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Characterization of a novel serine alkaline protease from *Bacillus atrophaeus* NIJ as a thermophilic hydrocarbonoclastic strain and its application in laundry detergent formulations

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ABSTRACT/RESUME

Abstract: *A thermophilic hydrocarbonoclastic bacterial strain Bacillus atrophaeus strain NIJ isolated from hydrocarbon-contaminated soil (Algerian desert, Hassi-Messaoud petroleum region) was screened from 100 isolates because of its efficiency in maximum protease production (31,000 U/mL). A novel extracellular serine alkaline protease named SAPNIJ was purified to homogeneity using ammonium sulphate fractionation (40-70%), heat-treatment (30 min at 90 °C), and gel filtration chromatography-HPLC (ZORBAX PSM 300 HPSEC) and biochemically characterized. Its molecular mass was ~28 kDa estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC The NH2-terminal 27 amino-acid sequence of SAPNIJ showed high homology with those of Bacillus-proteases. The pH and temperature optimal activities were 11 and 70 °C, respectively. The phenylmethanesulfonyl fluoride (PMSF) and diiodopropyl fluorophosphates (DFP) are the specific inhibitors of SAPNIJ which confirm that it belongs to the serine-proteases family. Non-ionic surfactants and oxidizing agents are the compatibles additives of SAPNIJ as catalyzer and stabilizer. This protease showed high stability and compatibility with some commercial laundry detergents than those of Savinase™ 16L, type EX (commercial enzyme), SAPHM from Bacillus licheniformis K7A, and SAPRH from Bacillus safensis RH12. Only 500 U/mL enzyme activity cans remove blood-stains at 40 °C for 30 min. This is the first report of protease from Bacillus atrophaeus, which can be a potential promising candidate for future applications in detergent formulations.*

I. Introduction

In various processes, such as digestion, cell growth and apoptosis, blood coagulation, as well as protein catabolism and so others, proteases are involved as catalyzers [1, 2]. More than 60% of commercially available enzymes worldwide are proteases [1]. At the beginning of 2017 the global enzymes market was evaluated at 7,082 US$. Hence, it recorded compound annual growth rate (CAGR) of 5.7% from 2018 to 2024 and it estimated to reach 10,519 US$ in 2024 [1]. Commercial enzymes have often been reported to be stable at elevated temperature and pH conditions. Most of them have, however, been criticized for their limited efficiency and stability in the presence of laundry detergents. Recent research has, therefore, become increasingly interested in the search for alternative sources particularly from bacterial origins. Among the so far reported bacterial organisms, *Bacillus* strains seem to offer a wide range of advantages, including (i) accelerated accumulation of biomass and enzymes and (ii) secrete extracellular proteases, which imply that (iii) the downstream purification and recovery of the enzyme may be simplified. The first commercial alkaline protease from *Bacillus licheniformis* was produced in the 60s of the last century, during that researches focused more and more of such *Bacillus* alkaline proteases whilst keeping low production cost and gifted with high catalytic activity and stability [3, 4]. As detergent additives [2], these latter are mostly used, [5-7] registering 89% of the overall sales [8].

Of particular interest, proteases from thermophilic and/or extremophilic strains gained more attention over their classical counterparts regarding their performances and broad applicability in extreme conditions (pH and/or temperature). In addition, these enzymes have high activity in non-physiological conditions, such as intensive calcium chelating agents, detergents, solvent-tolerance and substrate selectivity and stability. Due to these, harsh enzymes (e.g. 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 [9-12]. The increasing use of proteolytic enzymes in many industrial, especially detergent, tannery and wastewater treatment has prompted researchers to explore extreme biotopes such as the hydrocarbon-contaminated soil to obtained new proteases from hydrocarbonoclastic strains with potential biotechnological interest. Several reports have been conducted on the identification of extracellular thermostable serine alkaline proteases from hydrocarbonoclastic species such as *Aeribacillus pallidus* VP3 [10] and *Lysinibacillus fusiformis* C250R [9]. In the last 50 years, proteases and other enzymes in laundry detergents switched from being minor additives to key ingredients [13-15]. *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 [16]. *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus pumilus* are the most frequently used species in the industrial production of alkaline protease [17, 18]. As far as we know, the protease ability of *Bacillus atrophaeus* recorded as detergent biodditive hadn't been previously reported. Highlighting the extremophilic origin of *Bacillus atrophaeus* will increase the value of this protease in order to enhance its detergent exploitation in commercial purposes.

In this context, the present study reports, for the first time, on the purification and biochemical characterization of a detergent-stable protease (SAPNIJ) from *Bacillus atrophaeus* strain NIJ isolated from hydrocarbon contaminated soil in the south region of Algeria (Hassi Messaoud). It also provides basic information on the potential use of the SAPNIJ as a prospective candidate for future applications in detergent formulations.

II. Materials and Methods

II.1. Materials

Substrates, chemicals, and reagents, of the analytical grade or highest available purity, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The comparative detergent-stable alkaline serine proteases were: SAPHM from *Bacillus licheniformis* K7A [19]; SAPRH from *Bacillus safensis* RH12 [20]; and Savinase™ 16L, type EX (subtilisin 309) from *Bacillus clausii*, kindly supplied by Novozymes Biopharma DK A/S (Bagsvaerd, Denmark).

II.2. Strain Isolation and Culture Conditions

Soil samples were collected from the crude-oil-contaminated soil in desert of Hassi Messaoud petroleum region, Southeast of Ouargla in Algeria (GPS coordinates: 6° 04' 21" East, 31° 40' 57" North). Sampling steps and isolation of hydrocarbonoclastic strains were followed as describe by Eddouaouda et al. [21]. The protease enzymatic potential for strain isolates was qualitatively and quantitatively estimated using casein as protein hydrolysis substrate. Preliminary screening of protease activity was carried out using solid medium contains in g/L: Peptone 5, yeast extract 3, skimmed milk 250 mL, and bacteriological agar, 15 at pH 7.4. The culture plates were incubated at 45 °C for 12 h and upon

colonies surrounded by clear halos were selected as putative protease producers. Among these isolates, a strain coded NIJ, resulting in a large hydrolysis clear zone and a high proteolytic potency (5,000 U/mL) in a casein-based initial liquid medium (BILM) was selected for further experimental study. BILM (g/L): Casein, 15; yeast extract, 2; $CaCl_2$, 0.5; K_2HPO_4, 0.5; KH_2PO_4, 0.5, and 1% (v/v) petroleum. All media were autoclaved at 120 °C for 20 min. Cultivations were performed on a rotary shaker (180 rpm) for 46 h at 45 °C and in 1000 mL Erlenmeyer flasks with a working volume of 100 mL. Various carbon sources (CS) and nitrogen sources (NS) have been used (1) CS 5 g/L: Casein, gelatin, glucose, fructose, galactose, sucrose, and maltose with 5 g/L as CS and (2) NS 2 g/L: Beef extract, yeast extract, yeast peptone, soya peptone, $NH_4)_2SO_4$; ammonium chloride, NH_4Cl; sodium nitrate, and $NaNO_3$ with 10 g/L casein as NS. As best CS, casein was added at 5, 10, 15, 20, and 25 g/L in production broth under above conditions, to determine maximum production. Same, yeast extract at 2, 4, 6, 8, and 10 g/L. Finally, the optimized growth medium for protease production at pH 7.4 was composed of (in g/L): casein, 10 g; yeast extract, 4 g; K_2HPO_4, 1.5 g; KH_2PO_4, 1.5 g; $CaCl_2$, 1 g; and trace elements at 1% (v/v) [22].

II.3. Strain Identification

The strain NIJ was phenotypically characterized *via* key criteria of *Bacillus* genus. Then species identification has been assured by the *16S* rDNA gene sequencing through PCR. The two universal primers, one forward and the other reverse, designed from the conserved zones within the rRNA operon of *E. coli*. 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 1,541 to 1,525. NIJ-strain genomic DNA was purified employing the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The PCR amplification was conducted as follows: 35 cycles, 94 °C for 35 s denaturation, 65 °C for 40 s primer annealing, and 72 °C for 60 s extension. In this study, the amplified ~1.5 kb PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), leading to pFZR-16S plasmid, of the host strain *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA). Luria-Bertani (LB) medium added with Ampicillin (100 µg/mL), isopropyl-thio-β-D-galactopyranoside (IPTG) (0.4 mM), and X-gal (360 µg/mL) served to screen *E. coli* recombinant clones. DNA electrophoresis and purification, restriction, ligation, and transformation have been completed according to the method previously described by Sambrook [23].

