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Preliminary screening and characterization of novel proteolytic enzymes produced by extremophilic bacteria isolated from Tunisian and Algerian biotopes

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Abstract

The current paper reports on the production and characterization of extracellular thermostable proteases produced from thermophilic bacteria namely *Aeribacillus pallidus* VP3, *Anoxybacillus kamchatkensis* M1V, *Virgibacillus natechei* FarD^T, and *Melghiribacillus thermohalophilus* Nari2A^T strains isolated from different extremophlic biotopes in Tunisia and Algeria. The key challenge of the use of the different proteases in industrial applications is their efficiency under unconventional conditions. An optimization via one-factor-at-a-time (OFAT) methodology has been used to obtain 3,000 U.mL^{-1}; 4,600 U.mL^{-1}; 15,800 U.mL^{-1}; and 16,000 U.mL^{-1} of proteases activity from VP3, M1V, FarD^T, and Nari2A^T strains, respectively. Particularly, VP3 and FarD^T proteases showed a high tolerance to several organic solvents. In addition, the protease produced from FarD^T strain might be useful as potential candidate for application in detergency. The deproteinization of shrimp wastes by M1V and Nari2A^T proteases was successful with a high rate of deproteinization of about 48% and 82%. The obtained proteolytic hydrolysate obtained by M1V strain showed high biological activities with half maximal inhibitory concentration (IC50) of 71.52 µg.mL^{-1}, 107.67 µg.mL^{-1}, and 133.24 µg.mL^{-1}, respectively for the angiotensin, tyrosinase, and amylase inhibitory activities.

Keywords: Extremophilic bacteria; Thermostable proteases; Biotechnologically applications.

1. Introduction

Microbial life does not seem to be restricted to specific environments. Over the past few decades, it has become clear that microbial communities can be found in the most diverse conditions, including extremes of pressure, salinity, temperature, and pH [1]. These microorganisms, called extremophiles, produce biocatalysts mainly enzymes that are functional under extreme conditions. Accordingly, the unique properties of these biocatalysts have resulted in several novel applications of enzymes in industrial and biotechnological processes.

Biochemical properties of these enzymes demonstrated that they have a high optimum working temperature and pH, which could make them suitable for industries applications [2, 3]. Above all, proteases are enzymes catalyses the hydrolysis of peptide and iso-peptide bonds that join amino acids within proteins. Again, they can act near the ends of polypeptide chains [4]. They are differentiated according to their substrate specificity as amino-peptidases, which are cleaves the peptides at the N-terminus, and carboxypeptidases, which are degrades peptides at the C-terminus [5]. The unique catalytic activities of these enzymes make them an inexpensive choice for hydrolyzing peptide bonds for industrial uses [6].

Their ability to break down these compounds makes them excellent for stain removal. Proteolytic enzymes catalyze the hydrolysis of proteinaceous material and

show the highest abundance amongst industrial relevant biocatalysts [7, 8]. Proteolytic enzymes are found to have a large multiplicity in their applications, such as constituent in detergents [9], industrial processing of food [10], leather [11], peptide synthesis [12], and pharmaceutical products [13]. Additionally, the use of these proteases is highly efficient, by allowing the recovery of chitin and bioactive peptides from crustacean's bio-wastes [14, 15]. This was supported by cloning amino-acid sequences inspection and homology-modeling of the genes encoding extremozymes, which is endowed with a number of characteristics that are highly valued. Thus, these observations inspired us to explore other enzymes from the extremophile strains.

For particular interest, North Africa countries by their geographical position present a great diversity of extreme biotopes occupied by an important microbiological richness. Mainly, Tunisia and Algeria possess many extreme biotopes such as saline lakes, hydrothermal hot springs, and petroleum reservoirs. Furthermore, the family of *Bacillaceae* comprises the rod-shaped bacteria which form endospores, having a wide diversity of physiological characteristics. Their ability to form hardy spores enables them to be widely distributed in nature, from Arctic environments to hot springs and desert sands and from fresh water to salt or marine sediments. Very few reports are available on enzymes production from *Aeribacillus*, *Anoxybacillus*, *Virgibacillus*, and *Melghiribacillus* genera.

Keeping these points in view, the present contribution was undertaken the screening of bacteria isolated from different extreme ecological niches in Tunisian and Algeria and hyperproducing of proteolytic enzymes.

Additionally, the optimization through one-factor-at-a-time (OFAT) methodology and biotechnological application of these proteases, having expected proprieties for their potential uses as bio-additives with various laundry detergents and deproteinization of crustacean's by-products are also investigated.

2. Materials and Methods

2.1. Microorganisms

Different strains were isolated from different extreme ecological niches in Tunisia and Algeria (Figure 1). The biotopes are saline lakes, hydrothermal hot springs, and petroleum reservoirs. The ATAM, M1V, B5GN, HB14, and BA1 strains were isolated from Hammam Righa hot spring Ain Defla, in northwestern Algeria [16]. The heating system is characterized with a temperature of 68°C, a salinity of 150 $g.L^{-1}$ and a pH of 6.9. The VP3 strain is isolated from the production water of the Litayem oil-field managed by Thyna Petroleum Services[1], Sfax, Tunisia. The formation water was withdrawn from the oil-bearing horizons from depths of 1,300 m, with a temperature of 78°C, a salinity of 100 $g.L^{-1}$, and a pH 7.6, after passing through a pipeline of about 20 km. The $FarD^T$ strain is isolated from sediments of a saline lake located in Taghit, 93 km from Bechar, southwest of Algeria. The temperature at the sampling site was 39°C and pH was 6.8. The $Nari2A^T$ strain is isolated from the soil of Chott Melghir, an Algerian Salt Lake located at north-east of Biskra city, Algeria. The temperature at the sampling site was 39°C and the pH was 6.8. The soil is characterized by alkaline pH of 8.4 and a salinity of 112 $g.L^{-1}$. The C2SS100 and C2SS10 strains are isolated from the production water of Sercina petroleum reservoir, located near the Kerkennah Island, Tunisia. The production water is of about 30 km with a temperature of 25°C, a salinity of 71 $g.L^{-1}$, and a pH of 7.8. The positive strains were screened on skimmed milk agar plates (SMAP) as earlier reported [17].

