB) result in small-scale impact on enzyme activity. Based on these results, it can be concluded that our crude extracellular enzymes appertain to serine proteases families. In the existence of 2 mM EDTA and 10 mM EDTA like metalloprotease inhibitors, proteases have been found to keep 84% and 94% of their activity, respectively, indicating the non-existence of metal cofactors. The enzyme activity was conserved even after large-scale dialysis when the enzyme was opposed to buffer A containing 2 mM EGTA (results not presented). Insensitiveness against chelators is a beneficial quality with an important implication especially during detergent preparation since these agents were very useful like water softeners and spot eliminators (Rekik et al., 2019). As a matter of fact, moving of calcium out-off the powerful binding locations was related with a significant depletion in thermal stability since the serine protease family encloses two calcium binding sites (Lee and Jang, 2001).

Table 2. Effects of various inhibitors and reducing agents on the crude protease DZ28 stability. Protease activity measured in the absence of any inhibitor or reducing agent was taken as control (100%). Residual activity was measured at pH 12 at 60°C.

Inhibitor/reducing agent	Concentration	Residual protease activity (%) [a]
None	–	100 ± 2.5
PMSF	5 mM	0 ± 0.0
DIFP	5 mM	0 ± 0.0
SBTI	1 mg/mL	99 ± 2.5
TLCK	1 mM	97 ± 2.5
TPCK	1 mM	92 ± 2.2
Benzamidine	10 mM	101 ± 2.5
LD-DTT	10 mM	96 ± 2.4
2-ME	5 mM	95 ± 2.3
DTNB	5 mM	94 ± 2.3
NEM	2 mM	92 ± 2.2
Iodoacetamide	5 mM	82 ± 2.0
Leupeptin	50 µg/mL	93 ± 2.1
Pepstatin A	2 µg/mL	99 ± 2.5
EDTA	10 mM	94 ± 2.3
EGTA	2 mM	84 ± 2.1

[a] Values represent means of a three replicates, and ± standard errors are reported.

PMSF: phenylmethanesulfonyl fluoride; DIFP: diiodopropyl fluorophosphates; SBTI: soybean trypsin inhibitor; TPCK: *Na-p*-tosyl-L-phenylalanine chloromethyl ketone; TLCK: *Na-p*-tosyl-L-lysine chloromethyl ketone; LD-DTT: LD-dithiothreitol; 2-ME: 2-mercaptoethanol; DTNB: 5,5′-dithio-bis-2-nitro benzoic acid; NEM: *N*-ethylmalemide; EDTA: ethylene-diaminetetraacetic acid; EGTA: ethylene glycol-bis (b-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

The impacts of some metal ions on the extracted protease DZ28 stability were further studied (Table 3). Sequentially, crude protease activity was ameliorated by 145, 166, and 180%, following MgCl2, MnCl2, and CaCl2 at 1 mM addition in comparison with controls. Based on this results our enzyme is in need of Mn2+, Mg2+ and Ca2+ for its adequate action. In our experiment, adding Co2+ and Cu2+ have a positive impact on the extracted crude enzyme and a slight activity elevation was recorded. However, the enzyme was totally inhibited by Hg2+, Cd2 and Ni2+, and relatively within Fe2+. Including enzymes, ions recognized as toxic metal may bind to different organic ligands and will be toxic and responsible of proteins denaturation. Elevated crude protease activity using Mg2+, Mn2+, and Ca2+ may be explained by the protective results on the enzyme opposed to thermic denaturation by metal ions and consequently relax there principal character on keeping good activity at high level temperature degrees like was described by (Hadjidj et al., 2018).

Apparently, Ca2+ has a positive effect on the activity and stability of the crude protease DZ28 within preservation of its structure from autolysis. The data obtained are broadly consistent with the major trends of Bouacem et al describing the impacts of EDTA and Ca2+ on protease activity (Bouacem et al., 2015).

Table 3. Effects of some metal ions on the crude protease DZ28stability. The non-treated enzyme to which 2 mM EGTA were added was considered as 100%. Residual activity was measured at pH 12 at 60°C.

