Novel Research in Microbiology Journal (2023), 7(6): 2199-2209
Research Article

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/
DOI: 10.21608/nrmj.2023.325660

Quantification of thermo-halotolerant alkaline protease activity derived from *Bacillus licheniformis* strains isolated from extreme environments in Morocco

Taha Chouati[1,2]; Ounayssa Ayadi[1,3]; Mohammed Ajdig[1,4]; Lahcen Ouchari[1,4]; Bahia Rached[1,3]; Jamila El Alami[1]; Elmostafa EL Fahime[1,2*]

[1]Functional Genomic analysis platform and CCMM, National Center for Scientific and Technical Research (CNRST), Morocco; [2]Genopath Research Center, ERNN, Faculty of Medicine and Pharmacy, University Mohammed V of Rabat, Morocoo; [3]Laboratory of physical chemistry and biotechnologies of biomolecules and materials, University Hassan II Casablanca, FSTM, Mohammedia, Morocco; [4]Microbiology and Molecular Biology Team, Plant and Microbial Biotechnology, Biodiversity and Environment Center, Faculty of Sciences, Mohammed V University in Rabat, Morocco

[*]Corresponding author E-mail: m.elfahime@gmail.com

Received: 2 October, 2023; Accepted: 8 November, 2023; Published online: 11 November, 2023

Abstract

Proteases; especially alkaline proteases, constitute the most used group of enzymes in industry. The wide scope of their application requires varying properties; most commonly stability throughout the conditional changes that would occur during the various industrial processes. This study aimed to identify the potentially interesting bacterial strains obtained from Moroccan extreme environment, and evaluate their proteolytic activity under the various growing conditions. In this study, the impact of temperature and salinity on the alkaline protease activity (pH 11) of 33 *Bacillus* strains deposited in the Moroccan Coordinated Culture Collection, which originated from the various extreme environments from Morocco, was studied. Bacterial identification to the species level was performed using 16S rRNA gene sequencing and MultiLocus Sequence Typing (MLST), a technique that is much more precise and can identify the bacteria to the strain level. Strain B950 showed a relatively stable protease activity at the tested extreme conditions (*i.e.*, 130.8 U/ ml at 60 °C and 10 % NaCl). On the other hand, strain B961 displayed a better overall activity at either high temperature (140.8 U/ ml) or salinity (137 U/ ml); however, it was not as stable as when grown under both extreme conditions. Regarding identity of the bacterial strains, they were all representatives of *Bacillus licheniformis*; although B950 and B961 strains belonged to Sequence Types 3 and 5, respectively.

Keywords: Alkaline protease, *Bacillus licheniformis*, Extremo-tolerance, MultiLocus sequence typing scheme

1. Introduction

Proteases are hydrolytic enzymes that digest proteins by cleaving the peptide bonds among the amino acids. Their role in proteolysis makes them useful for a wide variety of industries such as waste management; food processing, and silver recovery (Zhu *et al*., 2019; Lin *et al*., 2022; Sujitha and Shanthi, 2023) Thus, they are commercially important industrial enzymes representing roughly 60 % of the total enzyme production; where 35 % of them are alkaline proteases (Hasan *et al*., 2022).

Alkaline proteases are enzymes of significant industrial importance due to their activity under various pH values that range from neutral to basic. Their structural stability; particularly their ability to endure extreme conditions such as high temperatures (Chauhan *et al*., 2021; Talhi *et al*., 2022) or salinity (Ugbede *et al*., 2023), is a key focus in enzyme research for industrial applications. They present a solution for several industrial processes that would normally denature the normal proteolytic enzymes, such as the detergent and leather production industries (Sellami-Kamoun *et al*., 2008; Hasan *et al*., 2022).

The proteases are found in plants; animals, and microbes (Barrett and McDonald, 1986). The latter are a much preferred source for the industrial protease production due to several characteristics; notably their ease of culture coupled with a high production yield, in addition to a relatively simpler genetic manipulation challenge (Lv *et al*., 2023). Although the mold fungi (Hashmi *et al*., 2022) and yeast fungi (De Brabander *et al*., 2023) can be used for protease production; however, the bacteria; especially members of *Bacillus* taxa, are the major producers of this enzymes, due to the multitude of novel enzymes they produce (Pham *et al*., 2019; Sedaghat *et al*., 2022).