2.2. Identification of microorganisms, DNA sequencing, and phylogenetic analysis

The VP3, M1V, $FarD^T$, and $Nari2A^T$ strains were identified previously based on both catabolic and molecular approaches [18-21]. For example, the species level identification of strain M1V was performed using microbiological characteristics and 16S rRNA (rDNA) gene sequence analysis. The colony morphologies were determined using cultures grown aerobically on nutrient agar (NA). Cell morphology and motility were examined microscopically in exponentially growing liquid cultures after 18 to 24 h of incubation at 60°C by means of conventional tests. Acid production from carbohydrates and hydrolyses of some polymers were determined using analytical profile index (API) strip tests API 20E and API 50 CHB (bioMérieux, SA,

[1] http://www.made-in-tunisia.net/vitrine/contact.php?tc1=lKmYnqyS

Marcy-l'Etoile, France) as recommended by the manufacturer. The temperature range for growth was determined by incubating the isolate at 30 to 80°C. The effect of NaCl on bacterial growth was studied in the presence of 1 to 5% (w/v) NaCl. The pH dependence of growth was tested in the pH range of 6 to 10. All the physiological tests were determined in NA medium, with the exception of the temperature growth at 80°C which was performed in Nutriment broth (NB). The polymerase chain reaction (PCR) amplification of the 16S rRNA gene was carried out with two universal primers, one forward and the other reverse, designed from the conserved zones within the rRNA operon of *E. coli* [22]. The forward primer was 5′-AGAGTTTGATCCTGGCTCAG-3′ extended from base position 8 to 27; the reverse primer was 5′-AAGGAGGTGATCCAAGCC -3′ extended from base position 1541 to 1525. The genomic DNA of the M1V strain was purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and then used as a template for PCR amplification. After denaturation at 95°C for 5 min, DNA samples were subjected to 35 cycles of amplification with denaturation at 95°C for 30 s, annealing at 61°C for 45 s, and extension at 72°C for 60 s, followed by a final elongation step at 72°C for 10 min. The PCR product (~1.5 kb) was then cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA), leading to the pM1V-16S plasmid. The *E. coli* DH5α was used as a host strain. All recombinant clones of *E. coli* were grown in Luria-Bertani (LB) media composed of ($g.L^{-1}$) the following: peptone, 10; yeast extract, 5; NaCl, 5 at pH 7.4, with the addition of ampicillin (100 $\mu g.mL^{-1}$), isopropyl-thio-β-d-galactopyranoside (IPTG) (0.67 mM), and X-gal (360 $\mu g.mL^{-1}$) for screening. DNA electrophoresis, DNA purification, restriction, ligation, and transformation were all performed according to the Sambrook method previously described [23].

2.3. Media composition and culture conditions

Enzyme production by microorganisms is generally affected by growth conditions and culture medium composition. Every strain has a particular optimized medium at pH 7.4.

The preculture of M1V and VP3 strains was carried out in a 1 L Erlenmeyer flask containing 100 mL of LB liquid medium at pH 7.4, then incubated at 45°C overnight. While, the preculture of $FarD^T$ strain was carried out on Sehgal and Gibbons medium (SG) containing the following (in $g.L^{-1}$): casamino acids, 7.5; yeast extract, 10; sodium glutamate, 1; trisodium citrate, 3; $MgSO_4 \cdot 7\ H_2O$, 20; KCl, 2; $FeSO_4 \cdot 7\ H_2O$, 0.036; and $MnCl_2 \cdot 4\ H_2O$, 0.00036 supplemented with 100 $g.L^{-1}$ of NaCl at pH 7 then incubated at 35°C overnight.

For $Nari2A^T$ strain, the preculture was carried out on International Streptomyces Project 2 medium (ISP2) containing the following (in $g.L^{-1}$): glucose, 4; yeast extract, 4; malt extract, 4; $CaCO_3$, 2; and 10% (w/v) NaCl then incubated at 55°C overnight. These pre-culture were used to inoculate different cultures, with initial A600 nm of 0.1 on optimized media for 22 h at 45°C (for VP3 strain), for 48 h at 45°C (for M1V strain), for 36 h at 35°C (for $FarD^T$), and for 52 h at 55°C ($Nari2A^T$ strain). The protease from *Aeribacillus pallidus* strain VP3 was produced using the optimized medium composed of ($g.L^{-1}$): gelatin, 15; soya flour, 2; NaCl, 10; K_2HPO_4, 1; KH_2PO_4, 1; $CaCl_2$, 2; $MgSO_4 \cdot 7H_2O$, 1; and 1% (v/v) trace elements. The alkaline protease from M1V strain was produced using the medium composed of ($g.L^{-1}$): gruel, 8; soy peptone, 4; KH_2PO_4, 1; K_2HPO_4, 1, $CaCl_2$, 0.2; $MgSO_4 \cdot 7\ H_2O$, 0.1 and 1% trace elements. The trace elements composed of ($g.L^{-1}$) the following: $ZnCl_2$, 0.4; $FeSO_4 \cdot 7H_2O$, 2; H_3BO_3, 0.065; and $MoNa_2O_4 \cdot 2H_2O$, 0.135. The media were autoclaved at 120°C for 20 min. The proteases from *Melghiribacillus thermohalophilus* $Nari2A^T$ and *Virgibacillus natechei* $FarD^T$ strains were produced using the optimized media containing only 40 and 60 $g.L^{-1}$ white shrimp shell by-product in the Erlenmeyer flasks. Before each assay, the cell debris was removed by centrifugation at 10,000 g for 30 min. Next, the obtained clear supernatant was used as a crude enzyme preparation.

