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Isolation and Characterization of Microorganisms for Protease Production from Soil Samples from Kosovo and Testing the Enzymes in Food Industry Application

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Abstract

Soil samples from different locations in Kosovo were screened for bacteria suitable for high-level production of various proteases. 5 isolates were found and selected to be promising candidates and further characterized by biochemical and morphological assays as well as 16S RNA sequencing and identified as *Bacillus* spp. The isolates were used for protease production via submerged fermentation. The produced enzymes were tested for different food industry applications, like meat, beer, milk, and feather degradation. The highest protease activity achieved was 0.63U mL-1 at 37ºC pH 4.7 from strain S10-1 which showed high potential for meat industry application.

Keywords: Bacillus spp.; Enzymes; Fermentation; Industrial biotechnology

1 Introduction

Enzymes are functional proteins, responsible for governing biochemical reactions in living cells by e.g. lowering the activation energy in biochemical reactions (1). These can selectively and effectively convert several substrates molecules to products even within complex matrix. Typically produced by fermentation and extracted from living cells, enzymes are biodegradable compounds making them also environmentally benign (2). Enzymes are used in many industries like pharmaceutical (3), detergent (4), leather (5), and food industry (6). Chief among the commercial enzymes are proteases, which hydrolyze peptide bonds within proteins. These are widely used, with over 60% of the enzyme market (7) and growing at a compound annual growth rate of 5.3% of global demand during 2014-2019 (6) and is expected to grow further (8).

Proteases are heavily used in food industry e.g. meat processing applications, where the focus is on providing tools to prepare ready-to-use, high-quality, tender meat and meat products. Meat tenderness is a function of the fibrous nature of the muscle among them myofibrils are responsible for meat toughness (9). Conventionally, aging the carcasses, mechanical tenderization, elevated-temperature storage, electrical stimulation, calcium chloride injection, are some of the techniques used by slaughterhouses to increases tenderness of meat. Alternative to these, enzymatic treatments provide affordable, less energy intensive and faster solution for the meat processing applications by hydrolyzing myofibrils due to proteolysis (2, 10).

For the beverage industry, proteases are used typically for clarification of wine, beer or juices, often in combination with other enzymes (11, 12). In particular for beer, haze in beer usually and mainly results from a reaction between beer proteins from grains (barley or wheat) and polyphenols, responsible for 40-75% of the beer haziness (13). The concentration of only 2mg L-1 of protein in beer causes haze formation. Enzymatic protein hydrolysis is one of the possibilities to enrich the beer with nutrients and prevent haze formation thereby improving organoleptic properties. In dairy industry proteases are mainly used in milk coagulation process by cleavage the peptide bond of κ-casein Phe105-Met106. Coagulated milk is destined for different type of cheese making. Lately, the effect of proteases in cheese ripening process and indication in texture and flavor of the cheese was reported (14, 15).

Proteases find application in treatment of byproduct from food industry. Poultry is one of these industries, where wastes are increasing every day with increment in the demand for poultry products. Feathers contain 91% keratin, which is an insoluble structural protein and is found in β-sheets form. Their

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biodegradation is challenging due to extensive disulfide bonds and cross-links, hydrogen bonds and hydrophobic interactions (16). Conventional methods include incineration, (excessive) use of alkaline solutions or burying. Proteolytic activity of enzymes provide environmentally benign solution to feather processing.

Ubiquitous in nature, proteases are industrially produced especially by animals, plants, and microorganisms. In particular, microorganisms are used due to available cultivation practice in large quantities in a short time as well as high and stable activity of the enzyme produced (17,18,19). Among those, bacteria are preferred organisms, since these typically grow and produce at higher rates, and have large substrate portfolio (20). Additionally, bacterial enzymes especially from *Bacillus* spp. show higher thermal stability than fungal counterparts (21). Furthermore, genome of bacteria is typically 2-5Mb, significantly smaller than the genome of fungi around 8-17Mb (22, 23, 24), rendering molecular characterization or manipulation easier to carry. Enzyme production using genetically modified organisms (GMOs) are also common practice due to available molecular biology toolbox, possibility of tailor-made products as well as well-established fermentation practice for known industrial workhorses, reaching high product titers (25,26). However, the risk for unpredicted outcome of transformation exists, additional DNA fragments inserted with the gene of interest or gene sequences rearrangement and deletion (27, 28). Owing to the highly regulated market rules for foods with GMO ingredients, the food industry endeavors to avoid using GMO technology if wild type strains are available (29). Following that, several studies have been performed for the isolation of protease production microorganisms from natural sources in an attempt to get production strains as well as to exploit natural diversity for enzymes of various functional, desired properties. Typically, extreme environments are explored to isolate microorganisms, including hot spring waters, high altitude areas, arable land, industrially polluted areas, etc.