II.4. DNA Sequencing and Bioinformatics Analysis

BigDye Terminator Cycle Sequencing Ready Reaction kits and the automated DNA sequencer ABI PRISM® 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA) were used to sequencing the cloned *16S* rDNA gene on both strands compared with sequences available in the public sequence databases and with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/), a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains. MEGA software v. 4.1. and the BioEdit version 7.0.2 software program and the ClustalW2 program available at the European Bioinformatics Institute server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were respectively used to perform phylogenetic and molecular evolutionary genetic analyses and multiple nucleotide sequence alignment.

II.5. Protease Activity Assays

The protease activity was assayed by a modified caseinolytic method [24] using Hammerstein casein (Merck, Darmstadt, Germany) as a specific substrate. Are added to 10 g/L casein, 0.5 mL diluted enzyme solution, 2.5 mL bicarbonate-NaOH 100 mM supplemented with $CaCl_2$ 2 mM (buffer, pH 11) (buffer A) and the reaction mixture was incubated for 15 min at 70 °C, after that, the reaction was stopped by adding 2.5 mL 20% TCA. Afterward, 30 min at room temperature the non-digested proteins were removed by centrifugation at 15,000 rpm for 20 min. Here after, were mixed 0.5 mL clear supernatant, 2.5 mL Na_2CO_3 500 mM and 0.5 mL Folin-Ciocalteu reagent, incubated 30 min at room temperature. The absorbance was measured at 660 nm against a blank control and the protease activity was measured by detecting the tyrosine released upon protease hydrolysis compared with a standard curve.

The protease activity present in the laundry detergent solution was evaluated, at 450 nm, by the method detailed by Boulkour Touioui et al. [22] using *N*,*N*-dimethylated casein (DMC) as a substrate and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) as a colour indicator.

II.6. Enzyme Purification

Five hundred milliliters of NIJ strain culture 46 h-under optimal conditions of protease production were centrifuged (30 min at 9,000*g*) to remove microbial cells. The supernatant was submitted to the following enzyme purification steps. (1) In two successive times, the addition of $(NH_4)_2SO_4$ (242 g/L and 202 g/L) until saturation (up to 40 and 70%, respectively) and the precipitate has been twice eliminated (9,000*g* for 30 min). (2) The obtained supernatant was re-suspended in a minimal volume of 50 mM HEPES buffer at pH 7.1 containing 2 mM $CaCl_2$ (buffer B), and dialyzed overnight against the repeated changes of the same buffer, and then, insoluble material was removed by centrifugation at 9,000*g* for 30 min. (3) The obtained sample was heat-treated for 30 min at 90 °C and insoluble material was removed by centrifugation for 30 min at 9,000*g*. The final supernatant was injected a high performance liquid chromatography (HPLC) gel filtration system using a ZORBAX PSM 300 HPSEC (26.2 mm × 250 mm), Agilent Laboratories, pre-equilibrated with 25 mM Tricine at pH 7.8 supplemented with 2 mM $CaCl_2$ (buffer C). Proteins were separated by isocratic elution at 280 nm and a flow rate of 45 mL/h with buffer C. The pooled fractions containing protease activity were concentrated in centrifugal micro-concentrators with 10-kDa cut-off membranes and stored at -20 °C in a 20% glycerol (v/v) solution for further analysis.

II.7. Analytic Methods

Based on the Bradford colorimetric [25] and Laemmeli [26] methods description, the protein concentration and molecular weight were respectively quantified for purified SAPNIJ. In our case a Dc protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA, USA) and SDS-PAGE under reducing conditions were employed as specific reagents. The native-PAGE (non-reducing conditions) of the purified SAPNIJ was performed using 10% resolving gel (stacking gel was omitted) in Tris-glycine buffer (pH 8.5) at 4 °C. The approximate molecular weight was determined by comparing with standard protein markers and also confirmed by size exclusion HPLC using Shodex Protein WK 802-5 column (8 mm × 300 mm), pre-equilibrated with buffer A and native protein markers of 669 to 1.35 kDa. Casein zymography staining was performed as described elsewhere [27] to visualized protein bands. Bands of purified SAPNIJ enzyme were separated on SDS gels and transferred to a ProBlott membrane, and the NH_2-terminal sequence analysis was determined by automated Edman's degradation using Applied Biosystems Protein sequencer ABI Procise 492/610A.

II.8. Biochemical Characterization of the Purified SAPNIJ

II.8.1. Effects of Inhibitors, Reducing Agents, and Metal Ions on Enzyme Stability

For studying SAPNIJ inhibitors, reducing agents, and metal ions requirement, different inhibitors and divalent metal ions have been tried under above conditions during 1 h at 40 °C.

II.8.2. Effects of pH on Protease Activity and Stability

The effect of pH on SAPNIJ activity was determined continuously at 70 °C over the pH range of 2-13 using casein as substrate and the following buffers at 100 mM, supplemented with 2 mM $CaCl_2$: 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), Na_2HPO_4-NaOH (pH 11.5-12), and KCl-NaOH (pH 12-13). Its pH stability was determined by pre-incubation in different buffer solutions with different pH values for 6 h at 40 °C. Aliquots were withdrawn, and residual enzymatic activity was determined as previously described.

II.8.3. Effects of Temperature on Protease Activity and Stability

In order to determine the SAPNIJ temperature effect, the enzyme activity was measured after incubation for 6 h at a wide range of temperatures (40-100 °C) and pH 11. The SAPNIJ thermal stability was determined after enzyme incubation at different high temperatures (70, 80, and 90 °C) and pH 11 for 6 h with and without 2 mM $CaCl_2$ and measuring the residual enzyme activity at a regular time intervals. The non-heated SAPNIJ, was considered as control (100%).

II.8.4. Effect(s) of Polyols and/or Calcium on Protease Thermostability

Proteases thermostability is often influenced by adding polyols and calcium, to do this, SAPNIJ was pre-incubation separately at 90 °C for 6 h (1) in the presence of PEG 1000, PEG 1500, PEG 6000, glycerol, sorbitol, mannitol, and xylitol at a final concentration of 100 g/L and (2) with 2 mM calcium alone (or plus 100 g/L PEG 1500). The residual activities were compared in the absence of polyols (I) and not incubated (NI) and without calcium addition.

II.8.5. Substrate Specificity and Kinetic Studies

Three types of protein substrates were used to determine substrate specificity profile of SAPNIJ: Natural (casein, albumin, gelatin, ovalbumin, and keratin) and modified (azo-casein, albumin azure, keratin azure, and collagen types I- and II-FITC conjugates from bovine articular cartilage) as well as synthetic peptides substrates , and esters. Enzymatic activities were determined on each substrate according to standard conditions previously described by Zaraî Jaouadi et al. [28]. Kinetic parameters were calculated from the initial rate activities of the purified and commercial enzymes at the optimum pH and temperatures (pH 11 and 70 °C for SAPNIJ; pH 10 and 70 °C for SAPHM; pH 9 and 60 °C for SAPRH; and pH 8 and 60 °C for Savinase™ 16L, type EX) using casein and synthetic peptide [*N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide]. The reaction was carried out in triplicate at different substrate concentrations ranging from 0 to 50 mM in assay buffer supplemented with 10% (v/v) dimethyl sulfoxide (DMSO) and 1% (v/v) Triton X-100. Kinetic parameters were estimated by Lineweaver–Burk plots. Kinetic constants, Michaelis–Menten constant (K_m) and maximal reaction velocity (V_{max}) values were calculated using the Hyper32 software package.