2.4. Protease activity assay

The protease activity was assayed by the addition of 0.5 mL of an appropriately diluted enzyme in 100 mM of the appropriate buffer, supplemented with 2 mM $CaCl_2$, to a 0.5 mL of 10 $g.L^{-1}$ casein. The reaction mixture was incubated for 15 min at the appropriate

temperature. The reaction was stopped by adding 0.5 mL of 200 $g.L^{-1}$ TCA. In fact, the liberated tyrosine from casein was measured using Kembhavi method [24]. One unit (U) of peptidase was defined as the amount of enzyme releasing 1 µg of tyrosine released under the assay conditions detailed. Protease activity present in the laundry detergent solution was determined through the method proposed by Boulkour Touioui et al. [25], which used the *N*,*N*-dimethylated casein (DMC) as a substrate and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) as a color indicator. One unit of protease activity was defined as the amount of enzyme required to catalyze the cleavage of 1 µmole of peptide bond from DMC per minute under the experimental conditions used. The absorbance was measured at 450 nm.

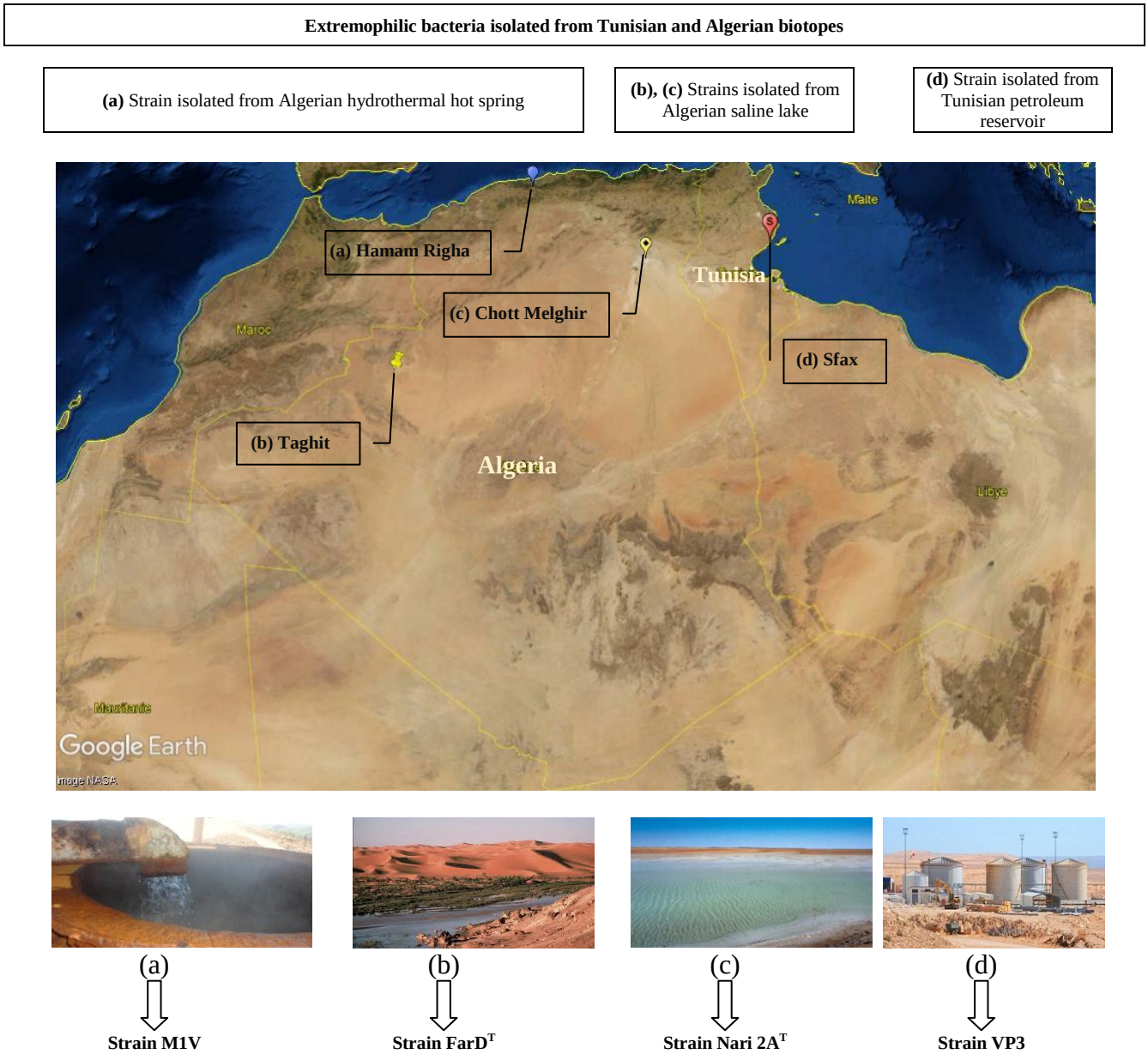

Figure 1: Geographical localization of the sites used for screening extremophilic bacteria in Algeria and Tunisia

2.5. Performance evaluation of the different proteases

2.5.1. Effects of organic-solvents on proteases activity and stability

Various organic solvents such as cyclohexane, chloroform, 1-buthanol, ethyl acetate, *iso*-propanol, ethanol, methanol, and dimethyl sulfoxide (DMSO), with different Log *P* values at 50% (v/v), were tested by shaking at 190 strokes per min and 37°C for 3 days to evaluate their effects on protease activity and stability, respectively. The relative and residual caseinolytic activities were assayed under the same conditions. The activity of each enzyme without any organic solvent was taken as 100%.