Kosovo is located in southeastern Europe, with predominantly continental climate influenced also by Mediterranean and Alpine climate. The country possesses diverse territory surrounded by mountains, arable fields, rich areas with thermal hot water springs, and mines with high deposit of lead, zinc, silver, nickel, and cobalt. On top of it, it is the third largest lignite reserve in Europe. To our knowledge this region is not that much explored for its microbial ecology, hence the aim of this research is to screen soil samples from different environments in Kosovo for bacteria with ability to produce protease extracellularly, and examine their application in different food industries.

2 Materials and methods

2.1 Isolation and selection

Soil samples from different locations corresponding to different environments (high altitude, heavy metal contaminated area, hot spring water, food industry zones) in Kosovo (Fig. 1) were collected and stored at +4ºC until further screening was performed. During the sampling, all used equipment were sterilized beforehand.

To isolate the microorganisms, one gram of soil sample was mixed with 9mL sterile 0.9% saline solution and incubated at 150rpm for 15min at room temperature. Out of this mixture, 100µL is further diluted and spread on the skim milk agar plates. The plates were incubated at 37ºC for 24h.

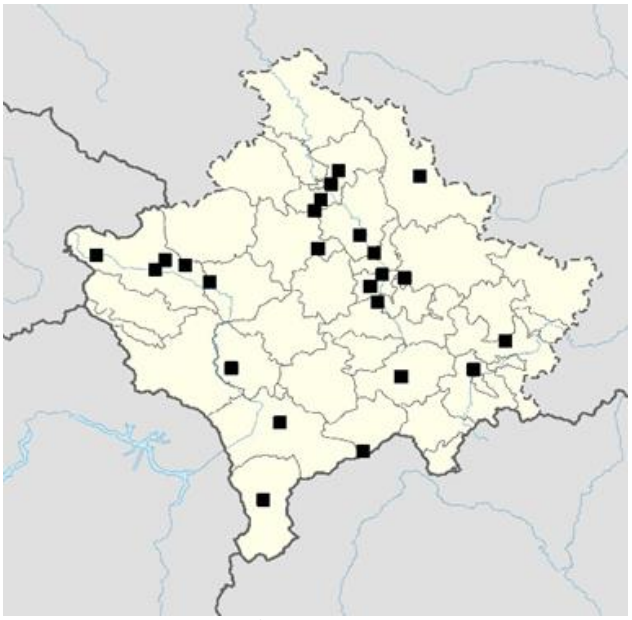


Figure.1: Map of soil sample points in Kosovo

Single colonies with surrounding clear zone around them were selected and streaked into fresh agar for further investigation (30, 31). All chemicals used are purchased from Sigma Aldrich or Merck. *Bacillus subtilis* 168 ATCC 23857 is used as reference microorganism.

2.2 Characterization of Bacillus spp. colonies Isolation and selection

Morphological and cultural characteristics were used to identify colonies. Each colony was examined using microscopy and rod shape cells identified as potential *Bacillus spp*. The Gram staining (Beveridge, 2001) (32) and catalase test (Taylor and Achanzar, 1972 (33) were used to further characterize the colonies. Semi-quantitative protease activity of each colony was measured in duplicate data, where colonies were inoculated with sterile needles on 5% skimmed milk agar. The radius of bacterial colony and radius of halo formed after 24h were measured using image processing software GIMP 2.10.12. The area of the halo around the culture was used to calculate protease index (Eq. 1).

$$PI = \frac{ATH}{AC} \quad (1)$$

where PI is the protease index, ATH is the area of total halo, AC the area of the bacterial colony. Colonies with large ATH and small AC are considered to be candidate host for further characterization using liquid culture.

