



## Purification and biochemical characterization of a novel detergent-stable serine alkaline protease from *Bacillus safensis* strain RH12

Hatem Rekik<sup>1,2</sup>, Nadia Zarái Jaouadi<sup>1,2</sup>, Fares Gargouri<sup>1</sup>, Wacim Bejar<sup>1</sup>, Fakher Frikha<sup>1</sup>, Najah Jmal<sup>3</sup>, Samir Bejar<sup>1,2</sup>, Bassem Jaouadi<sup>1,2,\*</sup>

<sup>1</sup> Laboratory of Microbial Biotechnology and Engineering Enzymes (LMBEE), Centre of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour Km 6, PO Box 1177, Sfax 3018, Tunisia: [rekik.hatem@hotmail.fr](mailto:rekik.hatem@hotmail.fr), [nedia.zarai@yahoo.fr](mailto:nedia.zarai@yahoo.fr), [fares.gargouri90@gmail.com](mailto:fares.gargouri90@gmail.com), [wacim.bejar@hotmail.fr](mailto:wacim.bejar@hotmail.fr), [frikha\\_fakher@yahoo.fr](mailto:frikha_fakher@yahoo.fr), [samir.bejar@cbs.rnrt.tn](mailto:samir.bejar@cbs.rnrt.tn), [bassem.jaouadi@yahoo.fr](mailto:bassem.jaouadi@yahoo.fr)

<sup>2</sup> Biotech ECOZYM Start-up, Business Incubator, Centre of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour Km 6, P.O. Box 1177, Sfax 3018, Tunisia [rekik.hatem@hotmail.fr](mailto:rekik.hatem@hotmail.fr), [nedia.zarai@yahoo.fr](mailto:nedia.zarai@yahoo.fr), [samir.bejar@cbs.rnrt.tn](mailto:samir.bejar@cbs.rnrt.tn), [bassem.jaouadi@yahoo.fr](mailto:bassem.jaouadi@yahoo.fr)

<sup>3</sup> STE JMAL (EJM)-Laundry Detergent Industry, Z.I. Avenue August 13, Z.I. Poudriere 1, P.O. Box 407, Boustene, Sfax 3000, Tunisia [ejm.direction@gmail.com](mailto:ejm.direction@gmail.com)

\* Authors to whom correspondence should be addressed; E-Mail: [bassem.jaouadi@cbs.rnrt.tn](mailto:bassem.jaouadi@cbs.rnrt.tn)

Tel.: +216 99 53 52 53

Received: / Accepted: / Published:

---

### Abstract:

A novel protease (SAPRH) was hyper-produced (9000 U/mL) from *Bacillus safensis* RH12, a newly isolated enzyme from a Tunisian offshore oil field. The enzyme was purified to homogeneity, using salt precipitation, heat-treatment and FPLC anion-exchange chromatography (Fast protein liquid chromatography). SAPRH was a monomer of molecular mass of ~28 kDa. The NH<sub>2</sub>-terminal 23 amino-acid sequence of SAPRH showed high homology with those of *Bacillus*-proteases. SAPRH displayed optimal activity at pH 9 and 60 °C. It was strongly inhibited by (PMSF, Phenylmethane sulfonyl fluoride) and (DFP, Diisopropylfluorophosphate), indicating that it belongs to the serine-proteases family. One of the most distinctive properties is its catalytic efficiency, which is higher than that of Alcalase 2.5 L, type DX (commercial enzyme) and SAPB from *Bacillus pumilus* strain CBS. Interestingly, the results of the wash performance analysis demonstrated considerably good de-staining at 40 °C for 30 min with low supplementation (500 U/mL). The *sapRH* gene, which encodes the serine alkaline protease SAPRH, from *Bacillus safensis* strain RH12, was isolated and its DNA sequence was determined. The highest sequence identity value (97%) was obtained with SAPB from *B. pumilus* strain CBS, with only 9 amino-acids of difference. The region, encoding SAPRH was

heterologously expressed in *E. coli* BL21- AI™ cells using GATEWAY™ pDEST™17 expression-vector.