2.5.2. Stability and compatibility of proteases with laundry detergents

The stability and compatibility of a variety of proteases with a wide range of commercial solid and liquid commercialized detergents were investigated. In order to check their stability and compatibility with detergents, each commercial detergent was diluted in tap water to obtain a final concentration of 7 $mg.mL^{-1}$ (in order to simulate washing conditions). The endogenous proteolytic enzymes present in these laundry detergents were inactivated by heating the diluted detergents for 1 h at 65°C, prior to the addition of each enzyme. A 500 $U.mL^{-1}$ of each protease was shake-incubated with each laundry detergent for 1 h at 40°C, and residual protease activity was determined at pH 10 and 60°C using DMC as a substrate. The enzyme activity of a control (without any detergent), incubated under similar conditions, was taken as 100%.

To estimate the stain removal capabilities of $FarD^T$ enzyme, Alcalase 2.4L FG, and Bioprotease N100L proteases, clean white cotton cloth pieces (5 cm × 5 cm) were soaked and dried with chocolate, egg, and blood. The stained cloth pieces were shake-incubated separately for 1 h with 1 L beakers containing a total volume of 100 mL of tap water, Class detergent (7 $g.L^{-1}$, in tap water), and detergent added with 500 $U.mL^{-1}$ of $FarD^T$ enzyme or with 500 $U.mL^{-1}$ of commercial proteases (Bioprotease N100L or Alcalase 2.4 L FG), followed by rinsing with water. Then, the washed cloth pieces were dried. Visual examination of various pieces was also carried out to show the effect of each used enzyme in the removal of proteinaceous stains. Then, untreated stained piece of cloth was taken as a control.

2.5.3. Deproteinization of crustacean's bio‑wastes

For the production of protease, the M1V strain was cultured in a 1 L Erlenmeyer flask with a working volume of 50 mL, containing 25 $g.L^{-1}$ shrimp waste at pH 7.4 for 48 h at 45°C in rotary shaker (200 rpm). Before each activity assay, the biomass was taken away by centrifugation at 10,000×g for 30 min, and the supernatant was used for the evaluation of protease activity and chitin extraction. Furthermore, the cleared supernatant was filtered through a coarse cloth followed by the use of a sieve (22 mesh size). Subsequently, the resulting filtrate (hydrolysate) was concentrated in a rotary evaporator at 40°C, freeze dried, and stored at -20°C. Next, the composition of the lyophilized hydrolysate and its biological activities were evaluated. The proteases efficiency in the deproteinization of shrimp, *Metapenaeus monoceros* shells by-products was conducted using different enzyme/substrate [E/S] [unit of enzyme/milligram protein] ratios ranging from 0 to 30. A 2 g of the shell homogenate was mixed with 50 mL distilled water at 75°C. The pH was adjusted to 10 with 5 N NaOH. The reactions were stopped by heating the solutions at 90°C for 24 h to inactivate the proteases activity. The solid phase was washed and then pressed manually through four layers of gauze [26]. The degree of deproteinization (DDP %) was calculated as described elsewhere [27].

2.6. Bioinformatics tools, DNA sequencing, and DNA sequence analyses

Phylogenetic and molecular evolutionary genetic analyses of ARNr 16S genes sequences of VP3, M1V, $FarD^T$, and $Nari2A^T$ strains were performed using the Molecular Evolutionary Genetics Analysis[2] (MEGA) software v. 4.1. Distances and clustering were calculated using the neighbor-joining method. The tree

[2] https://www.megasoftware.net

topology of the neighbor-joining data was evaluated by Bootstrap analysis with 100 re-samplings.

3. Results and discussion

3.1. Screening of protease-producing bacteria

In the current study, ten aerobic bacterial strains (*Aeribacillus pallidus* VP3, *Halomonas lutea* C2SS100Ciq, *Bacillus thermoamylovorans* ATAM, *Anoxybacillus kamchatkensis* M1V, *Lysinibacillus fusiformis* C250R, *Hydrogenophilus hirshii* B5GN, *Albidovulum inexpectatum* HB14, *Geobacillus thermodenitrificans* BA1, *Virgibacillus natechei* FarD^T, and *Melghiribacillus thermohalophilus* Nari2A^T strains) were studied. Among the ten strains, the VP3, M1V, FarD^T, and Nari2A^T strains were identified as protease hyper-producers based on their patterns of clear zone formation on casein-containing media at pH 7.4. On a basal media, the strains VP3, M1V, FarD^T, and Nari2A^T displayed 1,400; 3,000; 7,500, and 4,300 U.mL^{-1}, respectively (Table 1).

3.2. Taxonomy identification and molecular phylogeny of the microorganisms

The taxonomic position of the four selected strains was established by phenotypic characteristic and using 16S rRNA gene sequencing (Figure 2). VP3, M1V, FarD^T, and Nari2A^T were identified as related genera of *Bacillus*.