2.3 Cultivation process and determination of protease activity

A single colony from an agar plate is grown for 18hr in 10mL nutrient broth in a 50mL falcon tube to be used as inoculum. The final fermentation is carried in YPD medium with salt g L^{-1}: peptone, 10; glucose, 5; yeast extract, 5.5; KH_2PO_4, 1; K_2HPO_4 $3H_2O$, 3.7; $MgSO_4.7H_2O$, 0.1; (34, 35). To assess the effect of temperature, two different incubation temperatures (30 and 37ºC) are used while the pH was kept at 7; and to assess the effect of

pH, the fermentations are carried at three different pH (5, 7 and 9) while the temperature was kept at 37ºC. The cultures are sampled 24h for growth (measured by optical density at 600nm OD600) and protease activity. Protease activity was measured by casein method (36), with 0.65% casein (w/v) in potassium phosphate buffer at pH 7 or sodium acetate buffer at pH 5. The assay conditions were set to 10 min at 37ºC and terminated using 10% trichloroacetic acid. The tyrosine released is determined using Folin's phenol reagent (36). One unit of protease activity was defined as the amount of enzyme liberating 1µmol of tyrosine per minute under defined assay conditions.

2.4 16S rRNA sequencing and analysing

Sequencing ribosomal RNA (rRNA) 16S is well-accepted method to identify bacteria, yielding species-specific signature (37, 38). Bacterial DNA from the liquid culture was extracted using commercial kit (high pure PCR template preparation kit, Roche) and was used as a template for PCR amplification of 16S rRNA sequence. PCR reaction consist of: 10X Taq buffer Thermo-scientific (100mM Tris-HCl, 500mM KCl, 0.8% (v v^{-1}) Nonidet P40), 25mM $MgCl_2$, dNTP 0.4mM, universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGATACCTTGTTACGACTT-3') each 0.4µM, Taq polymerase 0.05U μL^{-1} and the DNA template 100ng. The regime of the temperature used was, first denaturing at 95ºC for 3min, followed by 31 cycles of denaturation at 95ºC for 0.30min, annealing at 55ºC for 1min, extension at 72ºC for 1min, and a final extension step at 72ºC for 10 min, then storage temperature at 4ºC (39). The amplified 16S sequence was purified using the Macherey-Nagel PCR purification kit and sequenced. The forward and reverse sequences were assembled using BioEdit version 7.0.5.3. The resulting sequences were BLAST'ed against GeneBank Database for 16S ribosomal RNA sequences (Bacteria and Archaea).

2.5 Application of produced enzymes in the food industry

The enzymes are tested in various food industry applications, ranging from meat to beer processing to feather meal preparation. The details are explained in this section.

2.5.1 Breakdown of meat myofibrils

Myofibril extraction was performed with fresh cow muscle tissue by using extracting buffer (100 mM KCl, 20 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 1 mM sodium azide) at 2ºC based on the method described by of OLSON & al. (40). 1.95mL of extracted myofibril at pH 5.5 (approximately the pH of the red meat) was aliquoted in a 15mL falcon tube and assayed with 50µL crude enzyme extracts. The hydrolysis takes place in an orbital shaker 150rpm at 37ºC for 1h and terminated with 2mL of 15% trichloroacetic acid. The mixture was cooled at room temperature for 15minute and centrifuged at 1000 xG for 15min. Finally the optical density of the supernatant was determined at 280nm (41). The same experiment is repeated with reference enzyme (Protease from *Aspergillus oryzae* Sigma cat Nr: P6110), where 50µL of enzyme is used instead of crude enzyme, keeping all other conditions same.

2.5.2 Beer haze treatment

45mL of hazy beer was treated with 5mL of crude enzyme (or distilled water for blank), incubated at 25ºC for 18h. Pre-weighted filter paper 0.45µm were used to filtrate the treated beer. After two hours of drying at 65ºC filter paper was re-weighed and the difference is noted as hydrolysed protein (13). The same experiment is repeated with reference enzyme (Protease from *Aspergillus oryzae* Sigma cat Nr: P6110), where 5mL of enzyme is used instead of crude enzyme, keeping all other conditions same.