Bacillus genus is highly versatile both phenotypically end genetically, which led to its frequent reclassifications, such as the case of *B. subtilis* group (Deng *et al*., 2023). *B. licheniformis* is a Gram (+); facultative aero-anaerobic, endospore forming bacterium. This bacterium is ubiquitous in the environment but is mainly found in the soil. Furthermore, *B. licheniformis* possesses the GRAS (Generally Regarded As Safe) status (de Boer *et al*., 1994), which makes *B. licheniformis* an ideal bacterium for research for the potentially useful enzymes (He *et al*., 2023). Furthermore, many strains of this species show interesting growth capabilities under the diverse conditions, which may reflect their enzymatic activities (Caulier *et al*., 2019).

The objectives of this study were to evaluate the thermal and halogenic stability of the alkaline protease activity of 33 *Bacillus* strains, and propose an updated identification for the interesting strains using 16S rRNA gene sequencing and MultiLocus sequence typing (MLST) scheme.

2. Material and methods

2.1. Strains origin and culture

This study concerned with 33 *Bacillus* strains that were deposited in the Moroccan Coordinated Collection of Microorganisms (CCMM). They were previously isolated from various extreme environments in Morocco and were identified as *B. aerius* (Aanniz *et al*., 2015). However, they were reclassified as *Bacillus* spp. in regard to the doubt over *B. aerius* classification (Dunlap, 2015). All these strains were revivified from cryo-conserved tubes containing 20 % glycerol. Fresh 24 h cultures of *Bacillus* spp. grown at 37 °C on Tryptone Soy Agar and Broth (TSA and TSB, respectively) (BIORAD, USA, CA), were prepared before each assay. Prior to this study, all *Bacillus* strains were subjected also to routine microbiologic assays; Gram staining, and detection of oxidase and catalase activities.

2.2. Protease production under different culture conditions

Skim milk agar (in g/ l; Skim milk powder: 28, casein enzymatic hydrolysate: 5, Yeast extract: 2.5, Dextrose: 1, and agar 15) was prepared with different pH values (6 - 10) using 1N NaOH. The selected strains were inoculated individually at each pH value, incubated at 37 °C and 55 °C, and monitored for 3 d. The protease activity of each bacterial strain was revealed by the formation of a clear zone around the cultures (Sulaiman *et al*., 2019). For each *Bacillus* strain, the size of the halos was measured and compared; those showing a clear zone equal or exceeding 8 mm in diameter after 3 d of incubation were selected for further assays.

2.3. Crude enzyme extraction

For crude protease enzyme extraction, the previously selected *Bacillus* strains were cultured individually in 15 ml TSB at 37 °C. After 24 h of incubation, the optical density (OD) of the obtained cultures was measured at 600 nm using EPOCH2 spectrophotometer (Agilent Technologies, CA, USA), to ensure that the growth was similar. Approximately, 2 ml of each broth culture was centrifuged at 12 000 x g for 10 min. at 4 °C. This process was repeated successively three times for each strain under the same conditions. Finally, the supernatants were filtered individually through a 0.22 µm Millipore filter (Millex GP, Germany), and the resulting filtrate was considered as the crude enzyme extract, which was stored at 4 °C, in reference to the previous methodology conducted by Liu *et al*., (2013).

2.4. *In vitro* estimation of the crude enzyme activity under different conditions

Crude enzyme activity was estimated *in vitro* using agar well diffusion assay (Ramadhan *et al*., 2021). SMA Petri plates were prepared and their pH values were adjusted to 9 and 10 using 1N NaOH solution (Sigma-aldrich, Germany). Using a sterile cork borer, 8 mm diameter wells were cut aseptically in the agar surface and 8 µl of the crude enzyme was inoculated in each well. The plates were incubated at 37 °C and 55 °C for each strain and monitored for 3 d. For each bacterial strain, the crude enzyme extract was prepared, this assay was performed in triplicates, and the mean diameter of the halo zone formed due to casein hydrolysis was measured using a calibrated ruler. The tested protease enzymes producing mean halo zone diameters of 5 mm or more were selected for further study.

2.5. Quantitative estimation of the crude enzyme activity

The crude enzyme activity of each tested *Bacillus* strain was measured quantitatively using a non-specific protease activity assay following the standard protocol reported by Cupp-Enyard, (2008). Briefly, 400 µl of each crude enzyme extract was added to 400 µl of casein solution (10 g/ l) as a substrate and incubated for 10 min. at the tested temperature. The reaction was stopped by adding 800 µl of TriChloro-Acetic acid (TCA). Afterwards, 200 µl of the reaction mixture was dispatched on a microplate, and 1 ml of Na_2CO_3 solution was added, followed by 200 µl of Folin's reagent. After 20 min. of incubation at 40°C, the produced L-tyrosine was estimated spectrophotometrically through measurement of the OD at 680 nm using EPOCH2 spectrophotometer (Agilent Technologies, CA, USA). The data was compiled; plotted using tyrosine as a standard, and finally the quantitative enzymatic activity was estimated. The assay was carried out at pH 11 under different conditions; mainly two incubation temperatures (55 and 60 °C) and two NaCl concentrations (0 % and 10 %) were tested for the casein solution (substrate). This led to obtaining four sets of conditions, including 55 °C and 0 % NaCl; 60 °C and 0 % NaCl; 55 °C and 10 % NaCl; 60 °C and 10 % NaCl).