II.9. Performance Evaluation of the Purified Used Enzymes

II.9.1. Effect of Detergent Additives and Compatibility of Enzymes with Laundry Detergents

The purified target enzymes (SAPNIJ; SAPHM; SAPRH; and Savinase™ 16L, type EX) were pre-incubated for 6 h at 40 °C in the presence of bleaches (H_2O_2, sodium perborate), surfactants (SDS, LAS, and sulfobetaine), non-ionic surfactants (Tween 20, 40, 80, and Triton X-100), anti-redeposition agents ($Na_2{\cdot}CO_3$, STPP, TAED, and Na_2·CMC) and other detergent additives. The residual activity was carried out in the optimum pH and temperature of each enzyme compared to 100% without any additive. H_2O_2 in 100 mM Borate-NaOH buffer was also assayed.

II.9.2. Stability and Compatibility of Enzymes with Laundry Detergents

A 500 U/mL of each purified protease previously tested with laundry detergents were shake-incubated (250 rpm) for 1 h at 40 °C in the presence of liquid and solid detergents (diluted in tap water at final concentration of 7 mg/mL [15] and beforehand heated for 1 h at 65 °C in order to inactivate endogenous proteases [29]), whose list is as follows: liquid [Dipex and Ecovax (Klin Productions, Sfax, Tunisia), Class (EJM, Sfax, Tunisia), Omino Bianco, Fairy, and iSiS Pril (Henkel, Algeria), and Skip (Unilever, France)], and solid [Ariel, Persil, and Tide (Procter & Gamble, Switzerland), OMO (Unilever, France), Dixan, Axion, and Nadhif (Henkel, Tunisia), and Det (Sodet, Sfax, Tunisia)]. Employing DMC as a substrate, residual activity was determined as mentioned before.

II.9.3. *Removal of Protein Stains from Cotton Fabrics*

The six enzymes, include the target, have been tested to remove blood stain contained on new cotton cloth pieces (10 cm × 15 cm) emerged in 1-L beakers containing a total volume of 100 mL of: tap water, iSiS Pril and commercial laundry detergent (7 mg/mL, in tap water) and detergent added with used enzyme (500 U/mL). After that, the washes were shake-incubated (250 rpm) at 40 °C for 30 min comparatively with untreated blood-stained piece of cloth. After treatment, the cloth pieces were taken out, rinsed with water, dried and submitted to visual observation to examine the stain removal effects of the enzymes.

II.10. Statistical Analysis

All experimentations were performed in three independent replicates by referring at the control experiment. The results were expressed with standard deviation (mean ± SD).

II.11. Nucleotide Sequences Accession Number

The nucleotide sequence data of *16S* rDNA (1510 bp) gene reported in this paper has been submitted to the GenBank/ENA/EMBL databases under accession number: **MG890424**.

III. Results and Discussion

III.1. Isolation and Screening of Protease-Producing Strains

In total, one hundred isolates were obtained from crude-oil-contaminated soil in Hassi Messaoud desert (Ouargla, Algeria) after successive subcultures on petroleum based medium. Six thermotolerant hydrocarbonoclastic bacterial isolates (2C, K2C, 7A, 7H, K2N, and NIJ) have

been screened as protease producers based on the ratio of the clear zone diameter (onto skimmed milk agar plates) and that of the colony (> 4 mm) which served as an indicator for the selection of strains with high extracellular protease production ability. Among the six strains, NIJ exhibited the highest ratio of 5.5 mm and the highest extracellular protease activity (about 5,000 U/mL) after 46 h incubation in a non-optimized medium and was, therefore, retained for all subsequent studies.

III.2. Strain Identification

The use of phenotypic and genotypic approaches allows an accurate bacterial identification. Isolate coded NIJ was initially attached to the genus *Bacillus* by referring to the methods described in the Bergey's Manual of Systematic Bacteriology [30] and taking into account the following results: Bacilli form, aerobic, spore-forming rods, Gram-positive, catalase-positive, oxidase-positive, motile, and colonies are round, undulate, dull white and non-luminescent. The strain metabolized citrate, malate, glycerol, L-arabinose, ribose, galactose, fructose, mannose, inositol, mannitol, maltose, sucrose, D-turanose, D-tagatose, gluconate, lactate, L-aspartate, and L-glutamate are readily utilized as energy sources in addition to other simple sugars. Lactose, sorbitol, glycogen, L-xylose, D-lyxose, starch, sorbose, erythritol, inulin, L-arabitol, capric acid, adipic acid, phenyl acetic acid, propionate, and glycine are not utilized as energy sources. A phylogenetic tree based on the *16S* rDNA gene (**Fig. 1A**) showed that the novel isolates clustered with members of the genus *Bacillus*, the nearest neighbour being *Bacillus atrophaeus* JCM 13347^T with an average similarity of 99% (accession no.: AB021181). Based on the results obtained in the course of the present study, we suggest the assignment of this isolate (accession no. **MG890424**) as *Bacillus atrophaeus* strain NIJ.

III.3. Protease Production

As extracellular enzymes proteases are induced and repressed in the absence or the presence of fermentable sugars, respectively. The ability to produce proteases is not necessarily related to a well-defined carbone or nitrogen source. For that, no defined medium has been established for the best production of proteases from different microbial sources [31]. This was confirmed in our case with the NIJ strain. In initial medium, the best carbon source for protease production was casein (5,000 U/mL) followed by gelatin (3,000 U/mL). However, enzyme production was significantly low (590 and 510 U/mL) when *Bacillus atrophaeus* strain NIJ was grown on glucose and sucrose, respectively. Otherwise, the optimum casein concentration as best carbon source was 10 g/L (12,500 U/mL). In parallel, yeast extract was the best organic nitrogen source (15,500 U/mL) followed by beef extract (13,225 U/mL). As inorganic nitrogen source $(NH_4)_2SO_4$ (10,528 U/mL) was the best in contrary with NH_4Cl (895 U/mL) and $NaNO_3$ (1,257 U/mL). 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 (18,752 U/mL), giving about 24.5-fold enzyme activity, compared to the medium without nitrogen sources (765 U/mL). In the medium containing (in g/L): Casein, 10; meat extract, 4; $CaCl_2$, 1; K_2HPO_4, 1.5; and KH_2PO_4, 1.5; the addition of trace elements at 1% (v/v) and 2% (v/v) petroleum significantly improved protease production by 1.65 folds, reaching 31,000 U/mL. Under this particular condition, the protease 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 46 h of cultivation (**Fig. 1B**).

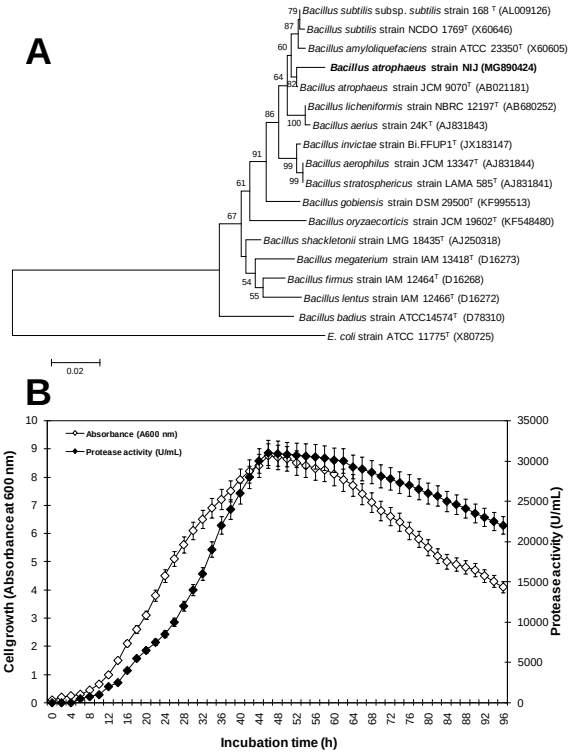


Figure 1. (A) *Phylogenetic tree based on 16S rDNA gene sequences showing the position of strain NIJ (accession n°. **MG890424**) within the radiation of the genus Bacillus. The sequence of E. coli ATCC 11775^T (accession n°. X80725) was chosen as an outgroup. Bar, 0.02 nt substitutions per base. Numbers at nodes (> 50%) indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps). NCBI accession numbers are*

presented in parentheses. ***(B)*** *Kinetic production of protease from strain NIJ. Time course of strain NIJ cell growth (◇) and SAPNIJ production (♦). Cell growth was monitored by measuring the absorbance at 600 nm and was converted to cell dry weight (g/L). Each point represents the mean (n = 3) ± standard deviation.*