2.5.3 Milk-clotting activity

The milk-clotting activity was determined according to the method of Iwasaki (42): 0.1mL of the crude enzyme was mixed well with 1mL of skim milk solution (10g skim milk powder in 100mL of 0.01M $CaCl_2$ solution) and incubated at 37ºC for 1h and 30min. Each 10 min tubes were checked for clotting formation. Obtained coagulation time is used for calculating milk clotting units, where one unit of milk clotting activity was defined as the amount of enzyme capable of coagulating 1mL of milk for 40 min, Eq. (2).

$$\frac{MCU}{mL} = \frac{2400s}{(Time(s) * volume of the\ enzyme\ in\ mL)} \quad (2)$$

2.5.4 Enzymatic treatment of feathers

The effect of the culture on feather degradation from isolated colonies was examined. The method was based on the work of Kaya and Nikerel (43) with small changes. In a 50mL of 48h grown culture, 1.2g of washed and autoclaved feather was added and the mixture is incubated at 150rpm, 37ºC for 12h. After 12h content was filtrated using filter paper, dried and the difference in the dry feather considered as degraded by the enzyme.

3 Result and discussion

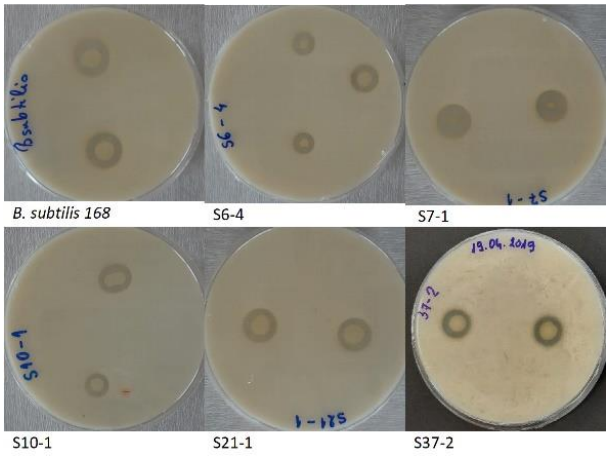
To isolate and identify *Bacillus* spp. capable of producing extracellularly protease, 102 soil samples from 34 different areas in Kosovo (Fig. 1) were screened. Out of initial sample pool, 66 single colonies were selected based on formation of clear halo in skim milk agar. The colonies were further identified as potential *Bacillus* spp. based on the form and color of colonies in nutrient agar, as well as based on Gram staining and catalase test as morphological tests. All identifications were compared with the reference strain *B. subtilis* 168. From the 66 colonies only 29 selected colonies resulted Gram-positive rod shape microorganisms and out of which, 28 colonies were catalase-positive (44). Remaining colonies were subjected to semi-quantitative protease activity measurement in skim milk agar, to select best extracellular protease producers.

Based on semi quantitative protease index results, five colonies were selected (Fig. 2). Protease index takes into account both the biomass formed and the proteolytic activity. S7-1 has small colonies, yet large halo resulting highest protease index, and despite S21-1 has about the same halo size, it also has largest biomass colony, therefore lowest protease index. Interestingly, protease index for S7-1 was 22 fold higher than the index of B. subtilis 168. These 5 colonies were further used for enzyme production by submerged fermentation, during which total DNA is isolated for final identification by sequencing genomic region encoding 16S rRNA.

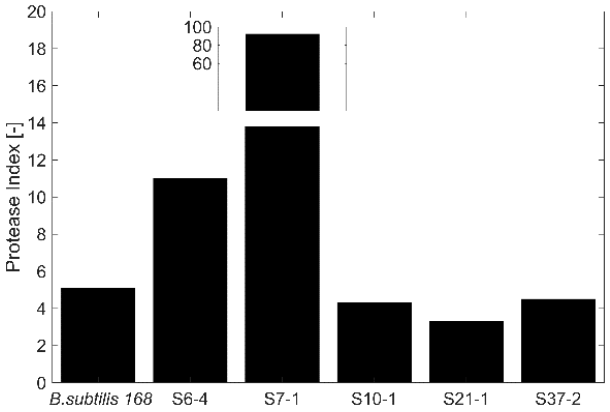
3.1 Identification by 16S sequencing

DNA samples were isolated and genomic regions encoding 16S rRNA were sequenced. Results sequences were BLAST'ed against 16S ribosomal RNA sequences (Bacteria and Archaea)

database in NCBI (Table 1), with all 0.0 e-value and percent identity ranging between 88-99%.