2.6. Identification of the tested *Bacillus* strains

The bacterial DNA of each *Bacillus* sp. was extracted using MAGPURIX robot and its quality was assessed using Nanodrop 8000 (Thermo-fisher Scientific, MA, USA). The DreamTaq kit was used to amplify a total of 7 fragments according to the

recommended manufacturer's instructions (Thermo-Fisher Scientific, MA, USA). The primers used for 16S rRNA gene sequencing were; fd4 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3'), as proposed by Weisburg *et al*., (1991); with an annealing temperature of 52 °C. The PCR products' quality was then assessed using gel electrophoresis on a 1 % agarose gel. For sequencing of the amplified DNA, the PCR products were purified using the EXOSAP-IT kit according to the manufacturer's instructions. The sequence reaction was carried out using the Big Dye Kit V3.1 following 25 cycles program of 96 °C for 1 min.; 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. This was then followed by a second purification using a no-wash Ethanol-EDTA precipitation method (Fujikura, 2015). Finally, the 3130XL Genetic analyzer was used for sequencing (Applied BioSystems, CA, USA).

2.7. MultiLocus sequence typing and phylogenetic analysis

The multiLocus sequence typing (MLST) is a method that uses the sequences of different house-keeping genes in order to provide a strain typing. This technique is precise concerning strain differences; however, it can only be applied to specific and/ or close species. For *B. licheniformis*, the followed MLST scheme relied on six house-keeping genes shown in Table (1), following the previous method conducted by Madslien *et al*., (2012). The process of amplifying and sequencing of each gene was conducted in a manner consistent with the methodology used in the previous subtitle. The sequences of the six genes were then modified and assembled using the UGENE software solution, followed by sequence alignment using MEGA XI. Following analysis of all sequences in the PubMLST database, the identification of alleles was conducted, and then the sequence types (ST) for each strain was retrieved.

2.8. Statistical analysis

The significance of the difference between the replicates was determined using a two-way ANOVA according to the studied parameters. A significant difference was considered only when those parameters had a significance level at $p < 0.05$.

3. Results

3.1. Screening for protease activity

The clear zone formed around the *Bacillus* cultures varied greatly. This preliminary screening revealed that 18 out of the 33 *Bacillus* strains displayed a halo zone of 8 mm diameter or more under the normal culture conditions (37 °C; pH 7). However, 11 *Bacillus* strains showed consistent results at the other tested conditions (37 °C, pH10 and 55 °C; pH10). These strains were selected for further tests.

3.2. Crude enzyme extract activity

Among the 11 *Bacillus* strains that were selected, a total of 7 strains exhibited *in vitro* encouraging outcomes, as indicated by the presence of a degrading halo zone exceeding 5 mm in diameter. These promising results were deduced either due to the substantial diameter of the halo zone displayed by the *Bacillus* strains and/ or their consistent manifestation across the different tested culture conditions. A summary of these findings is presented in Table (2). A two-way analysis of variance (ANOVA) was performed; indicating a statistically significant interaction between the temperature and pH in relation to the halo zone diameter, where F (3-10) = 6.09, p = 0.05.

3.3. Quantitative estimation of protease activity

The majority of *Bacillus* strains exhibited notable protease enzyme efficacy at pH 11 and at a temperature of 55 °C; however, B950 and B961 strains demonstrated superior performance compared with the other strains. When the temperature was increased to 60 °C or the concentration of NaCl was elevated to 10 %, the enzyme activity of the majority of the *Bacillus* strains decreased to differing extents; with the

Table 1: Details about the primer sets (*i.e*., forward and reverse) used for amplification of the target regions of 6 house-keeping genes used for MLST scheme