III.4. Purification and molecular weight determination of SAPNIJ

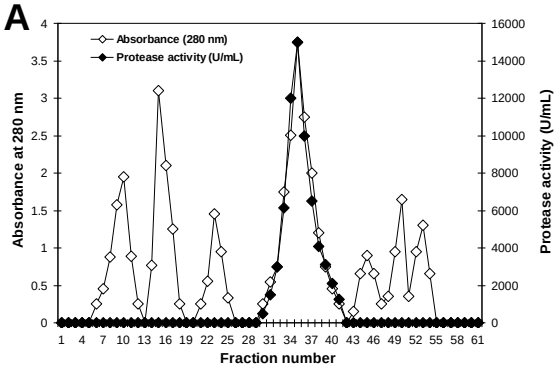
The results of purification are summarized in **Table 1** and **Fig. 2A**. A 25% yield was obtained after purification steps of SAPNIJ with 86,111 U/mg proteins as a specific activity and 34.7-fold enrichment.

Table 1. *Flow sheet for SAPNIJ purification from Bacillus atrophaeus strain NIJ.*

Purification step	Total activity (units) [a,b] × 10^3	Total protein (mg) [a,c]	Specific activity (U/mg of protein)	Activity recovery rate (%)	Purification yields (fold)
Crude extract	15,500 ± 95	6,250 ± 55	2,480	100	1
$(NH_4)_2SO_4$ Fractionation (40-70%)	12,865 ± 71	783 ± 37	16,430	83	6.6
Heat treatment (30 min at 90 °C)	7,905 ± 64	125 ± 26	63,240	51	25.5
HPLC (ZORBAX PSM 300 HPSEC)	3,875 ± 25	45 ± 2	86,111	25	34.7

[a] The experiments were conducted three times and ± standard errors are reported.
[b] One casein unit is defined as the amount of enzyme that hydrolyzed the substrate and produced 1 µg of amino-acid equivalent to tyrosine per minute under the experimental conditions used.
[c] Amounts of protein were estimated by the method of Bradford [25].

To finally get a pure product, the crude precipitate contaminated with proteins will require heating followed by HPLC separations [32]. The purification techniques used in our case were satisfactory as already reported in similar works [9, 27]. Compared to those previously reported for other microbial proteases, SAPNIJ has a significant specific activity which confirms its potential prospects in biotechnological and industrial bioprocesses. Approximately, SAPNIJ molecular mass was estimated at 28 kDa (**Figs 2B, 2C,** and **2D).** These **data** strongly suggested that SAPNIJ was a monomeric protein comparable to those previously reported for other proteases from *Bacillus* strains [28, 33].



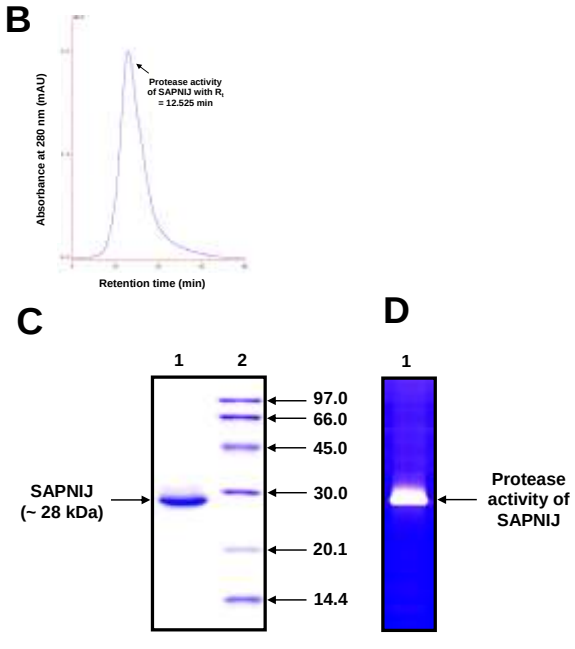


Figure 2. (A) *Gel filtration chromatography profile of the purified SAPNIJ protease on HPLC system using a ZORBAX PSM 300 HPSEC. The column (6.2 mm × 250 mm) (Agilent Technologies,*

*Lawrence, Kansas, MO, USA) was equilibrated with buffer C and assayed for protein content at 280 nm (◇) and protease activity (♦). **(B)** Chromatography profile of SAPNIJ on HPLC system using Shodex Protein WK 802-5 column (8 mm × 300 mm), pre-equilibrated with buffer C and native protein markers of 670, 158, 44, 17, and 13.5 kDa, shows a single peak of 28 kDa, approximately. Proteins were separated by isocratic elution at a flow rate of 30 mL/h with buffer C and detected using a UV-VIS Spectrophotometric detector at 280 nm. The pure SAPNIJ enzyme, with retention time (R_t) of 12.525 min, containing protease activity. **(C)** 12% SDS-PAGE of the purified SAPNIJ. Lane 1, purified SAPNIJ (50 µg) obtained after HPLC ZORBAX PSM 300 HPSEC chromatography (R_t = 12.525 min). Lane 2, Amersham LMW protein marker (GE Healthcare Europe GmbH, Freiburg, Germany). **(D)** Zymography with azo-casein staining of SAPNIJ activity. Lane 1, the purified SAPNIJ enzyme (50 µg).*

III.5. NH_2-Terminal Amino-Acid Sequence Determination of SAPNIJ

Comparing the first 27 NH_2-terminal amino acids of SAPNIJ, the highest identities, varying between 50.00 to 90.48%, were obtained with *Bacillus* proteases, in descending order: 90.48, 88.89, 85.71, and 80.95% identity of subtilisin E, subtilisin BM1, subtilisin Novo, and SAPB from *Bacillus subtilis* 168, *Bacillus mojavensis* A21, *Bacillus amyloliquefaciens* ATCC 23844^T, and *Bacillus pumilus* CBS, respectively (**Table 2)**.

***Table 2.** The alignment of the NH_2-terminal amino-acid sequence of the purified protease SAPNIJ from Bacillus atrophaeus strain NIJ with the sequences of other Bacillus proteases.*

Enzyme	Origin	NH_2-terminal amino acid[a]	Identity (%)[b]
SAPNIJ (this work)	*Bacillus atrophaeus* NIJ	AQSVPYGISQIKGPAVHSQGYTGSDVK	-
Subtilisin E	*Bacillus subtilis* 168	AQSVPYGISQIKAPALHSQGY	90.48
Subtilisin BM1	*Bacillus mojavensis* A21	AQSVPYGISQIKAPALHSQGYTGSNVK	88.89
Subtilisin Novo	*Bacillus amyloliquefaciens* ATCC 23844^T	AQSVPYGVSQIKAPALHSQGY	85.71
SAPB	*Bacillus pumilus* CBS	AQTVPYGIPQIKAPAVHAQGY	80.95
BPP-A	*Bacillus pumilus* MS-1	AQTVPYGIPQIKAPAVHAQGY	80.95
DHAP	*Bacillus pumilus* UN-31-C-42	AQTVPYGIPQIKAPAVHAQGY	80.95
APRMP1	*Bacillus licheniformis* MP1	AQTVPYGIPLIKAD	75.00
SAPRH	*Bacillus safensis* RH12	AQTVPYGIPQIKAPAIHAEGYKG	69.57
SAPDZ	*Bacillus circulans* DZ100	AQTVPYGMAQIKDPAVHGQGYKGAN	68.00
SAPLF	*Lysinibacillus fusiformis* C250R	VPSGPYGPIDIKADKVIEDGFKMDEYF	63.64
Subtilisin Carlsberg	*Bacillus licheniformis* NCIB 6816^T	AQTVPYGIPLIKADKVQAQGF	56.52
SAPHM	*Bacillus licheniformis* K7A	AQTVPQGIPLIKAEKVQAQGFDGARV	52.17
SPVP	*Aeribacillus pallidus* VP3	APSGPYGPQGIKADKVHAQGFKGAN	50.00

[a] Amino-acid sequences for comparison were obtained using the BLASTP (NCBI, NIH, USA) program database. The GenBank accession number is in parentheses.