a)



b)

Figure 2: a) Selected colonies in 5% skim milk agar for extracellular protease expression, b) Protease index of each colony calculated by using the area of colony and the total halo formed. The protease index for S7-1 is much higher than the rest and is shown with a different axis

3.2 Enzyme production and activity

Selected species are cultivated at different pH and Temperatures. Overall, the highest growth and activity was obtained from S10-1 grown at pH4.7 at 37ºC with the highest protease activity 0.63U mL-1 Fig. 3b). Protease activity at low pH is interesting for food industry applications, where acid proteases are always in high demand (45). Albeit being uncommon, production of acid proteases by Bacillus spp. is reported (46), even though Bacillus spp. are commonly known for neutral and alkaline protease production (47). S10-1 was isolated from agriculture soil used as source of forage grass from Bajgora region at an altitude of around 1800m. Soil from this region exceed the limits for heavy metal like Pb, Cd, and Zn as reported by Kelmendi (48). Under mesophilic conditions (pH 7, 37ºC) the highest activities were observed with strains S37-2, and S6-4 with 0.54 and 0.3U mL-1, respectively (Fig. 3d). Neutral proteases produced by Bacillus spp. are important due to generation of less bitterness hydrolysates from proteins. This is significant a major factor for food industry applications in particular for beverage industry where enzyme application significantly effects organoleptic characteristics.

Table 1: BLAST results of the assembled sequences with NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database

S. name	Closest match	e-value	(%) Identity
S6-4	*B. pumilus* ATCC 7061	0.0	97.92
S7-1	*B. albus* MCCC 1A02146	0.0	89.29
S10-1	*B. cereus* ATCC 14579	0.0	88.23
S21-1	*Bacillus albus* strain BPB24 16S	0.0	97.9
S37-2	*B. mobilis* MCCC 1A05942	0.0	99.02

The organism S37-2 is isolated from walnut plantation soil in Rahove in north part of Kosovo and S6-4 is isolated from highly mineralized hot spring water at Kllokoti with 3.6g L-1 minerals reported by Kosovo Environmental Protection Agency (2010)(49). Lastly, contrary to our expectations and several reports in literature on alkaline protease production by *Bacillus* spp., isolated strains failed to grow and produce well at pH 9 and 37ºC (Fig. 3. f).

3.3 Myofibril hydrolysis

To mimic meat processing application, the crude extract from culture supernatants at two different time points were used for myofibril hydrolysis and compared with the reference protease. Among the tested cultures, sample at the 48th hour from S10-1 culture exhibited the highest myofibril hydrolysis, nearly 10% of the commercial enzyme, which is considered to be promising as crude extract ought to be further purified before final use (Fig. 4a). The sample at 24th hour from S7-1 exhibited lowest myofibril hydrolysis, possibly due to low biomass level.

3.4 Hazy beer treatment results

Here, the supernatant from five different cultivations, at two different times (24 and 48h) were used as crude extracts for treatment of beer haziness (Fig. 4b). The total amount of proteinaceous material in untreated beer used for experiment was estimated to be between 346 to 525 mg L-1, in agreement with literature (13). Sample from cultivation of strain S37-2 at 48h showed the highest beer clarification action, with 212 mg/L haze protein degradation (nearly 61% decrease in residue) compared with the result of commercial enzyme with 335mg L-1 haze protein degradation.