**Keywords:** protease; *Bacillus safensis*; Offshore; oil field; Detergent; Wash performance.

**Mol2Net YouTube channel:** <http://bit.do/mol2net-tube>

**YouTube link:** please, paste here the link to your personal YouTube video, if any.

## 1. Introduction

Enzymes are fascinating the researchers owing to their enormous power of catalysis and eco-friendly nature. Since community demands are largely increasing, a continuous need to evolve enzymes is notably required. In fact, the techniques and computational tools have immensely developed keeping pace with the cutting-edge industries to meet the growing demands. Correspondingly, techniques such as protein engineering helps in the development of quality products by mutating the amino acids in order to make more stable and efficient product (Kumar et al., 2017). Enzymes are used in several environmental-friendly industrial purposes, as they are efficient, selective, and accelerate; they also speed up reactions by

forming transition-state complexes with their substrate, which reduces the activation energy of the reaction (Bharathiraja et al., 2017; Singh et al., 2016). Accordingly, the present study reports on the purification and biochemical characterization of a novel detergent stable protease (SAPRH) from *Bacillus safensis* strain RH12 isolated from offshore sediment in the Gulf of Gabes (Tunisia). It also provides basic information on the potential use of SAPRH as a prospective candidate for future applications in detergent formulations mainly with Class, a commercial liquid laundry detergent from the local company JMAL (EJM).

## 2. Results and Discussion

### Identification of the microorganism RH12

the morphological, biochemical, and physiological characteristics revealed that the RH12 isolate appeared in a bacilli form, and it was an aerobic, spore-forming rod, Gram-positive, catalase-positive, oxidase-positive, and motile, and colonies are round, undulate, dull white and non-luminescent. The carbohydrate profile of the isolate was further investigated using API 50 CH gallery tests. All the data obtained with regard to the physiological and biochemical properties of the isolate, therefore, strongly confirmed that the RH12 strain belonged to the *Bacillus* genus. In order to further support the findings related to the identification of strain RH12, the 16S rRNA gene sequence obtained was submitted to GenBank BLAST search analyses. Likewise, the results displayed strong homology with those of several cultivated strains of *Bacillus*, reaching a maximum sequence identity of 99%. The nearest *Bacillus* strains identified by the BLAST analysis were the *Bacillus safensis* strain SAFN-037 (accession no. AY167880) and *Bacillus safensis* strain FO-036bT (accession no. AF234854).

### SAPRH production and production

The protease production is composed of (g/L): galactose, 10; yeast extract, 4; CaCl<sub>2</sub>, 2; K<sub>2</sub>HPO<sub>4</sub>, 0.5; and KH<sub>2</sub>PO<sub>4</sub>, 0.5; the addition of trace elements at 2% (v/v) at pH 7.4. The protein elution profile obtained at the final purification step indicated that the protease was eluted at 220–300 mM NaCl. The results of the purification procedure are summarized in Table 1. Enzyme purity was estimated to be 43-fold higher than that of the crude extract. Under optimum assay conditions, the purified enzyme had a specific activity of 37000 U/mg, with a yield of about 19%.

To analyze the homogeneity and molecular weight of the purified SAPRH, native-PAGE (Polyacrylamide gel electrophoresis) and SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) were performed. The molecular mass of the purified enzyme was estimated to be approximately 28 kDa, as assessed by native-PAGE (data not shown). Further, single band by SDS-PAGE analysis showed the subunit molecular weight of approximately 28 kDa, indicating the high purity

of the enzyme. The SDS-PAGE analysis exhibited single band, indicating that the purified SAPRH enzyme was a monomeric protein consisted of one molecular weight subunit. Zymogram revealed one zone of caseinolytic activity corresponding to purified SAPRH.

#### Biochemical characterization of SAPRH

The findings indicated that enzyme activity was strongly inhibited by PMSF and DFP, two well-known inhibitors of serine proteases. Other inhibitors, such as TPCK (Tosyl phenylalanyl chloromethyl ketone) and TLCK (Tosyl-L-lysyl-chloromethane hydrochloride), a chymotrypsin alkylating agent, benzamidine, a trypsin competitive reagent, SBTI, and a soybean trypsin inhibitor, were used and observed to have no inhibitory effects on enzyme activity. Moreover, the thiol reagent (DTNB, (5,5-dithio-bis-(2-nitrobenzoic acid), NEM, (N-Ethylmaleimide) and iodoacetamide) had almost no influence on enzyme activity.