Metal ions at 1 mM	Origin	Residual protease activity (%) [a]
None	–	100 ± 2.5
Ca^{2+}	$CaCl_2$	180 ± 4.5
Mn^{2+}	$MnSO_4$	166 ± 4.1
Mg^{2+}	$MgSO_4$	145 ± 3.8
Cu^{2+}	$CuSO_4$	101 ± 2.5
Zn^{2+}	$ZnSO_4$	90 ± 2.1
Co^{2+}	$CoSO_4$	102 ± 2.5
Fe^{2+}	$FeSO_4$	75 ± 1.9
Ni^{2+}	$NiCl_2$	0 ± 0.0
Hg^{2+}	$HgCl_2$	0 ± 0.0
Cd^{2+}	$CdCl_2$	0 ± 0.0

[a] Values represent means of a three replicates, and ± standard errors are reported.

Effects of different pH and temperature on enzyme activity and stability

As shown in Figure 3a the crude protease DZ28 was active over a large pH range (2-13) and was optimal at pH 12. Respectively, a relative activity was observed 60% and 50% at pH 6.5 and 13. In our study, the effect of pH on enzyme stability profile exhibited that the extracted enzyme was very active in a large pH range from 8-13. Figure 3b. The crude protease DZ28 half-lives at different pH; 7, 8, 9, 10, 11 and 12 were 345, 300, 255, 210, 165 and 120 minutes, respectively. In comparison with the major and large commercial detergents enzymes our values were further competent as well as Alcalase ™ extrated from Bacillus licheniformis, with large activity between 8-9 pH values (Beg and Gupta, 2003) and the produced Savinase ™ from Bacillus lentus, with large activity between pH value of 8-10 (Beg and Gupta, 2003). These results further validate the good potentiality of the crude protease DZ28 in later manufacturing utilizations which requires enzymatic strength above large pH range (9-11) (Gupta et al., 2002). Furthermore, our crude protease DZ28 was ideally operative at 50 ° C in the presence of 1 mM Ca2+ At pH 12 and in the absence of CaCl2 and at 60 ° C applying casein like a substrate, (Fig. 3C). The extracted protease DZ28 half-lives away of additives were 255, 180 and 90 minutes at 50, 60 and 70 ° C (Fig. 3D). As appear in figure. 3D the crude protease DZ28 half-life rises to 300, 225, and 135 minutes at 50, 60, and 70 ° C in company of 1 mM CaCl2. As a matter of fact, Ca2+ cations were

reported in previous studies to enhance the stability and the activity of Aeribacillus pallidus VP3 proteases (Mechri et al., 2017a). The thermic stability and thermic activity of the extracted protease were high up than some previous reported other proteases extracted from Bacillus strain (Benkiar et al., 2013; Rekik et al., 2019).

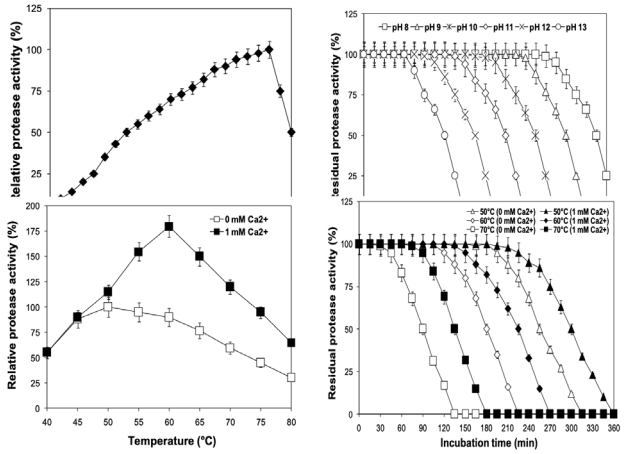

Figure 3. Effect of different pH values on (a) activity and (b) stability of the crude protease DZ28. The activity of the enzyme at pH 12 was supposed to be 100%. The buffers used for pH activity and stability are shown in Section 2. Effects of (C) thermoactivity and (D) thermostability of the crude protease DZ28. The enzyme was pre incubated at various temperatures of 50, 60, and 70 ° C in the absence or presence of 1 mM CaCl2. Remaining protease activity was measured from 0 to 6 hours at 15 minute intervals. The activity of the non-heated enzyme was assumed to be 100%.