The VP3 cells were motile rods, Gram-positive and spore forming. Catalase and oxidase reactions were positive. The temperature range for growth was 37°C to 65°C, with an optimum at 55°C. The salt concentration range for growth was between 0 g.L^{-1} and 80 g.L^{-1} NaCl, with an optimum at 10 g.L^{-1} NaCl [18]. Phylogenetic analysis revealed that strain VP3 was a member of *Firmicutes* phylum, family of *Bacillaceae* and was most closely associated to members of the genus *Aeribacillus* and in particular to the species *Aeribacillus pallidus*, with an average similarity of 99% (GenBank accession no.: KC581418).

For the M1V strain, cells were arranged singly or in pair, sporulated, motile, and rod-shaped bacterium. The isolate M1V was identified as a Gram-positive, aero-anaerobic facultative, catalase-positive, and oxidase-positive. The strain M1V was nitrate reduction positive and show negative results regarding tryptophane desaminase, lysine decarboxylase, H_2S, citrate, β-galactosidase, urease, indol, ornithine decarboxylases, and arginine dihydrolase production. The API 20E profile revealed that the M1V isolate could utilize gelatine. The API 50 CH tests revealed that it could utilize arabinose, inositol, and saccharose. Moreover, the strain M1V grew up to 80 °C with a pH range for growth between 7 and 9 and was able to grow in a range of 1% to 4% NaCl, suggesting its alkali-tolerance.

The 16S rRNA gene sequence from M1V was determined (GenBank accession no.: MH973636) and was 98% similar to that of *Anoxybacillus kamchatkensis* JW/VK-KG4T (GenBank accession no.: AF510985)

According to the phenotypic, morphologic, and molecular analysis, strain FarD^T is considered to represent a novel species of the genus *Virgibacillus* in the family *Bacillaceae* and order *Bacillales*, for which the name *Virgibacillus natechei* sp. nov., is proposed. The type strain of *Virgibacillus natechei* is FarD^T (DSM 25609^T or CCUG 62224^T) [20]. The FarD^T cells were rod-shaped, endospore forming, and motile. Growth occurred at 15°C to 40°C (optimum, 35°C), pH 6 to 12 (optimum, 7) and in the presence of 1% to 20% NaCl (optimum, 10 %).

While, the *Melghiribacillus thermohalophilus* strain Nari2A^T gen. nov., sp. nov., is a novel filamentous, endospore-forming, thermophilic and moderately halophilic bacterium [21]. The type strain Nari2A^T was Gram-positive, aerobic, catalase-negative and oxidase positive. Optimum growth occurred at 50 to 55°C, 7 to 10 % (w/v) NaCl, and pH 7 to 8. The strain exhibited 95.4%, 95.4%, and 95.2% 16S rRNA gene sequence (GenBank accession no.: KC845574) similarity to *Thalassobacillus devorans* strain G19.1^T, *Sediminibacillus halophilus* strain EN8d^T, and *Virgibacillus kekensis* strain YIM-kkny16^T, respectively.

Table 1
Retained proteolytic bacteria, their origins and initial protease activity.

Microorganism	Origin	Initial protease activity ($U.mL^{-1}$)	Optimized protease activity ($U.mL^{-1}$)
Anoxybacillus kamchatkensis strain M1V	Hammam Righa hot spring Ain Defla, Algeria	3,000	4,600
Aeribacillus pallidus strain VP3	Thyna Petroleum Services, Sfax, Tunisia	1,400	3,000
Melghiribacillus thermohalophilus strain sp. nov., gen. nov., Nari2A^T	Sahara salt lake, Chott Melghir, Algeria	4,300	15,800
Virgibacillus natechei strain sp. nov., FarD^T	Sediments of a saline lake, Taghit, Bechar, Algeria	7,500	16,000