#### Influences of additive on protease stability

When SAPRH was pre-incubated for 1 h at 40 °C in the presence of several commercially available laboratory non-ionic surfactants, denaturing agents or anionic surfactants and bleach agents, a higher stability in the presence of 15% Tween 20, Tween 40 or Triton X-100 and the strong anionic surfactants, particularly SDS and LAS was observed compared to SAPB; and Alcalase 2.5 L, type DX. SAPRH was also highly stable against bleaching agents since it retained 160 and 85% of its initial activity after treatment with 15% hydrogen peroxide and 5% sodium perborate, respectively, vs 109 and 61% for SAPB and 150 and 83% for Alcalase 2.5 L, type DX, respectively. This stability is of interest since only a few wild-type proteases have been reported to be oxidant, surfactant and bleach stable. KERAB, keratinase from *Streptomyces* sp. strain AB1, was remarkably stable in the presence of 5% Tween 40, 1.5% SDS, and 15% H<sub>2</sub>O. The data to compared to Alcalase 2.5 L, type DX and SAPB, SAPRH is extremely stable and compatible with the commercial liquid and solid laundry detergents used at a concentration

of 7 mg/mL, retaining 100% of their initial activity with Class, Ecovax, and Dipex (vs 88, 100, and 91% for SAPB and 75, 90, and 90% for Alcalase 2.5 L, type DX, respectively). The data illustrated in **Fig. 1** also indicate that SAPRH exhibited high stability, i.e. N88% with Nadhif (vs 81% for SAPB and 80% for Alcalase 2.5 L, type DX) even after 1 h incubation at 40 °C. The alkaline proteases SAPRH and SAPB were noted to be less stable in the presence of Ariel (75 and 67%) and Skip (83 and 74%).

#### Stains removal from cotton fabrics

Several pieces of stained cotton cloth were incubated at different conditions and used to evaluate the performance of SAPRH and Alcalase 2.5 L, type DX in terms of their ability to remove blood, egg, and chocolate stains. As shown in **Fig. 2**, a limited washing performance was observed with detergent (Class) only.

The supplementation of SAPRH or commercial protease Alcalase 2.5 L, type DX in detergent seems to improve the cleaning process as evidenced by rapid blood stain removal when compared to detergent alone. In fact, SAPRH facilitated the release of proteinaceous materials in a much easier way than the currently used Alcalase 2.5 L, type DX protease. Furthermore, the combination of SAPRH with the Class liquid detergent resulted in the complete stain removal (**Fig. 2**).

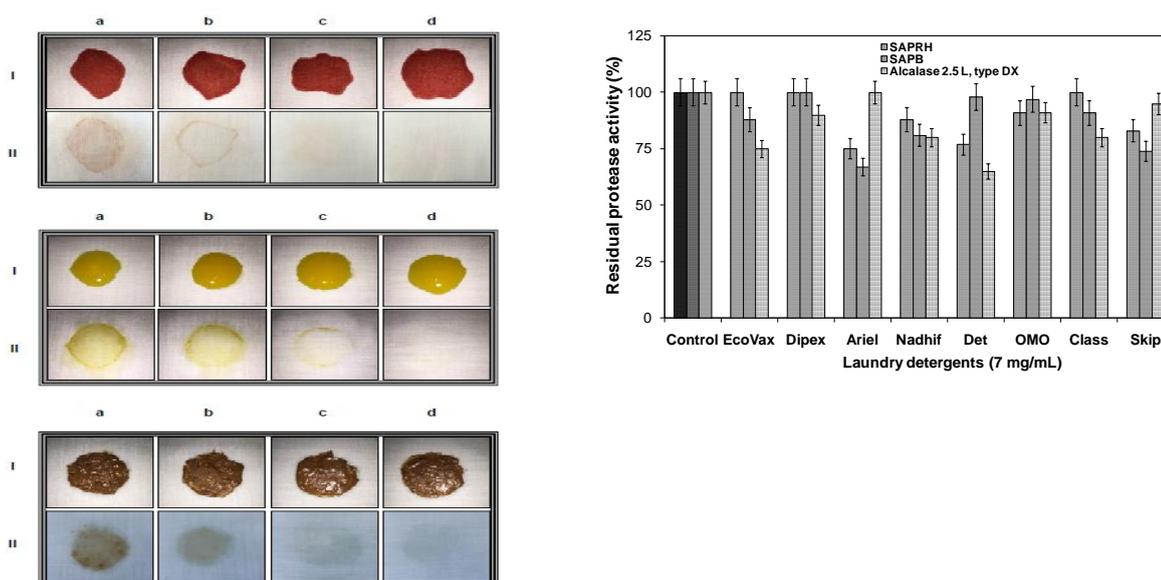
#### Molecular cloning and DNA analysis of the *sapRH* gene encoding SAPRH