[b] Residues not identical with the protease SAPNIJ from *Bacillus atrophaeus* strain NIJ are indicated in black box

III.6. Biochemical Characterization of Purified SAPNIJ

III.6.1. Effects of Inhibitors, Reducing Agents, and Metal Ions on Enzyme Activity and Stability

The findings in **Table 3** indicated that enzyme activity was totally inhibited by PMSF and DFP, two well-known specific inhibitors of serine proteases. On the other hand, non-serine inhibitors have activated the enzyme. The thiol reagent had almost no influence on enzyme activity. In favor of metalloprotease inhibitors experiments and chelators insensitivity, 10 mM EDTA and 2 mM

EGTA well-preserved 93 and 83% SAPNIJ activities, respectively, which suggested that no metal cofactors were required, and consequently, these properties, are requirements for potential application in detergent formulations because these agents are inserted as water softeners and stain removers [20, 34].

Table 3. *Effects of various inhibitors, reducing agents, and metal ions on SAPNIJ stability. Protease activity measured in the absence of any inhibitor or reducing agent was taken as control (100%). The non-treated enzyme to which 2 mM EGTA were added was considered as 100% for metal ion assay. Residual activity was measured at pH 11 at 70 °C.*

Inhibitor/reducing agent/metal ions	Concentration	Residual protease activity (%) [a]
None	–	100 ± 2.5
PMSF	5 Mm	0 ± 0.0
DFP	5 mM	0 ± 0.0
SBTI	1 mg/mL	98 ± 2.5
TLCK	1 mM	99 ± 2.5
TPCK	1 mM	90 ± 2.2
Benzamidine	10 mM	103 ± 2.6
LD-DTT	10 mM	97 ± 2.4
2-ME	5 mM	96 ± 2.4
DTNB	5 mM	95 ± 2.3
NEM	2 mM	93 ± 2.2
Iodoacetamide	5 mM	84 ± 2.1
Leupeptin	50 µg/mL	92 ± 2.1
Pepstatin A	2 µg/mL	101 ± 2.5
EDTA	10 mM	93 ± 2.2
EGTA	2 mM	83 ± 2.1
Ca^{2+} ($CaCl_2$)	2 mM	170 ± 4.0
Mn^{2+} ($MnSO_4$)	2 mM	133 ± 3.1
Mg^{2+} ($MgSO_4$)	2 mM	156 ± 3.5
Cu^{2+} ($CuSO_4$)	2 mM	102 ± 2.5
Zn^{2+} ($ZnSO_4$)	2 mM	85 ± 2.1
Co^{2+} ($CoSO_4$)	2 mM	105 ± 2.6
Fe^{2+} ($FeSO_4$)	2 mM	73 ± 1.9
Ni^{2+} ($NiCl_2$)	2 mM	0 ± 0.0
Hg^{2+} ($HgCl_2$)	2 mM	0 ± 0.0
Cd^{2+} ($CdCl_2$)	2 mM	0 ± 0.0

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

PMSF: phenylmethanesulfonyl fluoride; DFP: diiodopropyl fluorophosphates; SBTI: soybean trypsin inhibitor; TPCK: *N*α-*p*-tosylL-phenylalanine chloromethyl ketone; TLCK: *N*α-*p*-tosylL-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 (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid.

Several metal ions were also assayed for their effects on SAPNIJ stability (**Table 3**). Improvement of the enzyme activity was: in a very high way by the addition of $MnCl_2$, $MgCl_2$, and $CaCl_2$ at 2 Mm, with a lesser degree by Co^{2+} and Cu^{2+}, and far by Fe^{2+}. However, it was completely inhibited by Ni^{2+}, Hg^{2+}, and Cd^{2+}. Likewise, the purified proteases SAPHM [19], SAPRH [20], and SAPDZ [35] were previously reported to be totally activated by Ca^{2+}, Mg^{2+}, and Mn^{2+} but strongly inhibited by Cd^{2+}, Ni^{2+}, and Hg^{2+}. The toxic metal ions exerted toxicity by binding to a variety of organic ligands, causing the denaturation of proteins, including enzymes. This observation copes with findings published by Bouacem et al.

who reported the effect of EDTA and Ca^{2+} on protease activity [36].