Combined effect(s) of some polyols and/or calcium on the enzyme thermal stability

As shown in Figure 4a, addition of some polyols appears to improve the thermic stability of the enzyme in comparison to standard. As matter of fact, residual activities were 90, 70, 66, 60, 55, 41 and 35% in the company of mannitol, sorbitol, glycerol, PEG 1000, PEG 150, PEG 6000 and Xylitol respectively, 6 hours after the incubation at 70 ° C. Away from polyols the activity was 50% after incubation for 50-6 hours. The results shown in Figure 4a indicated that the elevated levels of enzyme activities were registered in the presence of mannitol like additive at a concentration of 100 g/L. In accordance to some reports in the literature, the thermic stabilities of the alkaline proteases of Bacillus safensis RH12 (Rekik et al., 2019) and Lysinibacillus fusiformis C250R (Mechri et al., 2017b) were improved in the presence of glycerol, mannitol, and some polyethylene glycol.

Data shown in Fig. 4b indicate that the addition of mannitol or CaCl2 relatively enhance the thermostability. Take note of, addition of mannitol only was better to CaCl2 solo, with half-lives all over 180 and 135 minutes, appropriately. Nevertheless, the presence of both additives ameliorated the half-lives at 70 ° C to be 225 minutes, designating a positive blend effect of mannitol and CaCl2 on the thermic stability of the extracted DZ28 strain protease. The addition of polyols causes changes in enzyme's micoenvironment which can boost and elevate their thermal stability (Steuer et al., 2009). The hydroxyl groups of polyols stand on their protective effect (Omrane Benmrad et al. 2019). In actual fact, polyols addition protects against enzyme molecules denaturation by elevating the hydrophobic interactivities inside the protein fragments that come further resistant to thermic inactivation (Joo and Chang, 2005).

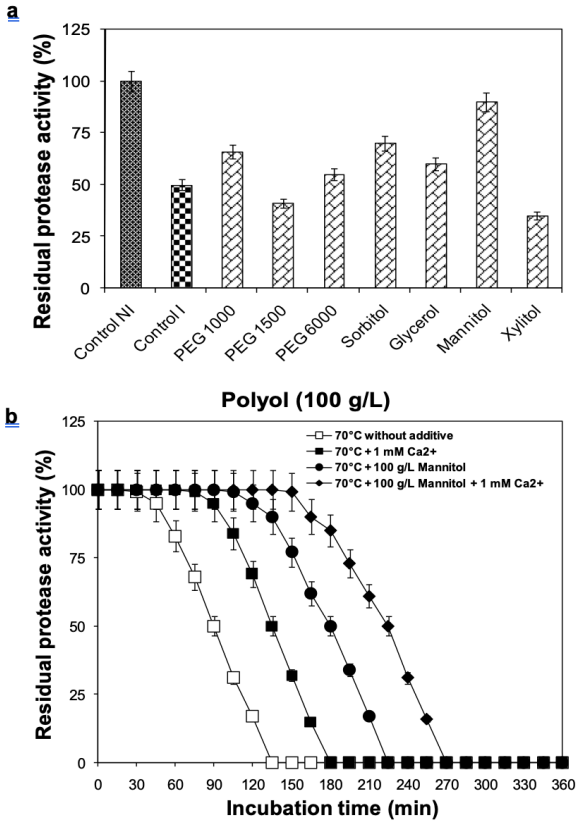

Figure 4(a). The effect of some polyols on the thermal stability of proteases. The crude protease DZ28 was heated to 70 ° C for 6 hours in the absence or presence of various polyols (10%). The remaining enzyme activity was determined under optimal conditions. The vertical bar indicates the standard error of the mean (n = 3). Fig. 4. (b) Combined effect of mannitol and calcium on the thermal stability of the crude protease DZ28 at 70 ° C. The enzyme was preincubated in the absence (□) or presence of additives: 1 mM Ca2+ (■); 100 g / L mannitol (•); and 1 mM Ca2+ and 100 g /L mannitol (♦). Remaining protease activity was determined from 0 to 360 minutes at 15 minute intervals. The activity of the non-heated enzyme was considered to be 100%. Each point represents the mean (n = 3) ± standard deviation.