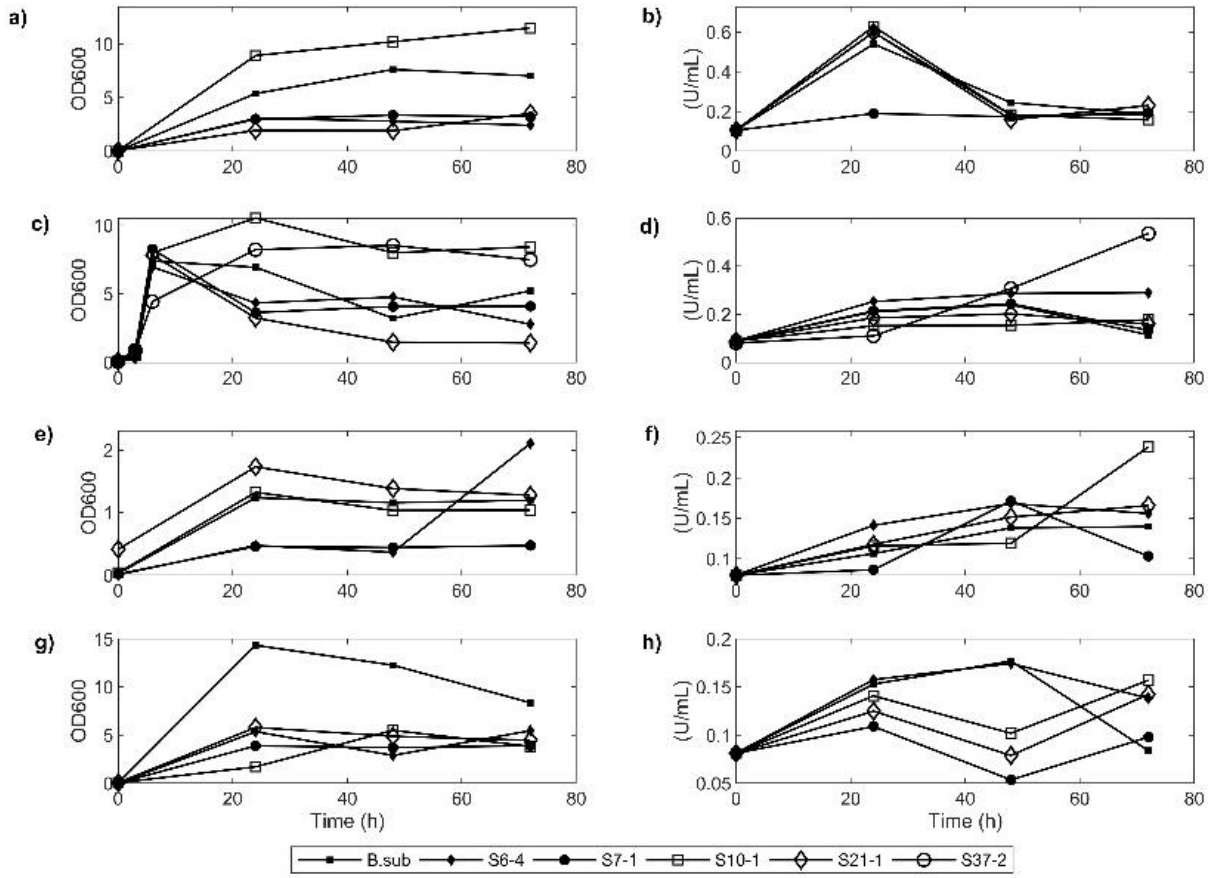


Figure 3: a) growth and b) activity at pH 4.7 and 37ºC; c) growth and d) activity at pH 7 and 37ºC; e) growth and f) activity at pH 9 and 37ºC; g) growth and h) activity at pH 7 and 30ºC

3.5 Milk clotting results

Milk clotting assay was performed with supernatant from isolated strains, crude enzyme form cultures S7-1 and S21-1 coagulated the milk. However, the time of coagulation was 1h 30min calculated in milk clotting unit 4.44MCU mL-1. While, for supernatant from *B. subtilis* culture took only 45minutes to coagulate the milk or 8.89MCU mL-1 calculated in milk clotting unit.

3.6 Enzymatically feather degradation

In our research we assessed the ability of 48h grown cultures form five isolated strains for degradation of feathers. Results showed that S6-4 biodegraded the highest amount of feather above 41% (Fig. 4c). This shows a good possibility for this culture to find application in feather biodegradation from poultry industry.