III.6.2. ***Effects of pH and Temperature on Protease Activity and Stability***

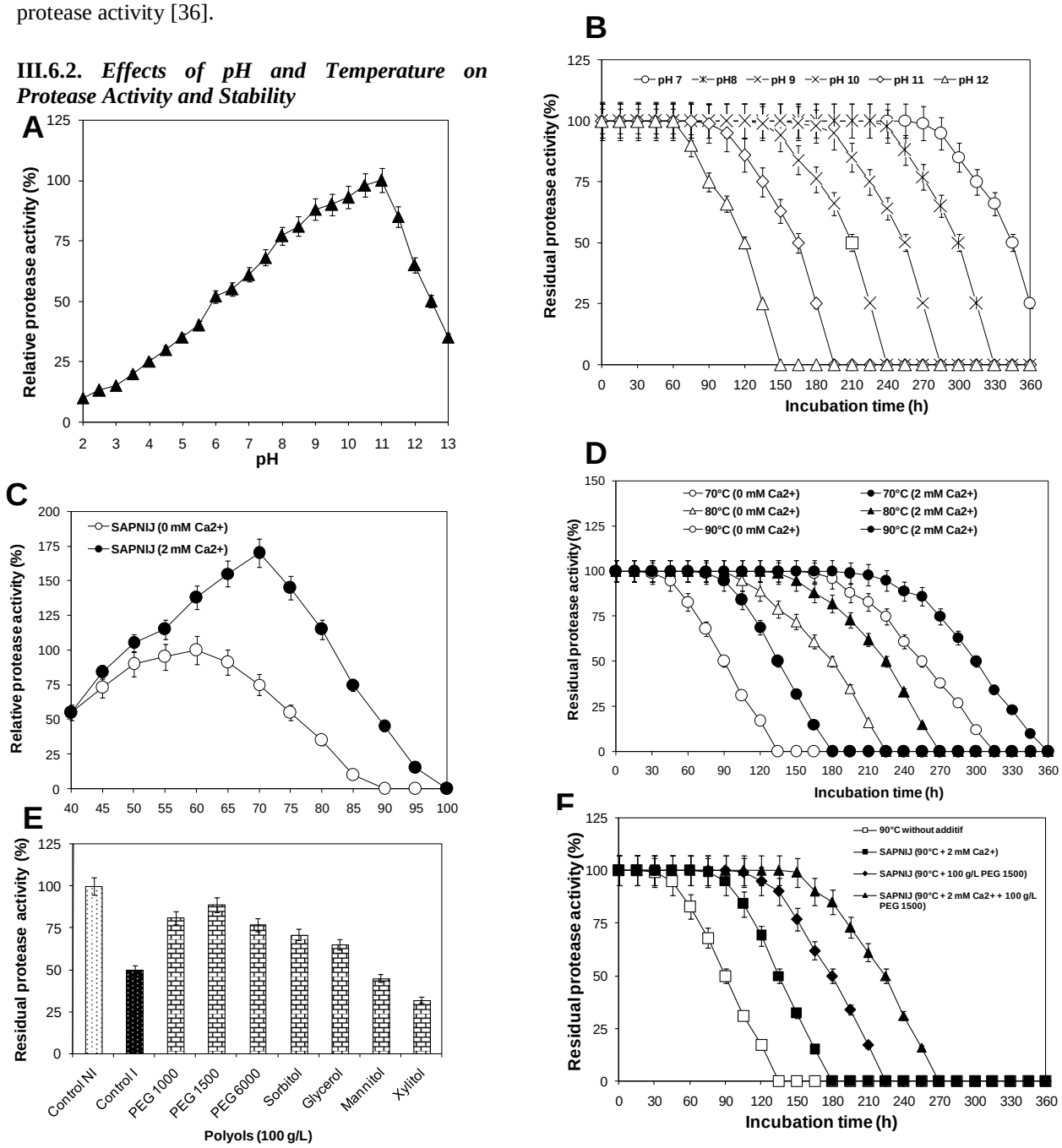


Figure 3. *Effects of pH on the* ***(A)*** *activity and* ***(B)*** *stability of SAPNIJ. The activity of the enzyme at pH 11 was taken as 100%. Effects of the* ***(C)*** *thermoactivity and the* ***(D)*** *thermostability of SAPNIJ. The enzyme was pre-incubated in the absence or presence of $CaCl_2$ at various temperatures 70, 80, and 90 °C. The residual protease activity was determined from 0 to 6 h at 15 min intervals. The activity of the non-heated enzyme was taken to be 100%.* ***(E)*** *Effect of polyols on protease thermostability. Purified SAPNIJ was heated at 90 °C for 6 h in the absence or presence of various polyols (10%). Residual enzyme activity was determined under optimal conditions. Vertical bars indicate standard error of the mean (n = 3).* ***(F)*** *Combined effect of PEG 1500 and calcium on the thermostability of SAPNIJ at 90 °C. The enzyme was pre-incubated in the absence (□) or presence of additive: 2 mM Ca^{2+} (■); 10% PEG 1500 (♦); and 2 mM Ca^{2+} and 10% PEG 1500 (▲). The residual protease activity was determined from 0 to 360 min at 15 min intervals. The activity of the non-heated enzyme was considered as 100%. Each point represents the mean (n = 3) ± standard deviation.*

The enzyme was constantly active in a wide range of pH (2-13) with a maximum activity at pH 11 (**Fig. 3A)**, taking into account the relative activities 52% (pH 6) and 50% (pH 12.5), the pH stability interval (7-12) (**Fig. 3B**) and the half-life times at pH 7, 8, 9, 10, 11, and 12 were 345, 300, 255, 210, 165, and 120 min, respectively. SAPNIJ was noted to be more efficient at alkaline pH in comparison to the maximal activity of major commercial detergent enzymes, including Alcalase™ from *Bacillus licheniformis* (8 and 9) [37] and Savinase, produced by *Bacillus lentus* (8 to 10) [37] and the alkaline microbial enzymes studied, 9 and 12 for SAPRH [20] and SAPDZ [35], respectively. This confirmed the promising potential of SAPNIJ for future industrial application, which requires enzyme stability in wide pH (9-11) range [38]. The thermostability has been improved in the presence of 2 mM Ca^{2+} at optimal conditions (**Fig. 3C**). As shown in **Fig. 3D**, the half-life times of SAPNIJ at 70, 80, and 90 °C increased to 300, 225, and 135 min in the presence of 2 mM $CaCl_2$. In fact, Ca^{2+} was previously reported to improve the activity and stability of the *Aeribacillus pallidus* VP3 protease [10]. The thermoactivity and the thermostability of SAPNIJ were higher than several other proteases previously reported from *Bacillus* strains [20, 35].

III.6.3. Combined Effect(s) of Polyols and/or Calcium on the SAPNJJ Thermostability

As indicated in **Fig. 3E**, some polyols have allowed the improvement of the enzyme thermostability compared to the controls. Indeed, the residual activities after 6 h incubation at 90 °C were 89, 81, 77, 71, 65, 45, and 32% in the presence of PEG 1500, PEG 1000, PEG 6000, sorbitol, glycerol, mannitol, and xylitol, respectively, whereas the activity was 50% after 6 h of incubation in the absence of polyols. In the same context, glycerol, mannitol, and polyethylene glycol improved the thermal stability of the alkaline proteases SAPRH from *Bacillus safensis* RH12 [20] and SAPLF from *Lysinibacillus fusiformis* C250R [9]. Of the same, the presence of $CaCl_2$ or PEG 1500 partially improved the thermostability. Note that the effect of PEG 1500 alone is better than that of only $CaCl_2$, the half-life times being of the order of 180 and 135 min, respectively (**Fig. 3F**). However, the addition of the two additives simultaneously, improves the half-life at 90 °C to be 225 min demonstrating the beneficial effect of the combination of $CaCl_2$ and PEG 1500 on the thermostability of the purified SAPNIJ. The addition of polyols can improve enzymes thermostability by the protective effect their hydroxyl groups [39, 40].

III.6.4. Substrate Specificity Profile and Kinetic Parameters Determination

The results presented in Table 4 show that SAPNIJ was able to degrade natural substrates to different degrees. The casein was the preferred SAPNIJ natural substrate compared to albumin, gelatin, ovalbumin, and keratin. Similar results were obtained for SPVP produced by *Aeribacillus pallidus* VP3 [10] For alkaline protease produced by *Brevibacillus brevis* US575, there is not a favorite substrate. It was found to be active over a broad range of substrates such as keratin followed by gelatin, elastin, casein, myoglobin, and albumin [28]. SAPNIJ showed high level of hydrolytic activity with all modified substrates tested with highest specificity for azo-casein and albumin azure, however, displaying no activity towards collagen types I and II-FITC conjugates (**Table 4**). The purified enzyme was noted to exhibit both amidase and esterase activities on C-terminal protected and L-tyrosine N-terminal such as BTEE and ATEE, but not on N-terminal and C-terminal protected L-arginine and cysteine: BAEE, BCEE, and TAME respectively. Among the used synthetic peptides, the substrate specificity profile suggested that SAPNIJ enzyme largely preferred hydrophobic substrates, with aromatic residues occupying the P_1 and P_4 positions of *p*NA substrates. Additionally, the relative activity of SAPNIJ is 9% with Suc-A-A-V-*p*NA and 53% with Suc-A-A-F-*p*NA when alanine residue is placed in P_1 position. These behaviors, also reported for other serine from *Bacillus* origins [9, 10, 19] indicated that the SAPNIJ protease was closely similar to serine protease not only in terms of specificity for position P_1, but also with regard to the effects of amino-acids residues neighboring the cleavage site. Rather, some differences were observed inside chain specificity at P_2, which could presumably indicate the presence of an extended active site. Proline was also noted to promote hydrolysis at the P_2 position in SAPNIJ enzyme, a feature that was not observed for protease, subtilisin E from *Bacillus subtilis* 168 [41]. The SAPNIJ, SAPHM, SAPRH, and Savinase™ 16L, type EX exhibited the classical kinetics of Michaelis-Menten for the two used substrates. With casein as a natural substrate, the k_{cat}/K_m values exhibited by SAPNIJ were 10.70 4.26, and 1.34 times higher than those of Savinase™ 16L, type EX; SAPRH; and SAPHM; respectively. When Met Suc-$(Ala)_2$-Pro-Phe-*p*NA was used as a synthetic substrate, SAPNIJ was also noted to exhibit k_{cat}/K_m values that were 3.70, 2.12, and 1.37 times higher than those of Savinase™

16L, type EX; SAPRH; and SAPHM; respectively (**Table 5**).