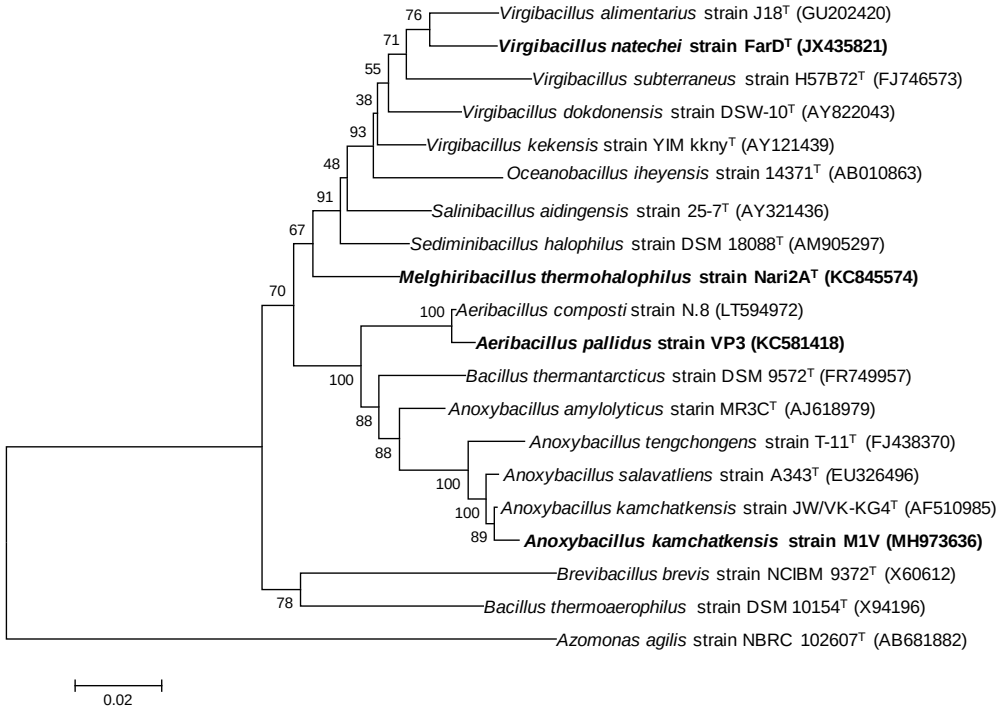

Figure 2: Phylogenetic tree based on 16S rRNA gene sequences showing the position of VP3, M1V, FarD^T, and Nari2A^T strains within the radiation of the other bacterial genus. The sequence of *Azomonas agilis* strain NBRC 102607^T (accession no: AB61882) was chosen arbitrarily as an outgroup. Bar, 0.02 nt substitutions per base. Numbers at nodes (>50%) indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 100 bootstraps). NCBI accession numbers are presented in parentheses.

3.3. Optimization via OFAT methodology

A classical method OFAT, which involves changing one independent variable, while fixing others at certain levels, was adopted to optimize the proteases production by VP3, M1V, FarD^T, and Nari2A^T strains.

OFAT optimization strategy of the medium involves changing one independent variable, such as carbon and azote sources, temperature, and pH, while fixing others at certain levels. Some researchers have shown that OFAT method can be more effective under certain conditions since, the number of runs is limited, and experimental error is not large compared to factor effects, which must be additive and independent of each other. Currently, the optimization of protease production from VP3, M1V, FarD^T, and Nari2A^T strains is currently underway through design of experiments approaches (DOE, DOX, or experimental design) by the means of Taguchi, Box-Behnken, and Plackett-Burman designs.

For example, for the *Aeribacillus pallidus* strain VP3, the best carbon source and nitrogen sources were chosen. Various carbon sources at a concentration of 5 $g.L^{-1}$ (casein, gelatin, glucose, sucrose, xylose, and

maltose) were tested. A best activity was achieved of 1,400 $U.mL^{-1}$ within gelatin. Then, the gelatin concentration was optimized by varying the concentration from 0 $g.L^{-1}$ to 25 $g.L^{-1}$. So, an activity of 2,795 $U.mL^{-1}$ has been achieved at 15 $g.L^{-1}$ gelatin. Different organic and inorganic nitrogen sources at a concentration of 2 $g.L^{-1}$ (yeast extract, soya flour, meat extract, beef extract, $(NH_4)_2SO_4$, and $(NH_4)_3FeSO_3$) were tested.

A best activity of 2,920 $U.mL^{-1}$ was recorded with soya flour. After that, the soya flour concentration was optimized by varying the concentration from 0 $g.L^{-1}$ to 6 $g.L^{-1}$. Hence, an activity of 3,000 $U.mL^{-1}$ has been achieved at 2 $g.L^{-1}$ soya flour. So, the strain VP3 displayed the highest extracellular protease activity (~ 3,000 $U.mL^{-1}$) after 22 h incubation in an optimized medium composed of $g.L^{-1}$ by gelatin, 15; soya flour, 2; NaCl, 10; K_2HPO_4, 1; KH_2PO_4, 1; $CaCl_2$, 2; $MgSO_4 \cdot 7H_2O$, 1; and 1% (*v*/v) trace elements.

For the M1V strain a maximum protease activity of 4,600 $U.mL^{-1}$ was achieved at pH 7.4 using the medium composed of ($g.L^{-1}$): gruel, 8; soy peptone, 4; KH_2PO_4, 1; K_2HPO_4, 1, $CaCl_2$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1 and 1% trace elements. The cultivation was performed for 48 h at 45°C. While, the strain Nari2A^T was cultured in Erlenmeyer in an initial medium, containing 20 $g.L^{-1}$ of powder bio-waste from shrimp or crab f or 52 h at 55°C. Powder from shrimp by-product as the sole energy, carbon, and nitrogen sources gave an acceptable level of proteases production, with (7,500 $U.mL^{-1}$) followed by the powder from crab by-product with (3,200 $U.mL^{-1}$).

Therefore, the effect of various concentrations of powder from white shrimp bio-waste on protease production by strain Nari2A^T was studied. The optimum for the production of protease activity (15,800 $U.mL^{-1}$) was obtained with 40 $g.L^{-1}$ of shrimp by-product. The strain FarD^T was cultured for 36 h at 35°C in Erlenmeyer flasks in optimized media. A high level of peptidase production, (16,000 $U.mL^{-1}$) was obtained with this bio waste as the sole carbon, and nitrogen sources.

3.4. Performance evaluation of the different proteases

3.4.1. Effects of organic-solvents on protease activity and stability

The effects of organic solvents on the activity and stability of the protease's activities from VP3 and FarD^T strains are shown in table 2. These enzymes exhibit high proteases activities in the presence of organic solvents. So, they would be very useful for organic synthetic reactions [9].

In upgrading to environmentally mild chemical synthesis, proteases are foreseen as superior enzymes as proteases in non-aqueous media offers various novel characteristics compare to traditional aqueous enzymatic transformation [28, 29]. The proteolytic enzymes have attracted a high deal of attention in organic synthesis because they have many advantages associated with the application of enzymes for the synthesis of peptides and esters [30-32]. Very few research works are published in literature concerning the screening of microorganisms which produce organic solvent-stable proteases [33]. Poor stability and low catalytic activity of enzymes are the major limitations of their use in organic solvents, which tend to strip water from protein and thereby disrupt non-covalent forces and decrease enzyme activity and stability.