Using the protease gene sequences of *Bacillus* strains, two primers, called F-RH50 and R-RH51, were designed and used to amplify a fragment of about 1.4 kb that could contain the *sapRH* gene. This PCR fragment was purified and cloned in a pCR-Blunt cloning vector using an *E. coli* Top10 host strain, thus leading to the recombinant plasmid pHR1.

The recombinant thermostable serine alkaline protease (His6-rSAPRH) from *B. safensis* strain RH12 was expressed, purified, and characterized in *E. coli* strain BL21-AI™ using the GATEWAY™ Cloning Technology.

**Table 1.** Flow sheet for SAPRH purification from *Bacillus safensis* strain RH12

Purification step	Total activity (units) <sup>a,b</sup> × 10 <sup>3</sup>	Total protein (mg) <sup>a,c</sup>	Specific activity (U/mg of protein)	Activity rate (%)	recovery	Purification factor (fold)
Crude extract	4,500 ± 75	5250 ± 90	857 ± 135	100		1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Fractionation (45-65%)-dialysed	3,735 ± 49	1,233 ± 21	3,029 ± 243	83		3.53
Heat treatment (30 min at 65 °C)	1,800 ± 21	135 ± 10	13,334 ± 451	40		15.55
FPLC (UNO Q-6)	851 ± 10	23 ± 2	37,000 ± 898	19		43

**Figure 1.** Washing performance analysis test of SAPRH.

### 3. Materials and Methods

#### Experimental

##### Isolation and protease-producing

Isolations were made at 37 °C from marine sediment in the deep Mediterranean Sea. They were freshly collected from a petroleum reservoir in “Hasdrubal”, an offshore oil and gas site belonging to the BritishGas Tunisia Ltd. company. The Hasdrubal oil and gas field is located 100 km offshore Tunisia in the Gulf of Gabes.

##### Classical and molecular identification RH12

The morphological, cultural, physiological, and biochemical characteristics of the bacterium were investigated as well described by a previous detailed study (Jaouadi et al., 2008).

##### Assay of proteolytic activity:

Protease activity assay was carried (Kembhavi et al., 1993), using Hammerstein casein (Merck, Darmstadt, Germany) as a substrate. The proteolytic activity present in the laundry detergent solution was evaluated as described elsewhere (Touioui et al., 2015) using N,N-dimethylated casein (DMC) as a substrate.

##### Protease purification:

Five hundred milliliters of a 22-h culture of *Bacillus safensis* strain RH12 was centrifuged for 30 min at 9000 g to remove microbial cells. The supernatant containing extracellular protease was used as the crude enzyme preparation and submitted to the following purification steps. It was initially saturated up to 45-65% and dialyzed overnight against the repeated changes of the same buffer. The clear supernatant was incubated for 30 min at 65 °C. The supernatant was loaded and applied to an FPLC system, using a 12 mm ×

53 mm UNO Q-6 chromatography column equilibrated with buffer B. The elution of proteins was carried out with a flow rate of 1 mL/min by using a linear NaCl gradient ranging from 0 to 500 mM in the same buffer and detected using a UV/VIS Spectrophotometric detector at 280 nm.

#### Gene cloning and expression of the protease

Two oligonucleotides were synthesized, based on the high degree of sequence homology published for the protease gene of *Bacillus* strains and utilized for the isolation and determination of the sapRH encoding gene sequence (Benkiar et al., 2013; Jaouadi et al., 2008).