Table 4. *Substrate specificity profile of SAPNIJ enzyme from Bacillus atrophaeus strain NIJ.*

Substrate		Concentration	Absorbance (nm)[a]	Relative protease activity (%)[a]
Natural protein	Casein	30 g/L	600	100 ± 2.5
	Albumin	30 g/L	600	89 ± 2.1
	Gelatin	30 g/L	600	55 ± 1.4
	Ovalbumin	30 g/L	600	35 ± 1.1
	Keratin	30 g/L	600	25 ± 0.9
Modified protein	Azo-casein	25 g/L	440	100 ± 2.5
	Albumin azure	25 g/L	440	77 ± 2.1
	Keratin azure	25 g/L	440	26 ± 0.9
	Collagen type	10 g/L	440	0 ± 0.0
	Collagen type II	10 g/L	440	0 ± 0.0
Ester	BTEE	10 mM	253	100 ± 2.5
	ATEE	10 mM	253	96 ± 2.4
	BAEE	10 mM	253	0 ± 0.0
	BCEE	10 mM	253	0 ± 0.0
	TAME ↓	10 mM	253	0 ± 0.0
Synthetic peptide (*p*NA)	P4 - P3 - P2 - P1 -P'1			
	Suc-F-*p*NA	5 mM	410	58 ± 1.6
	Benz-Y-*p*NA	5 mM	410	41 ± 1.4
	Met-*p*NA	5 mM	410	35 ± 1.1
	Ac-L-*p*NA	5 mM	410	32 ± 1.0
	Pro-*p*NA	5 mM	410	0 ± 0.0
	Ac-A-*p*NA	5 mM	410	0 ± 0.0
	Benz-R-*p*NA	5 mM	410	0 ± 0.0
	Suc-Y-L-V-*p*NA	5 mM	410	0 ± 0.0
	Suc-A-A-A-*p*NA	5 mM	410	0 ± 0.0
	Suc-A-A-V-*p*NA	5 mM	410	9 ± 0.3
	Suc-A-A-F-*p*NA	5 mM	410	53 ± 1.5
	Benz-F-V-R-*p*NA	5 mM	410	0 ± 0.0
	Suc-A-A-P-F-*p*NA	5 mM	410	100 ± 2.5
	Suc-F-A-A-F-*p*NA	5 mM	410	99 ± 2.5
	Suc-A-A-V-A-*p*NA	5 mM	410	92 ± 2.3
	Suc-A-A-P-M-*p*NA	5 mM	410	80 ± 2.1
	Suc-A-A-P-L-*p*NA	5 mM	410	77 ± 2.1
	Suc-L-L-V-Y-*p*NA	5 mM	410	65 ± 1.6
	Ac-Y-V-A-D-*p*NA	5mM	410	0 ± 0.0

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

BTEE: *N*-benzol-L-tyrosine ethyl ester, ATEE: *N*-acetyl-L-tyrosine ethyl ester monohydrate, BAEE: *N*-benzol-L-arginine ethyl ester, BCEE: *S*-benzyl-L-cysteine ethyl ester hydrochloride, TAME: N_{α}-*p*-tosyl- L-arginine methyl ester hydrochloride. *N*-succinyl-L-Phe-*p*-nitroanilide, *N*-benzoyl-L-Tyr-*p*-nitroanilide, *N*-acetyl-L-Leu-*p*-nitroanilide, L-Met-*p*-nitroanilide, L-Pro-*p*-nitroanilide trifluoroacetate salt, *N*-acetyl-L-Ala-*p*-nitroanilide, L-Val-*p*-nitroanilide hydrochloride, *N*-benzoyl-L-Arg-*p*-nitroanilide, *N*-succinyl-L-Tyr-L-Leu-L-Val-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Phe-*p*-nitroanilide *N*-succinyl-L-Ala-L-Ala-L-Val-*p*-nitroanilide, *N*- benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide, *N*-succinyl-L-Phe-L-Ala-L-Ala-L-Phe-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Val-L-Ala-*p*-nitroanilide, *N*-succinyl-L-Leu-L-Leu-L-Val-L-Tyr-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Met-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide, *N*-acetyl-L-Tyr-L-Val-L-Ala-L-Asp-*p*-nitroanilide.

Table 5. *Kinetic parameters of purified proteases: SAPNIJ; SAPHM; SAPRH; and Savinase™ 16L, type EX for the hydrolysis of natural protein and synthetic peptide and as substrate.*

Substrate	Enzyme	K_m (mM) [a]	V_{max} (U/mg) [a]	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	Catalytic efficiency relative to SAPNIJ
Casein[b]	SAPNIJ	0.362 ± 0.01	87,450 ± 315	58,300	161.050	1.00
	SAPHM	0.453 ± 0.02	81,560 ± 283	54,373	120,029	0.74
	SAPRH	0.621 ± 0.03	35,250 ± 515	23,500	37,842	0.23
	Savinase™ 16L, type EX	0.945 ± 0.09	21,332 ± 476	14,221	15,048	0.09
Met Suc-$(Ala)_2$-Pro-Phe-*p*NA[c]	SAPNIJ	0.577 ± 0.05	186,570 ± 950	124,380	215,563	1.00
	SAPHM	0.755 ± 0.06	178,200 ± 852	118,800	157,350	0.72
	SAPRH	0.787 ± 0.07	120,125 ± 890	80,083	101,757	0.47
	Savinase™ 16L, type EX	0.935 ± 0.08	81,595 ± 605	54,397	58,179	0.26

[a] Values represent means of a three replicates, and ± standard errors are reported.
[b] For the natural substrate, one protease unit was defined as the amount of enzyme that hydrolyzed the substrate and produced 1 µg of amino-acid equivalent to tyrosine per min at 660 nm under the assay conditions used.
[c] For the synthetic peptide substrate, the amount of released *p*-nitroanilide (*p*NA) was recorded at 410 nm. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 µmole of *p*NA under standard assay conditions.

III.7. Performance Evaluation of the Purified Used Proteases

III.7.1. Effects of Additive on Protease Stability

It should be noted that a remarkable stability of the SAPNIJ activity has been obtained in the presence of 15% Tween 20 and 40, or Triton X-100 and the strong anionic surfactants, particularly SDS and LAS compared to those of SAPHM; SAPRH; and Savinase™ 16L, type EX (**Table 6**). Also, SAPNIJ has given an excellent stability compared to its counterparts by retaining 200 and 99% of its initial activity after treatment with 15% hydrogen peroxide and 5% sodium perborate, respectively, 175 and 73% for SAPHM; 160 and 85% for SAPRH; and 144 and 75% for Savinase™ 16L, type EX; respectively. This remarkable stability is similar to some proteases as KERAB in the presence of 5% Tween 40, 1.5% SDS, and 15% H_2O_2, [42], BM1 and BM2 in the presence of 1% Triton X-100, 0.1% SDS, and 1% H_2O_2 [43, 44].
Bleach stability was also attained through protein engineering [45]. For this purpose, this remarkable SAPNIJ stability is of interest since only a few wild-type proteases have been reported to be oxidant, surfactant and bleach stable.

Table 6. *Effect of some detergents on the enzyme stability. The purified enzymes (SAPNIJ; SAPHM, SAPRH, and Savinase™ 16L, type EX) 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]			
		SAPNIJ	SAPHM	SAPRH	Savinase™ 16L, type EX
None	–	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 2.5
Tween 20	10% (v/v)	155 ± 3.7	130 ± 3.4	127 ± 3.3	142 ± 3.8
	15%	140 ± 3.8	121 ± 3.3	119 ± 3.2	137 ± 3.6
Tween 40	10% (v/v)	145 ± 3.9	119 ± 3.2	138 ± 3.4	129 ± 3.3
	15%	133 ± 3.3	111 ± 2.7	129 ± 3.3	110 ± 2.7
Tween 80	10% (v/v)	130 ± 3.4	110 ± 2.7	117 ± 3.1	108 ± 2.7
	15%	122 ± 3.2	105 ± 2.6	108 ± 2.6	106 ± 2.6
Triton X-100	10% (v/v)	170 ± 4.0	162 ± 3.9	159 ± 3.7	130 ± 3.2
	15%	153 ± 3.7	144 ± 3.4	141 ± 3.4	120 ± 3.2
SDS	1% (w/v)	88 ± 2.4	75 ± 2.2	90 ± 2.3	80 ± 2.0
	5%	71 ± 2.1	50 ± 1.5	60 ± 1.5	61 ± 1.5
LAS	1% (w/v)	98 ± 2.5	81 ± 2.0	91 ± 2.2	96 ± 2.3
	5%	89 ± 2.2	66 ± 1.8	65 ± 1.7	73 ± 1.9
Sulfobetaine	50 mM	101 ± 2.5	86 ± 2.4	94 ± 2.3	105 ± 2.6
H_2O_2*	10% (v/v)	240 ± 6.5	198 ± 5.2	187 ± 4.1	155 ± 3.6
	15%	200 ± 5.5	175± 4.0	160 ± 3.7	144 ± 3.5
Sodium perborate	2% (w/v)	115 ± 2.8	80 ± 2.0	112 ± 2.6	89 ± 2.1
	5%	99 ± 2.5	73 ± 2.1	85 ± 2.3	75 ± 2.1
TAED	10% (w/v)	124 ± 3.2	119 ± 3.2	135 ± 3.3	114 ± 2.7
$Na_2{\cdot}CO_3$	50 mM	99 ± 2.5	78 ± 2.0	98 ± 2.4	121 ± 3.2
$Na_2{\cdot}$CMC	10% (w/v)	97 ± 2.5	101 ± 2.5	112 ± 2.6	101 ± 2.5
STPP	25 mM	95 ± 2.4	83 ± 2.1	80 ± 2.0	92 ± 2.2
Zwittergent 3-12	15 mM	115 ± 2.7	105 ± 2.6	121 ± 3.2	113 ± 2.6
CHAPS	25 mM	96 ± 2.4	89 ± 2.2	103 ± 2.5	125 ± 3.2
CTAB	25 mM	103 ± 2.6	99 ± 2.5	116 ± 3.1	110 ± 2.7
TTAB	25 mM	108 ± 2.7	115 ± 2.8	105 ± 2.6	106 ± 2.6
Zeolithe	1% (w/v)	105 ± 2.6	88 ± 2.2	108 ± 2.7	90 ± 2.2
Perfume	1% (v/v)	121 ± 3.2	111 ± 2.7	105 ± 2.6	97 ± 2.5