The ability to use enzymes in non-aqueous solvents expands the potential applications of biocatalysts in chemical transformations which is useful for many industries. Enzymatic reactions in organic solvents provide numerous industrially attractive advantages, such as increased solubility of non-polar substrates, reversal of the thermodynamic equilibrium of hydrolysis reactions, suppression of water-dependent side reactions, alternation of substrate specificity and enantio-selectivity, and elimination of microbial contamination.

Table 2
Effects of organic-solvents on protease activity and stability.

Organic solvents (50% v/v)	**Relative activity (%)**			**Residual activity (%)**		
	Crude extract from strain VP3	Crude extract from strain FarD^T	Thermolysin	Crude extract from strain VP3	Crude extract from strain FarD^T	Thermolysin
Control	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 25	100 ± 2.5
Cyclohexan	304 ±7.6	320 ± 8.02	138 ± 3.4	421 ±10.5	280 ± 1.6	125± 3.5
Chloroform	196 ± 4.9	174 ± 6.4	93 ± 2.3	141 ± 3.5	126 ± 1.4	66 ± 1.9
1-buthanol	162 ± 4.0	120 ± 2	43 ± 1.2	135 ± 3.6	81 ± 1.25	15 ± 1.0
Ethyl acetate	180 ± 4.5	74 ± 3	0 ± 0.0	164 ± 3.6	65 ± 0.3	0 ± 0.0
Iso-propanol	86 ± 2.1	90 ± 1.2	101 ±2.5	67 ± 1.6	52 ± 0.2	91 ±2.3
Ethanol	0 ± 0,0	40 ± 1,5	71 ± 2.0	0 ± 0,0	20 ± 0.2	35 ± 1.2
Methanol	163 ± 4,0	120 ± 2.7	92 ± 2.4	126 ± 3.1	84 ± 1.9	75 ± 2.0
DMSO	481± 12	180 ± 3.2	155 ± 4.1	370 ± 1.8	121 ± 3.2	27± 3.5

3.4.2. Stability and compatibility of proteases with laundry detergents

The data presented in table 3 show that different proteases are stable and compatible with different detergents even after 1 h incubation at 40°C. It exhibited better stability with Class. Again, the addition of FarD^T enzyme or commercial proteases (Alcalase™ Ultra 2.5 or savinase™ 16L, type EX L) in Class seems to improve the cleaning process as evidenced by rapid stain removal. In fact, protein stains like chocolate, egg, and blood have been hard to eradicate with commercial detergent. Some stains could only be dealt with at high temperatures and even then, the stain was only partly removed. Detergent enzymes particularly with proteases offered a key and are being persistently improved to digest proteinaceous stains.

The ability of proteases to hydrolyze the various proteinaceous stains has attracted the interest of industrialists in detergent market. The visual comparison of the washed cloth revealed that the combinations of every enzyme individually with commercial detergents (Class) yielded fairly good results of its ability to remove blood, egg, and chocolate stains. In fact, a limited washing performance was observed with tap water only or with detergent (Class) only.

The supplementation of FarD^T enzyme or commercial proteases (Bioprotease N100L or Alcalase 2.4 L FG) in Class detergent seems to enhance the cleaning process as evidenced by rapid stain removal. Thus, FarD^T enzyme was characterized by its strong hydrolytic effect against blood and chocolate which are recalcitrant stains. Similarly, protease from strain VITP14 demonstrated its potential use as a bio-additive detergent in the removing of the blood stains from cotton cloth [34].

Although reported the usefulness of thermostable alkaline peptidase from *Bacillus* and related genus for the removal of protein stains from cotton cloth in the presence and absence of detergents [9, 35, 36], we believe that the FarD^T enzyme form is more effective.

Table 3
Stability and compatibility of proteases with laundry detergents

	Residual activity (%)				
Commercial Detergents	Bioprotease NL100	Alcalase 2.4 L FG	VP3 enzyme	M1V enzyme	FarD^T enzyme
EcoVax	60	75	100	81	65
Dipex	62	90	100	97	92
Det	70	88	65	92	83
Ariel	85	90	81	71	71
Nadhif	82	85	100	100	90
OMO	83	100	92	52	65
Dixan	60	90	100	71	81
Skip	71	75	100	83	80
Class	86	80	53	52	100
iSiS	60	74	27	80	74
Dipex	62	90	51	86	92
Alyss	74	64	100	52	60
Detech	67	81	100	73	40
Judy	51	73	60	45	27

3.4.3. Deproteinization of crustacean's bio-wastes

Powder from shrimp and crab bio-wastes at 20 g.L^{-1} gave an acceptable level of proteases production with 7,500 U.mL^{-1} and 3,200 U.mL^{-1}, respectively by the strain *Melghiribacillus thermohalophilus* Nari2A^T. Particularly, the *Anoxybacillus kamchatkensis* strain M1V was found to produce a high level of proteolytic activity when grown in a media containing shrimp bio-waste powder as a sole carbon and nitrogen source. In order to ameliorate proteases production by strain M1V, several concentrations of speckled shrimp by-product powder were applied. Since the protease activity was considerably detected and measured in the initial medium containing only 10 g.L^{-1} of shrimp by-product, with a significant yield (1,200 U.mL^{-1}), the investigation of medium by the classical method (OFAT) involves changing one independent variable (such as the agitation, temperature, and pH) while fixing others at certain levels. The protease production increased linearly up to 25 g.L^{-1} shrimp byproduct where it reaches its maximum (2,000 U.mL^{-1}) (Table 4).