4 Conclusion

Five different strains were isolated from different region soils in Kosovo and identified as a good production host of protease enzyme. All isolated strains were characterized as Bacillus sp. strain by using both morphological and molecular techniques. Interestingly, there is no single strain that produces best protease for all applications, rather all five strains are promising for different applications. S10-1 has shown good activity for meat processing, while protease from S37-2 performed better in haze treatment in beer. Strains S7-1 and S21-1 showed moderate milk clotting activity, while S6-4 showed promising results in feather treatment. In conclusion these five strains have a potential for industrial application.

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Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that

submitted work is original and has not been published elsewhere in any language.

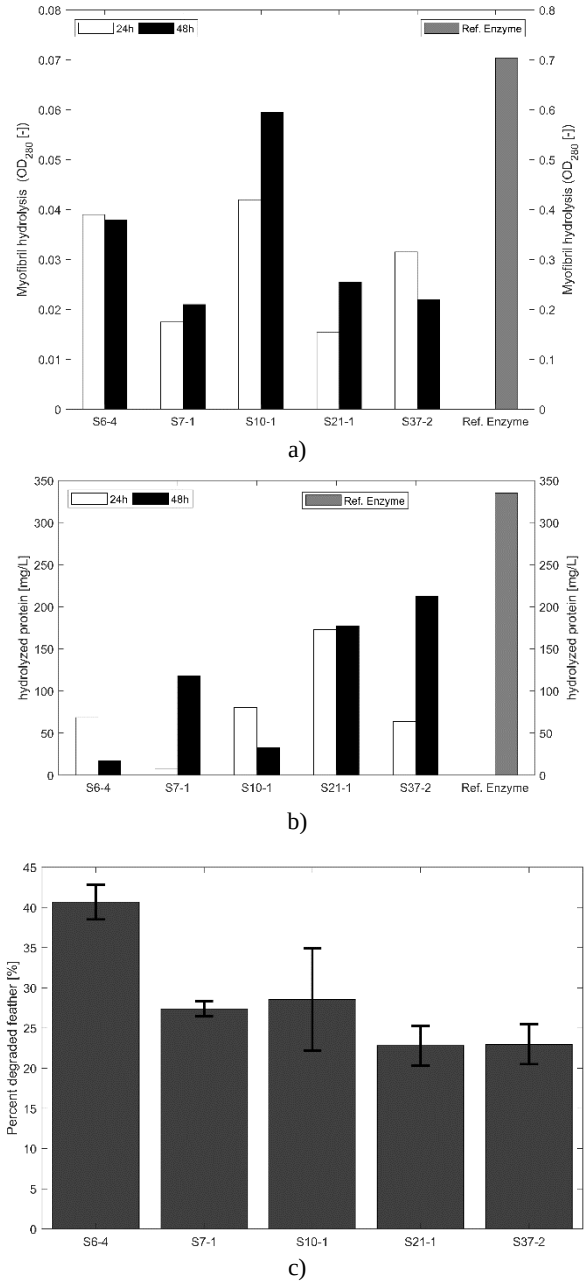

Figure 4: a) Breakdown of myofibril by the protease enzymes produced by each organism. In the right-most column, CE: commercial enzyme. b) Haze beer treatment by protease. c) Grams of feathers degraded by 48h culture from different cells

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing

References

1 Agarwal, P.K. Enzymes : An Integrated View of Structure, Dynamics and Function. *Microbial Cell Factories*. 2006; 12: 1-12.

2 Gupta, R., Beg, K.Q., and Lorenz P. Bacterial Alkaline Proteases : Molecular Approaches and Industrial Applications. *Applied Microbiology and Biotechnology*. 2002; 59: 15-32.

3 Vellard, M. The Enzyme as Drug: Application of Enzymes as Pharmaceuticals. *Current Opinion in Biotechnology*. 2003; 14(4): 444-450.

4 Hasan, F., Shah, A.A., Javed, S., and Hameed, A. Enzymes Used in Detergents: Lipase. *African Journal of Biotechnology*. 2010; 9(31): 4836-4844.

5 Choudhary, R.B., Jana, A.K., Jha, M.K. Enzyme Technology Applications in Leather Processing. *Indian Journal of Chemical Technology* Ind. Jour. of Chem. Technol. , 11(5), 659, 671 (2004).