Substrate specificity profile

As shown in Table 4, natural substrates were degraded on different degrees by the extracted proteases. More effectively the extracted protease of the DZ28 strain was in case of casein more than ovalbumin, albumin, keratin and gelatin. These results are in good agreement with other studies which have shown that protease extracted from Aeribacillus pallidus VP3, degraded preferably, casein followed by other substrate such as keratin, ovalbumin, gelatin, and BSA, which were slightly degraded by the extracted crude enzyme (Mechri et al. 2017a). Furthermore, alkaline protease manufactured by Brevibacillus brevis US575 demonstrated large range of activity against multiple substrates lake keratin, come after gelatin, casein, myoglobin, elastin and albumin (Jaouadi et al., 2013). Once the crude proteases from the DZ28 strain were examined against varied proteins substrates, elevated amounts of hydrolytic activity was registered with large number of substrates and the highest specificity were recorded with azocasein and albumin.

Performance evaluation of the crude protease from strain DZ28

Effects of some additive on enzyme stability

The crude protease DZ28 activity and stability, extracted from medium grown in the existence of anti redeposition agents and 1% perfume, mainly cationic (TTAB and CTAB),10% Na2•CMC, and zwitterionic (Zwittergent 3-12 and CHAPS) detergents agent, overall measurement results are summarized in (Table 5). Various commercially convenient modifiers or anionic surfactants, nonionic surfactants, and bleaching factors to 15% Tween 20 and powerful anionic surfactants, mainly LAS and SDS, were tested as opposed to Alcalase ™ 1 and 40 or Triton X100 by incubating at 40 °C for 6 hours the extracted protease DZ28 (Table 5). Our crude enzyme DZ28 kept 250 and 125% of its native action in comparison to 144 and 75% of Alcalase ™ following dealing with 15% hydrogen peroxide and 5% sodium perborate, as a consequence after bleaching, however the crude protease stay highly strong. Such protease activity and stability were fascinating because to our knowledge, extremely little wild-type proteases notified to be invariable in opposition to detergents, bleaches and oxidants. According to Jaouadi et al. (2010) Streptomyces p the AB1 strain stability was notably higher with the existence of 1.5% SDS, 15% H2O2 and 5% Tween 40. Furthermore the two proteases (BM1 and BM2) shared good quality in the existence of 1% Triton X100 and manifested solidity in the existence of 1% H2O2 and 0.1% SDS (Gupta et al., 1999; Haddar et al., 2009). Bleach balance has additionally been accomplished with the aid of using protein engineering (Radha and Gunasekaran, 2008). Our data presents several solutions to detergent formulations with cleaning bio-additives using the potential of crude proteases from the DZ28 strain.

Stability and compatibility of crude enzyme with commercial laundry detergents

As follows from the Fig. 5a, in comparison to Alcalase™, our crude protease DZ28 was exceptionally strong as much as the stable washing detergents and the commercial product tested at a concentration of 7 mg/mL, their starting activity was retained 100% in presence of ISIS and Pril (vs 90% within Alcalase™) and 98, 97, and 95 with OMO, Ariel, and Dixan, respectively (vs 90, 100, and 88% for Alcalase™, respectively). Additionally, fig. 5a shown the high stability of the crude protease DZ28 exceeding 90% in Det (as opposed 66% of Alcalase ™) despite 6 hours of incubating at 40 ° C as illustrated. Nevertheless, a low stability of the crude protease DZ28 was recorded in the existence of Axion and Persil (77%), while on the contrary Alcalase ™ maintained 100% and 94% of the initial action of Axion and Persil, respectively. It has been reported that Proteases VM10 and SSR1 and protease SSR1 (Venugopal and Saramma, 2006; Singh et al., 2001) retained at most 42% and 37% of their initial action, respectively, during incubation in the existence of Ariel below the identical conditions. Our observations, furthermore hold-up the effectiveness of the extracted crude protease DZ28 in some hereafter manufacturing implementations like a cleaning bio-additive for detergent preparations.