Beyond this concentration, the proteolytic activity decreased sharply by losing more than 25% of its maximum between 30 and 40 g.L^{-1} shrimp substrate. Obviously, these results demonstrate that *Anoxybacillus kamchatkensis* strain M1V obtains its carbon, nitrogen, and salts supplies immediately from shrimp by-product. The protease production by strain M1V was undertaken in the optimal medium. The extent of protein hydrolysis during fermentation by *Anoxybacillus kamchatkensis* strain M1V was measured by assessing the proteolytic activity and hydrolysate composition. The hydrolysate has a maximum amount of protein (42 ± 1.02%), which coincides with the optimum of proteolytic production (2,000 U.mL^{-1}) after 48 h of culture. The sugar level in the hydrolysate was about 2.5 ± 0.12% of sugar and a very low amount of fats (2 ± 0.06%).

Table 4
Optimization of protease activity by M1V strain

Shrimp by-product concentration (g.L^{-1})	10	15	20	25	30	35	40
Protease activity (U.mL^{-1})	1,200	1,600	1,850	**2,000**	1,400	1,150	1,000
Biomass (g.mL^{-1})	1,8	3	6,07	**7**	5	5,04	3
Yield (protease/g bio-waste)	120	106,66	37	**80**	46,66	32,85	25

The obtained hydrolysate was noticeable due to its high protein content. Several studies have apprised that the controlled enzymatic digestion is the broadly applied method for the production of protein hydrolysates [37]. Within the time course of hydrolysis, a large variety of peptides and free amino-acids are generated depending on the protease specificity. Definitely, the level, the changes in size, and the composition of peptides and free amino-acids modulate the biological activities. Here, a few investigations have been conducted on the generation of biologically active proteins using microbial fermentation. In fact, the high protease activity (2,000 $U.mL^{-1}$) reflects the generation of small sized bioactive peptides in the hydrolysate mixture. Indeed, the hydrolysate was assessed for its antioxidant and enzyme inhibitory capacities using various tests. In the present investigation, a stronger angiotensin inhibitory activity (ACEI) was recorded for shrimp shell hydrolysate compared to captopril activity. The corresponding half maximal inhibitory concentration (IC50) was 71.52 ± 1.48 $\mu g.mL^{-1}$ and 85.33 ± 1.26 $\mu g.mL^{-1}$ for the hydrolysate and captopril, respectively. Again, the shrimp by-product hydrolysate showed an important tyrosinase inhibitory effect even though the IC50 was higher than the value determined for Kojic acid, 107.67 ± 33 $\mu g.mL^{-1}$ and 4.05 ± 0.25 $\mu g.mL^{-1}$, respectively. Finally, the shrimp hydrolysate also displayed a promising activity against amylase; it's IC50 (133.24 ± 0.67 $\mu g.mL^{-1}$) was remarkably lower than that obtained with acarbose (570.16 ± 1.82 $\mu g.mL^{-1}$) (Figure 3). For the deproteinization of shrimp bio-wastes, a percent of 48% and 82% has been recorded after by M1V and Nari2A^T proteases.

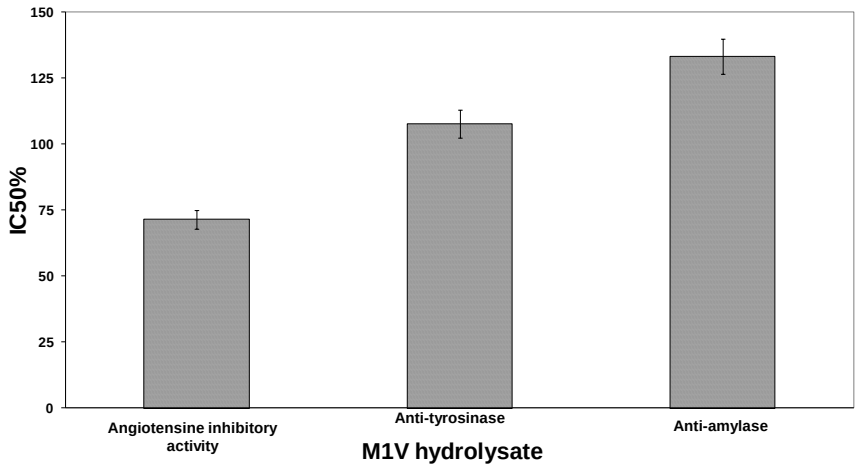

Figure 3: Assessment of enzyme inhibitory potential.

4. Conclusion

Four extremophilic strains, identified as *Aeribacillus pallidus* strain VP3, *Anoxybacillus kamchatkensis* M1V, *Virgibacillus natechei* strain FarD^T, and *Melghiribacillus thermohalophilus* Nari2A^T were selected as producers of extracellular proteases with high activity (3,000 $U.mL^{-1}$ to 16,000 $U.mL^{-1}$) and solvent-stability (residual activities varying attaining 481%) at alkaline pH ranges and high temperatures. The crude preparations showed a great for enhancing the washing performance various solid and liquid detergents with excellent washing performance when added to laundry detergents. In this study, we also demonstrated that the culture supernatant can deproteinize shrimp wastes, with a high deproteinization degree (82%). More interestingly, the recuperated protein hydrolysate showed an interest remarkable biological activity. Again, these crude preparations showed high stability in the presence of organic solvents Thus, the present study describes for the use of proteases from extremophilic bacteria as a new and promising opportunity in various bioprocesses and biotechnological researches.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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