[a] Values represent means of a three replicates, and ± standard errors are reported.
* Values were determined in presence of 100 mM Borate-NaOH buffer and 2 mM $CaCl_2$ at optimum pH and temperature of each enzyme.
Sulfobetaine: *N*-dodecyl-*N*-*N'*-dimethyl-3-ammonio-1-propane sulfonate; Tween: poly (oxyethylene) sorbitan monolaurate; Triton: octyphenolpoly (ethylene glycolether); TAED: tetraacetyl ethylene diamine; STPP: sodium tripolyphosphate; CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; CTAB: hexadecyl trimethyl ammonium bromide; TTAB: tetradecyl trimethyl ammonium bromides.

III.7.2. Stability and Compatibility of Proteases with Various Commercial Laundry Detergents

The results presented in **Fig. 4A** show that SAPNIJ is extremely stable and compatible with the commercial laundry detergents used at a concentration of 7 mg/mL, retaining 100% of its initial activity with Dixan, iSiS Pril, and Ariel (vs 88, 90, and 100% for SAPNIJ; 90, 100, and 95% for Savinase™ 16L, for type EX; and 61, 85, and 75% for SAPRH, respectively). We recorded on the same figure that SAPNIJ exhibited high stability and more than 90% with Det (vs 77, 66, and 55% for SAPRH; Savinase™ 16L, type EX; and SAPHM, respectively) even after 6 h incubation at 40 °C. Incubated under the same conditions in the presence of Ariel, protease VM10 [46] and protease SSR1 [47] retained only 42 and 37% of their initial activities, respectively. In contrary, this alkaline proteases SAPNIJ (same thing for SAPRH) was noted to be less stable in the presence of Axion (77 and 68%) and Persil (74 and 90%) compared to Savinase™ 16L, type EX and SAPHM (100 and 85% (for Axion) and 94 and 100% (for Persil) of their initial activity, respectively.

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III.7.3. Stains removal from cotton fabrics

Blood stain was rapidly eliminated by the combination of SAPNIJ (or crude enzyme) or commercial protease Savinase™ 16L and type EX in detergent comparatively to detergent alone. In the case of the used of SAPNIJ with the Ariel solid detergent resulted in the complete stain removal (**Fig. 4B**). These findings advise the usefulness of SAPNIJ in future industrial applications as a cleaning bio-additive in detergent formulations.
Although Jaouadi et al. [27], Bouacem et al. [36], and Hadjidj et al. [19] reported the usefulness of alkaline proteases: SAPB [27], SAPCG [36], and SAPHM [19] respectively for the removal of blood stains from cotton cloth. In addition, the protease SAPLF from an hydrocarbonoclastic strain, displayed good washing performance at 40 °C and alkaline pH range (pH 8-11) [9].

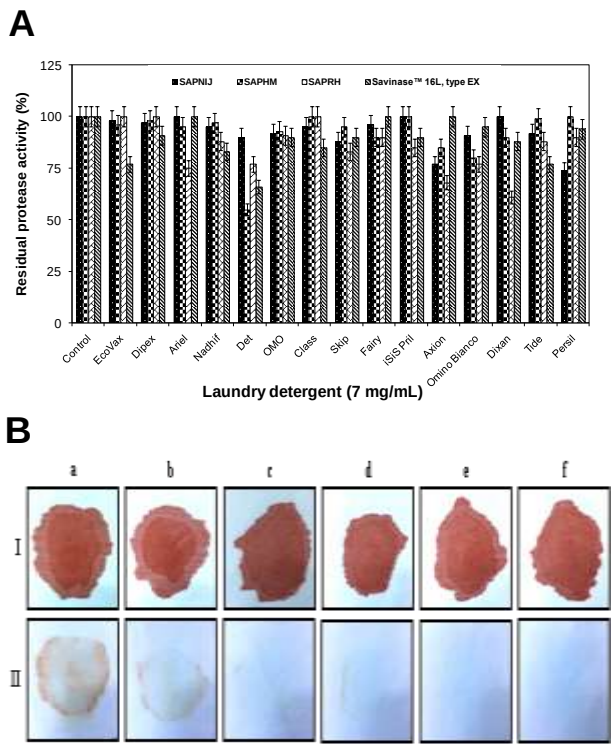

Figure 4. (A) *Stability of SAPNIJ; SAPHM; SAPRH; and Savinase™ 16L, type EX proteases in the presence of liquid and solid detergents. Enzyme activity of the control sample, which contained no additive and was incubated under similar conditions, was taken as 100%. Each point represents the mean of three independent experiments. Vertical bars indicate standard error of the mean (n = 3). One unit of protease activity was defined as the amount of enzyme required to catalyze the liberation of 1 µmole of peptide bond from DMC per min under the experimental conditions used.* ***(B)*** *Example of washing performance analysis test of SAPNIJ. Stained cloth pieces with blood. (I) Control: untreated stained cloth pieces, (II) stained cloth pieces washed with: (**a**) distilled water, (**b**) Ariel commercial laundry detergent (7 mg/mL), (**c**) SAPRH (500 U/mL) + Ariel detergent (7 mg/mL); (**d**) SAPHM (500 U/mL) + Ariel detergent (7 mg/mL); (**e**) Savinase™ 16L, type EX (commercial enzyme, 500 U/mL) + Ariel detergent (7 mg/mL), and (**f**) SAPNIJ (500 U/mL) + Ariel detergent (7 mg/mL).*

IV. Conclusion

Differently to other *Bacillus* proteases, this study confirmed that the novel extracellular serine alkaline protease (SAPNIJ) has attractive biochemical characteristics as a purified detergent-stable protease from *Bacillus atrophaeus* NIJ which not documented on protease activity. Its catalytic efficiency was higher than those of Savinase™ 16L, type EX (commercial enzyme), SAPHM from *B. licheniformis* K7A, and SAPRH from *B. safensis* RH12. Characterization of SAPNIJ demonstrates also its stability under broad temperature and pH range even in the presence of non-ionic surfactants, oxidizing and bleaching agents, and some commercial liquid and solid detergents than already described proteases. The wash performance analysis supported its candidature in detergent industry, even at low temperature at very low level of supplementation. Since enzyme was able to effectively remove food stain at low temperature, it can find application in cleaning sensitive clothes, which may not be washed at high temperature. Considering the better stability at high pH and with different commercial detergents, SAPNIJ could have applications in the laundry industry. This allows for this novel protease encouraging properties for biotechnological applications such as the stability: at high pH values, in the presence of certain metal ions, detergents and detergent additives. These traits mean that this enzyme could be considered to be suitable for a variety of industrial applications especially in detergent formulations, apart from the possibility of its application for the laundry industry.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Statement All procedures performed in studies involving animals bloods were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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