Gene	Name	Forward primer 5'-3' sequence	Reverse primer 5'-3' sequence	Temp. (ºC)
adk	Adenylate kinase	GGT AAA GGG ACA CAG GCT GA	TCG AGT AAA GGC TGG GTT TG	58
ccpa	Transcriptional regulator	TAT GAT GTA GCA CGC GAA GC	TAT CCC CAA GCG CTC TTT TA	58
recF	Recombination protein F	ACG GTT CTG TTC CCA TTC AG	CAT CAC GGC CAT TGA CAT AG	58
sucC	Succinyl-CoA synthetase, subunit beta	GGG TCC CGA CGG CCA ACA AA G	GGC CGG TTC CCC TCC GTA GT	58
rpoB	DNA-directed RNA polymerase, subunit beta	AGG TCA ACT AGT TCA GTA TGG ACG	AAG AAC CGT AAC CGG CAA CTT	58
Spo0A	Transcriptional regulator	GAA GTG CTT GGT GTC GCA TA	TGT GTA GCC GAA AAG TGA CG	58

Where; the table contains the names of the genes; their abbreviations, the forward and reverse sequences, as well as the annealing temperature

Table 2: Halo diameters (mm) for each selected Bacillus strain after 72 h incubation at 37 °C with pH 9 and pH 10, as well as at 55°C with pH 9 and pH 10

Temp. (C °)	**37 °C**		**55 °C**	
pH	9	10	9	10
Strain no.	Halo diameter (mm)	Halo diameter (mm)	Halo diameter (mm)	Halo diameter (mm)
B956	4.7 ± 2.1	1,7 ± 1.7	5.0 ± 1.4	5.0 ± 0.8
B958	7.0 ± 0.8	6.0 ± 1.4	6.0 ± 1.6	3.0 ± 2.2
B959	3.3 ± 2.6	2.0 ± 0.8	4.0 ± 2.4	3.7 ± 0.9
B967	7.0 ± 1.4	7.0 ± 1.6	6.3 ± 2.1	2.0 ± 0.8
B773	5.0 ± 2.2	4.7 ± 2.1	5.7 ± 1.7	2.0 ± 2.2
B780	5.0 ± 0.8	2.0 ± 1.4	3.0 ± 2.2	2.0 ± 0.8
B814	5.0 ± 2.2	4.7 ± 1.9	4.0 ± 1.6	2.7 ± 2.1
B821	5.0 ± 1.6	6.0 ± 1.6	5.0 ± 1.6	5.0 ± 2.2
B950	7.0 ± 1.4	7.3 ± 0.9	7.0 ± 1.4	4.0 ± 1.6
B961	6.0 ± 0.8	8.0 ± 2.2	7.0 ± 1.6	2.7 ± 1.2
B963	3.0 ± 1.4	3.0 ± 2.2	4.0 ± 2.8	4.3 ± 2.1

Where; each value represents the mean of three replicates; with the error margins (±) represented in the column. Names in bold represent the strains that produced halos of 5 mm or more at 55°C; regardless of the pH value

exception of B956 and B961, which displayed an increase and little variance in the enzyme activity, respectively. The obtained results presented in Fig. (1) demonstrated that the tested experimental conditions including a pH of 11, 10 % NaCl, and a temperature of 60 °C, had a detrimental impact on the enzyme activity of all *Bacillus* strains; with the exception of B956 and B950, which unexpectedly displayed improved outcomes. All these results were statistically significant ($p < 0.05$).

3.4. 16S rRNA identification of the selected *Bacillus* strains

The genomic DNA from the selected 7 Bacillus strains was extracted, and the 16S rRNA gene sequence as well as the 6 additional housekeeping genes were amplified using PCR. The results of the 16S rRNA gene sequencing showed that all strains belong to *B. licheniformis* and their accession numbers were assigned, as summarized in Table (3).

3.5. MultiLocus sequence typing

The MLST analysis showed that the 7 *Bacillus* strains did not share the same sequence type (ST). B950 was from ST 3 while B961 was from ST 5. However, both were confirmed to belong to *B. licheniformis*.

4. Discussion

The search for bacterial proteases is a well-documented and a broadly studied subject; owing to their multiple uses and ease of production. From the industrial point of view, the production of stable enzymes that can withstand multiple extreme conditions is even more interesting. Therefore, the screening and identification reported in this study aimed to search for the potentially interesting *Bacillus* strains originating from regions that were poorly or insufficiently studied.