These findings advise on the usefulness of SAPRH in future industrial applications as a cleaning bioadditive in detergent formulations. This is the first report pertaining to the cloning sapRH gene from *Bacillus safensis*, encoding a serine alkaline protease (SAPRH), which is endowed with a number of properties that are highly valued for the detergent industry. Further studies, some of which are currently underway in our laboratories, are needed to hyperproduce the SAPRH enzyme in *Pichia pastoris* system and to explore its structure-function relationships using site-directed mutagenesis approach.

## 4. Conclusions

### Acknowledgments

The authors would like to thank to Mrs. N. Masmoudi, Mrs. N. Kchaou, and Mr. K. Walha (Analysis Unit-CBS) for their technical assistance. We would also like to express their gratitude to Pr. H. Belghith and Dr. W. Saibi (CBS) for their constructive suggestions and discussions. Special thanks are also due to Pr. W. Hariz from the English Department at the Sfax Faculty of Science, University of Sfax (Sfax, Tunisia) for language polishing services and constructively proofreading.

### Author Contributions

Conceived and designed the experiments: BJ.

Performed the experiments: HR, NZJ.

Analyzed the data: NZJ, HR, SB, BJ.

Contributed reagents/materials/analysis tools: NZJ, HR.

Wrote the text of the paper: HR, NZJ.

Critical revision of manuscript: HR, NZJ, SB, BJ.

Proofreading and polishing the language of the present paper: WH, BJ.

### Conflicts of Interest

The authors declare that they have no conflict of interest.

### Abbreviations

FPLC: Fast protein liquid chromatography

PMSF: Phenylmethane sulfonyl fluoride

DFP: Diisopropylfluorophosphate

PAGE: Polyacrylamide gel electrophoresis

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TPCK: Tosyl phenylalanyl chloromethyl ketone

TLCK: Tosyl-L-lysyl-chloromethane hydrochloride

DTNB: 5,5-dithio-bis-(2-nitrobenzoic acid)

NEM: N-Ethylmaleimide

KERAB: keratinase from *Streptomyces* sp. strain AB1

SAPB: Serine Alkaline Protease from *Bacillus pumilus* CBS

## References and Notes

- Benkiar, A., Zarái Jaouadi, N., Badis, A., Rebzani, F., Soraya, B.T., Rekik, H., Naili, B., Ferradji, F.Z., Bejar, S., Jaouadi, B. 2013. Biochemical and molecular characterization of a thermo-and detergent-stable alkaline serine keratinolytic protease from *Bacillus circulans* strain DZ100 for detergent formulations and feather-biodegradation process. *International Biodeterioration & Biodegradation*, **83**, 129-138.
- Bharathiraja, S., Suriya, J., Krishnan, M., Manivasagan, P., Kim, S.-K. 2017. Production of enzymes from agricultural wastes and their potential industrial applications. in: *Advances in food and nutrition research*, Vol. 80, Elsevier, pp. 125-148.
- Jaouadi, B., Ellouz-Chaabouni, S., Rhimi, M., Bejar, S. 2008. Biochemical and molecular characterization of a detergent-stable serine alkaline protease from *Bacillus pumilus* CBS with high catalytic efficiency. *Biochimie*, **90**(9), 1291-1305.
- Kembhavi, A.A., Buttle, D.J., Knight, C.G., Barrett, A.J. 1993. The two cysteine endopeptidases of legume seeds: purification and characterization by use of specific fluorometric assays. *Archives of biochemistry and biophysics*, **303**(2), 208-213.
- Kumar, V., Baweja, M., Liu, H., Shukla, P. 2017. Microbial enzyme engineering: applications and perspectives. in: *Recent advances in Applied Microbiology*, Springer, pp. 259-273.
- Singh, R., Kumar, M., Mittal, A., Mehta, P.K. 2016. Microbial enzymes: industrial progress in 21st century. *3 Biotech*, **6**(2), 174.
- Touioui-Boulkour, B., Zarái Jaouadi, N., Boudjella, H., Ferradji, F.Z., Belhoul, M., Rekik, H., Badis, A., Bejar, S., Jaouadi, B. 2015. Purification and biochemical characterization of two detergent-stable serine alkaline proteases from *Streptomyces* sp. strain AH4. *World Journal of Microbiology and Biotechnology*, **31**(7), 1079-1092.