Stains removal from cotton fabrics

In the aim to evaluate the crude proteases DZ28 and Alcalase ™ for their ability to remove blood stains performances, several pieces of stained cotton cloth

Table 4. Substrate specificity profile of the crude protease DZ28.

Substrate	Concentration		Absorbance (nm)[a]	Relative protease activity (%)[a]
Natural protein	Casein	30 g/L	600	100 ± 2.5
	Albumin	30 g/L	600	90 ± 2.1
	Gelatin	30 g/L	600	50 ± 1.2
	Ovalbumin	30 g/L	600	38 ± 1.0
	Keratin	30 g/L	600	20 ± 0.8
Modified protein	Azo-casein	25 g/L	440	100 ± 2.5
	Albumin azure	25 g/L	440	87 ± 2.3
	Keratin azure	25 g/L	440	23 ± 0.9

[a] Values represent the means of three replicates, and ± standard errors are reported.

Table 5. Effect of some detergents on the crude protease DZ28 stability. The crude protease DZ28 and Alcalase™ were pre-incubated with each detergent additive for 6 h at 40°C and the residual activity were measured under the each assay standard conditions of each used enzyme. The activity is expressed as a percentage of the activity level in the absence of additives

Detergent	Concentration	Residual protease activity (%) [a]	
		Crude protease DZ28	Alcalase™
None	–	100 ± 2.5	100 ± 2.5
Tween 20	10% (v/v)	135 ± 3.6	142 ± 3.8
	15%	144 ± 3.9	137 ± 3.6
Tween 40	10% (v/v)	140 ± 3.8	129 ± 3.3
	15%	130 ± 3.4	110 ± 2.7
Tween 80	10% (v/v)	136 ± 3.4	108 ± 2.7
	15%	128 ± 3.3	106 ± 2.6
Triton X-100	10% (v/v)	180 ± 4.5	130 ± 3.2
	15%	165 ± 4.1	120 ± 3.2
SDS	1% (w/v)	91 ± 2.3	80 ± 2.0
	5%	72 ± 2.1	61 ± 1.5
LAS	1% (w/v)	99 ± 2.5	96 ± 2.3
	5%	93 ± 2.2	73 ± 1.9
Sulfobetaine	50 mM	102 ± 2.5	105 ± 2.6
H_2O_2*	10% (v/v)	250 ± 6.9	155 ± 3.6
	15%	190 ± 5.1	144 ± 3.5
Sodium perborate	2% (w/v)	125 ± 2.9	89 ± 2.1
	5%	105 ± 2.6	75 ± 2.1
TAED	10% (w/v)	134 ± 3.6	114 ± 2.7
$Na_2{\cdot}CO_3$	50 mM	98 ± 2.5	121 ± 3.2
$Na_2{\cdot}CMC$	10% (w/v)	101 ± 2.5	101 ± 2.5
STPP	25 mM	96 ± 2.4	92 ± 2.2
Zwittergent 3-12	15 mM	111 ± 2.7	113 ± 2.6
CHAPS	25 mM	98 ± 2.5	125 ± 3.2
CTAB	25 mM	104 ± 2.6	110 ± 2.7
TTAB	25 mM	107 ± 2.7	106 ± 2.6
Zeolithe	1% (w/v)	109 ± 2.7	90 ± 2.2
Perfume	1% (v/v)	141 ± 3.8	97 ± 2.5