As demonstrated in Fig (1), the *Bacillus* strains B950 and B961 represented clear standouts under all the tested conditions. B961, specifically; showed the best yield across normal conditions (*i.e*., 55 °C and 0 % NaCl) and displayed slightly better results at 60 °C and 10 % NaCl alone. However, this strain showed a marked decline in activity when tested at both 60 °C and 10 % NaCl, in agreement with the previously

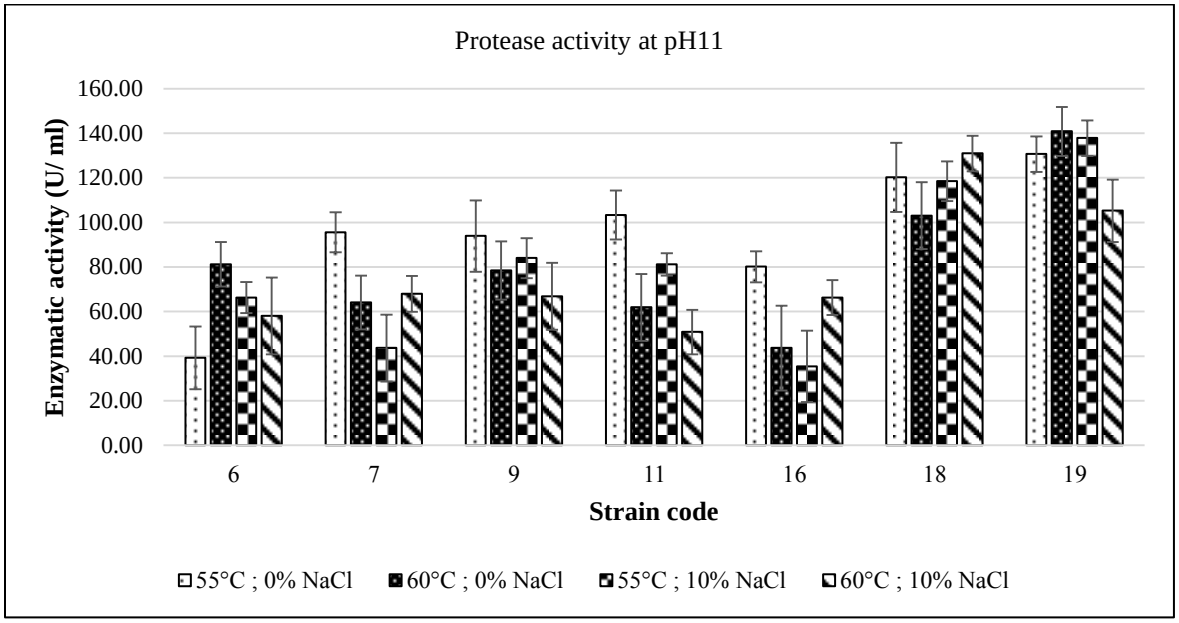

Fig. 1: Protease activity (U/ ml) of crude protease enzyme extract at pH 11 for each selected *Bacillus* strain under four different tested conditions, including 55 °C and 0 % NaCl, 60 °C and 0 % NaCl , 55 °C and 10 % NaCl, 60 °C and 10 % NaCl. The selected seven *Bacillus* strains were codified as follow; 6: B956, 7: B958, 9: B967, 11: B773, 16: B821, 18: B950, and 19: B961. The Error bars represent the standard deviation of the three replicates

Table 3: BLAST results of *Bacillus* strains identification through amplifying the 16S rRNA gene sequence, showing the selected Bacillus strains number, their size, species identification, identity percentage, and their assigned accession numbers

Strains	Size (bp)	Closest relative	Identity (%)	Accession number
B956	1484	*Bacillus licheniformis*	99.93	OR512954
B958	1484	*Bacillus licheniformis*	99.87	OR512955
B967	1470	*Bacillus licheniformis*	99.80	OR512957
B773	1484	*Bacillus licheniformis*	99.26	OR512959
B821	1432	*Bacillus licheniformis*	99.58	OR512963
B950	1482	*Bacillus licheniformis*	99.87	OR512966
B961	1484	*Bacillus licheniformis*	99.87	OR512967

recorded *Bacillus* strains, such as *B. megaterium* (Manavalan *et al*., 2020) and *B. amyloliquefaciens* (Hashmi *et al*., 2022), which had alkali-thermotolerant activity.

Interestingly, strains B958, B821, and B950 showed better results at 60 °C and 10 % NaCl than in the presence of either condition alone. Similarly, the previous study reported by Dammak *et al*., (2016) presented a *Halorubrum ezzemoulense* strain that had a thermostable halo-alkaline protease activity, and was isolated form solar saltern in Tunisia. However, the low yield for both *Bacillus* strains; mainly B958 and B821 (67.94 and 66.29 U/ ml, respectively) made them poor candidates for further uses. Meanwhile, B950 surprisingly showed the best results of protease enzyme activity at 60 °C and 10 %; expressing a better stability under these conditions.

The tolerance and stability exhibited by B950 and to a lesser extent by B961 are rarely reported among the *B. licheniformis* representatives, although these species otherwise had good output as enzyme producers (Díaz-Cornejo *et al*., 2023). However, a previous study reported by Asitok *et al*., (2022) observed that the ability of a bacterial strain to withstand multiple extreme conditions, such as high pH, temperature, and salinity is crucial; as the enzyme yield can be reasonably optimized.

In this study, molecular identification of the 7 *Bacillus* strains through 16S rRNA gene amplification showed that they all belong to *B. licheniformis*. However, this is mitigated by the high genetic closeness of *B. licheniformis* and *B. paralicheniformis* (Dunlap *et al*., 2015), in addition to the fact that only *B. licheniformis* strain possesses the previously mentioned "Generally Regarded As Safe" status, which means that it poses no threats to the human health (Zeigler, 2005). Different methods have been proposed for differentiation between *B. licheniformis* and *B. paralicheniformis*; resulting in mixed results (Jeong *et al*., 2018; Olajide *et al*., 2021). Although MLST scheme has been designed for ST determination; however, it can be a robust tool for species identification (Madslien *et al*., 2012). While the use of such scheme helped us to identify the strains of B950 and B961 as *B. licheniformis*; however, it should be mentioned that this method uses a combination of 6 house-keeping genes, which makes it much more time and resource consuming; especially if being used for large numbers of bacterial strains.

Conclusion

This research identified *Bacillus licheniformis* strains that can tolerate high temperature, salt, and pH from the Moroccan Coordinated Collection of Microorganisms. Best-performing proteases were dosed using a modified Lowry technique. Using 16S rRNA gene sequencing and MLST, the intriguing bacteria were identified as *B. licheniformis*. B950 demonstrated high stability at pH 11, 60 °C, and 10 % NaCl, which made it a prime candidate for further study, while B961 demonstrated greater yield under alkaline circumstances with high temperature (60 °C) or salt concentration (10 %), but not both.

Acknowledgments

The authors acknowledge all members of the Coordinated Collections of Moroccan Microorganisms (CCMM) for supporting this research work.

Conflict of interests

The authors declare no conflicts of interest among them.

Funding source

This work did not receive a special funding; however, it was conducted in accordance with the working budget of the CCMM.

Ethical approval

Non-applicable.

Author's Contributions

Conceptualization, T.C., J.E. and E.E.; Data curation, T.C., O.A. and M.A.; Formal analysis, B.R. and L.O.; Investigation, T.C., O.A. and M.A.; Methodology, T.C, O.A. and M.A.; Project administration, B.R., L.O. and J.E.; Software, M.A.; Resources, L.O.; Supervision, J.E. and E.E.; Visualization, T.C, J.E and E.E.; Validation, L.O., B.R. and E.E.; Roles/Writing - original draft, T.C. and O.A.; Writing/ reviewing and editing, B.R. and E.E.

5. References

Aanniz, T.; Ouadghiri, M.; Melloul, M.; Swings, J.; Elfahime, E.; Ibijbijen, J. et al. (2015). Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. Brazilian Journal of Microbiology. 46(2): 443-453. https://doi.org/10.1590/S1517-838246220140219

Asitok, A.M.; Ekpenyong, I.; Takon, S.; Antai, N.; Ogarekpe, R.; Antigha, P. et al.; (2022). Overproduction of a thermo-stable halo-alkaline protease on agro-waste-based optimized medium through alternate combinatorial random mutagenesis of *Stenotrophomonas acidaminiphila*. Biotechnology Reports 35:e00746. https://doi.org/10.1016/j.btre.2022.e00746

Barrett, A.J. and McDonald, J.K. (1986). Nomenclature: Protease, proteinase and peptidase. The Biochemical Journal. 237(3): 935. https://doi.org/10.1042/bj2370935

Caulier, S.; Nannan, C.; Gillis, A.; Licciardi, F.; Bragard, C. and Mahillon, J. (2019). Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. Frontiers in Microbiology. 10: 302. https://doi.org/10.3389/fmicb.2019.00302

Chauhan, J.V.; Mathukiya, R.P.; Singh, S.P. and Gohel, S.D. (2021). Two steps purification, biochemical characterization, thermodynamics and structure elucidation of thermostable alkaline serine protease from *Nocardiopsis alba* strain OM-5. International Journal of Biological Macromolecules. 169: 39-50. https://doi.org/10.1016/j.ijbiomac.2020.12.061