were incubated under different conditions. As presented in Fig. 5b, the only limited cleaning performance was registered with ISIS detergent. When the commercial protease Alcalase ™ or the crude protease DZ28 were added on, the washing agent appears to ameliorate the cleaning procedure, like indicated by the quick elimination of blood stains in opposition to the solely washing agent. As a matter of fact, the crude protease DZ28 further help the proteinaceous substances elimination more easily compared the Alcalase ™ enzyme actually used. Besides, the mixture of crude protease DZ28 and solid ISIS detergent offered total stain elimination (Figure 5b). An important implication of these findings is that crude protease DZ28 may have the usefulness in future industrial applications as a cleaning bio-additive for detergent formulations. The data obtained are broadly consistent with the major tendencies reported the usefulness of alkaline proteases in improving the elimination of blood stains from cotton cloth, such as proteases extracted from Bacillus pumilus CBS (Jaouadi et al., 2008), Caldicoprobacter guelmensis D2C22T (Bouacem et al. 2015), and Bacillus licheniformis K7A (Hadjidj et al., 2018). As well, the oil-contaminated metagenomic sludge library protease Pro2T21opti seems to be more effective at alkaline pH range (pH 8-11) and low temperatures (30 ° C) (Gong et al., 2017). For all these reasons and others, we believe that the crude protease DZ28 is further successful.

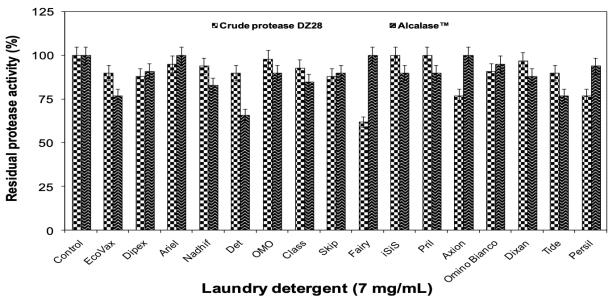

Figure 5a. Stability of crude proteases DZ28 and Alcalase ™ in the presence of liquid and solid detergents. The enzyme activity of the control sample, which contained no additives and was incubated under similar conditions, was considered to be 100%. Each point represents the average of three independent experiments. The vertical bar indicates the standard error of the mean (n = 3). One unit of protease activity was defined as the amount of enzyme required to catalyze the release of 1 µmole peptide bond from the DMC under the experimental conditions used.

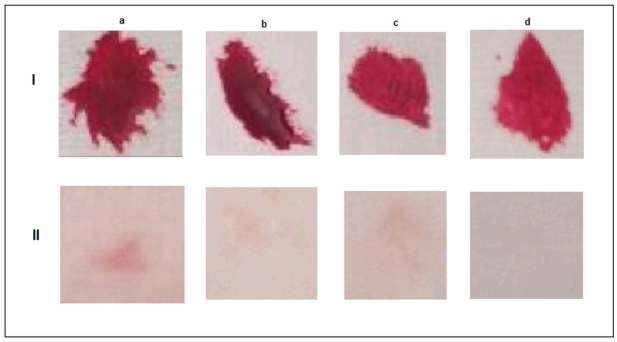

Figure 5b. Cleaning performance analysis test of our crude protease in the presence of the commercial detergent ISIS. (a) A cloth soiled with blood washed with tap water. (b) Blood stain cloth washed with ISIS detergent (7 mg / ml), (c) Blood stain cloth washed with ISIS containing Alcalase ™ 2.5 L (commercially available enzyme, 500 U / ml), (d) Blood stain cloth, washed with ISIS added with crude protease DZ28 (500 U/ml). I: untreated cloths (control) and II: treated cloths.

CONCLUSION

In summary, this study assesses the potential of the detergent-stable crude alkaline protease DZ28 by examining its interesting biochemical properties. This crude protease has exhibited some encouraging characteristics for biotechnologies and their applications that vary from other Bacillus proteases. Furthermore, to our knowledge, this is the first study to deal with the protease activity of Halobacillus salinus strain DZ28, isolated from an Algerian lake sediments. The results documented in our experiment indicate that by optimization and characterization of crude enzymes, stability at high pH values in the presence of specific metal ions, detergents, and detergent additives, this crude enzyme have some industrially useful properties. As a matter of fact, this enzyme may be suitable for a diversity of industrial uses, such as detergent synthesis, besides its potential applications in the laundry industry.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declared that they have no conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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