Cupp-Enyard, C. (2008). Sigma's Non-specific Protease Activity Assay-Casein as a Substrate. Journal of Visualized Experiments. 19: 899. https://doi.org/10.3791/899

Dammak, D.F.; Smaoui, S.M.; Ghanmi, F.; Boujelben, I. and Maalej, S. (2016). Characterization of halo-alkaline and thermostable protease from *Halorubrum ezzemoulense* strain ETR14 isolated from Sfax solar saltern in Tunisia. Journal of Basic Microbiology 56(4): 337–346. https://doi.org/10.1002/jobm.201500475

de Boer, A.S.; Priest, F. and Diderichsen, B. (1994). On the industrial use of *Bacillus licheniformis*: A review. Applied Microbiology and Biotechnology. 40(5): 595–598. https://doi.org/10.1007/BF00173313

De Brabander, P.; Uitterhaegen, E.; Delmulle, T.; De Winter, K. and Soetaert, W. (2023). Challenges and progress towards industrial recombinant protein production in yeasts: A review. Biotechnology Advances. 64: 108121. https://doi.org/10.1016/j.biotechadv.2023.108121

Deng, F.; Chen, Y.; Xiao, H.; Yao, C.; Chen, J.; Zhao, Y. et al. (2023). *Bacillus pfraonensis* sp. Nov., a new strain isolated from a probiotic feed additive with low cytotoxicity and antimicrobial activity. Research in Microbiology. 174(7): 104090. https://doi.org/10.1016/j.resmic.2023.104090

Díaz-Cornejo, S.; Otero, M.C.; Banerjee, A. and Gordillo-Fuenzalida, F. (2023). Biological properties of exopolysaccharides produced by *Bacillus* spp. Microbiological Research. 268: 127276. https://doi.org/10.1016/j.micres.2022.127276

Dunlap, C.A. (2015). The status of the species *Bacillus aerius*. Request for an Opinion. International

Journal of Systematic and Evolutionary Microbiology. 65(7): 2341. https://doi.org/10.1099/ijs.0.000271

Dunlap, C.A.; Kwon, S.W.; Rooney, A.P. and Kim, S.J. (2015). *Bacillus paralicheniformis* sp. Nov., isolated from fermented soybean paste. International Journal of Systematic and Evolutionary Microbiology. 65(10): 3487–3492. https://doi.org/10.1099/ijsem.0.000441

Fujikura, K. (2015). No-wash ethanol precipitation of dye-labeled reaction products improves DNA sequencing reads. Analytical Biochemistry. 468: 39–41. https://doi.org/10.1016/j.ab.2014.09.010

Hasan, M.J.; Haque, P. and Rahman, M.M. (2022). Protease enzyme based cleaner leather processing: A review. Journal of Cleaner Production. 365: 132826. https://doi.org/10.1016/j.jclepro.2022.132826

Hashmi, S.; Iqbal, S.; Ahmed, I. and Janjua, H.A. (2022). Production, Optimization, and Partial Purification of Alkali-Thermotolerant Proteases from Newly Isolated *Bacillus subtilis* S1 and *Bacillus amyloliquefaciens* KSM12. Processes. 10(6): 6. https://doi.org/10.3390/pr10061050

He, H.; Yu, Q.; Ding, Z.; Zhang, L.; Shi, G. and Li, Y. (2023). Biotechnological and food synthetic biology potential of platform strain: *Bacillus licheniformis*. Synthetic and Systems Biotechnology. 8(2): 281–291. https://doi.org/10.1016/j.synbio.2023.03.008

Jeong, D.W.; Lee, B.; Lee, H.; Jeong, K.; Jang, M. and Lee, J.H. (2018). Urease Characteristics and Phylogenetic Status of *Bacillus paralicheniformis*. Journal of Microbiology and Biotechnology. 28(12): 1992–1998. https://doi.org/10.4014/jmb.1809.09030

Lin, H.; Zhou, B.; Zhao, J.; Liao, S.; Han, J.; Fang, J. et al. (2022). Insight into the protein degradation during the broad bean fermentation process. Food Science & Nutrition. 10(8): 2760-2772. https://doi.org/10.1002/fsn3.2879

Liu, Q.; Sun, S.; Piao, M. and Yang, J.Y. (2013). Purification and Characterization of a Protease Produced by a *Planomicrobium* sp. L-2 from Gut of *Octopus vulgaris*. Preventive Nutrition and Food Science. 18(4): 273–279. https://doi.org/10.3746/pnf.2013.18.4.273

Lv, X.; Li, Y.; Xiu, X.; Liao, C.; Xu, Y.; Liu, Y. et al. (2023). CRISPR genetic toolkits of classical food microorganisms: Current state and future prospects. Biotechnology Advances. 69: 108261. https://doi.org/10.1016/j.biotechadv.2023.108261

Madslien, E.H.; Olsen, J.S.; Granum, P.E. and Blatny, J.M. (2012). Genotyping of *B. licheniformis* based on a novel multi-locus sequence typing (MLST) scheme. BMC Microbiology. 12: 230. https://doi.org/10.1186/1471-2180-12-230

Manavalan, T.; Manavalan, A.; Ramachandran, S. and Heese, K. (2020). Identification of a Novel Thermostable Alkaline Protease from *Bacillus megaterium-TK1* for the Detergent and Leather Industry. Biology. 9(12): 472. https://doi.org/10.3390/biology9120472

Olajide, A.M.; Chen, S. and LaPointe, G. (2021). Markers to Rapidly Distinguish *Bacillus paralicheniformis* From the Very Close Relative, *Bacillus licheniformis*. Frontiers in Microbiology. 11(11): 596828. https://doi.org/10.3389/fmicb.2020.596828

Pham, J.V.; Yilma, M.A.; Feliz, A.; Majid, M.T.; Maffetone, N.; Walker, J.R. et al. (2019). A Review of the Microbial Production of Bioactive Natural Products and Biologics. Frontiers in Microbiology. 10: 1404. https://doi.org/10.3389/fmicb.2019.01404

Ramadhan, A.R.; Bachruddin, Z.; Widodo; Erwanto, Y. and Hanim, C. (2021). Isolation and selection of proteolytic lactic acid bacteria from colostrum of dairy cattle. IOP Conference Series: Earth and Environmental Science. 788: 012077. https://doi.org/10.1088/1755-1315/788/1/012077

Sedaghat, S.; Yazdi, F.T.; Mortazavi, A. and Shahidi, F. (2022). Enhancement of alkaline protease production of *Bacillus* strains isolated from dairy sludge under cold, salt and ultrasound stress. International Dairy Journal. 129: 105335. https://doi.org/10.1016/j.idairyj.2022.105335

Sellami-Kamoun, A.; Haddar, A.; Ali, N.E.H.; Ghorbel-Frikha, B.; Kanoun, S. and Nasri, M. (2008). Stability of thermostable alkaline protease from *Bacillus liche*niformis RP1 in commercial solid laundry detergent formulations. Microbiological Research. 163(3): 299–306. https://doi.org/10.1016/j.micres.2006.06.001

Sujitha, P. and Shanthi, C. (2023). Importance of enzyme specificity and stability for the application of proteases in greener industrial processing- a review. Journal of Cleaner Production. 425: 138915. https://doi.org/10.1016/j.jclepro.2023.138915

Sulaiman, N.A.; Mahadi, N.M. and Ramly, N.Z. (2019). Biochemical Properties and Potential Application of Proteases from Alkalophilic *Bacillus lehensis* G1. Tropical Life Sciences Research. 30(2): 2. https://doi.org/10.21315/tlsr2019.30.2.3

Talhi, I.; Dehimat, L.; Jaouani, A.; Cherfia, R.; Berkani, M.; Almomani, F. et al. (2022). Optimization of thermostable proteases production under agro-wastes solid-state fermentation by a new thermophilic *Mycothermus thermophilus* isolated from a hydrothermal spring Hammam Debagh, Algeria. Chemosphere. 286(1): 131479. https://doi.org/10.1016/j.chemosphere.2021.131479

Ugbede, A.S.; Abioye, O.P.; Aransiola, S.A.; Oyewole, O.A.; Maddela, N.R. and Prasad, R. (2023). Production, optimization and partial purification of bacterial and fungal proteases for animal skin dehairing: A sustainable development in leather-making process. Bioresource Technology Reports. 24: 101632. https://doi.org/10.1016/j.biteb.2023.101632

Weisburg, W.G.; Barns, S.M.; Pelletier, D.A. and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 173(2): 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Zeigler, D.R. (2005). Application of a *recN* sequence similarity analysis to the identification of species within the bacterial genus *Geobacillus*. International Journal of Systematic and Evolutionary Microbiology. 55(3): 1171-1179. https://doi.org/10.1099/ijs.0.63452-0

Zhu, X.; Li, Y.; Yang, G.; Lv, M. and Zhang, L. (2019). Covalent immobilization of alkaline proteinase on amino-functionalized magnetic nanoparticles and application in soy protein hydrolysis. Biotechnology Progress. 35(2): e2756. https://doi.org/10.1002/btpr.2756