6 Raveendran, S., Parameswaran, B., Ummalyma, S.B., Abraham, A., Mathew, A.K., and Madhavan, A. Applications of Microbial Enzymes in Food Industry. *Food Technolgy and Biotechnology*. 2018; 56(1):16-30.

7 Hosseini, S.V., Saffari, Z., Farhanghi, A., Atyabi, S.M., and Norouzian, D. Kinetics of Alkaline Protease Production by Streptomyces Griseoflavus PTCC1130. *Iran Journal of Microbiology*. 2016; 8(1): 8-13.

8 Karigar, C.S., and Rao, S.S. Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. *Enzyme Research*, 1, 1, 11 (2011).

9 Dinh, N.T.T. Meat Quality: Understanding of Meat Tenderness and Influence of Fat Content on Meat Flavor. *Tạp chi Phát triển Khoa học và Công nghệ.* 2008; 9(12): 65-70.

10 Pinelo M., Zeuner B., and Meyer A.S. Food and Bioproducts Processing Juice Clarification by Protease and Pectinase Treatments Indicates New Roles of Pectin and Protein in Cherry Juice Turbidity. *Food Bioproducts Processing*. 2009; 88(2–3): 259–265.

11 Yadav. D.N., Vishwakarma, R.K., Borad, S., Bansal, S., Jaiswal, A.K., and Sharma, M. Development of Protein Fortified Mango Based Ready-to-Serve Beverage. *Journal of Food Science and Technology*. 2016; 53(10): 3844–3852.

12 Lopez. M., Edens, L. Effective Prevention of Chill-Haze in Beer Using an Acid Proline-Specific Endoprotease from Aspergillus Niger. *Journal of agricultural and Food chemistry*. 2005; 53: 7944–7949.

13 Steinier, E., Becker, T., and Gasti, M.E. Turbidity and Haze Formation in Beer – Insights and Overview. *Journal of the Institute of Brewing*. 2010; 116(4): 360-368.

14 Mohanty, A.K., Mukhopadhyay, U.K., Grover, S., and Batish, V.K. Bovine Chymosin : Production by rDNA Technology and Application in Cheese Manufacture. *Biotechnology advances*. 1999; 17: 205–217.

15 Sousa, M.J., Ardö, Y., and McSweeney, P.L.H. Advances in the Study of Proteolysis During Cheese Ripening. *International Dairy Journal*. 2001; 1(4-7): 327-345.

16 Abdel-Fattah AM, El-Gamal MS, Ismail SA, Emran M.A., Hashem, A.M. Biodegradation of Feather Waste by Keratinase Produced from Newly Isolated Bacillus Licheniformis ALW1. *Journal of Genetic Engineering and Biotechnology*. 2018; 16(2): 311–318.

17 Anbu, P., Gopinath, S.C.B., Chaulagain, B.P., and Lakshmipriya, T. LAKSHMIPRIYA, Microbial Enzymes and Their Applications in Industries and Medicine 2016. Biomed Res. Int., 2017, (2017).

18 Kasana, R.C., Salwan, R., and Yadav, S.K. Microbial Proteases: Detection, Production, and Genetic Improvement. *Critical Review in Microbiology*. 2011;37(3):262–276.

19 Lü, J., Wu, X., Jiang, Y., Cai, X., Huang, L., and Yang, Y. et al. An Extremophile Microbacterium Strain and Its Protease Production

Under Alkaline Conditions. *Journal of Basic Microbiology*. 2014;54(5):378–385

20 Gopinath SC, Anbu P, Arshad MK, Lakshmipriya T, Voon CH, and Hashim U., et al. Biotechnological Processes in Microbial Amylase Production. *Biomed Research International*. 2017; 2017.

21 da Silva, R.R., Bacterial and Fungal Proteolytic Enzymes: Production, Catalysis and Potential Applications. *Applied Biochemistry and Biotechnology*. 2017; 183(1): 1-19.

22 Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., and Azevedo, V. et al. The Complete Genome Sequence of the Gram-Positive Bacterium Bacillus Subtilis. *Nature*. 1997; 390(6657): 249–256.

23 Ma, J., Wang, C., Wang, H., Liu, K., Zhang, T., and Yao, L., et al. Analysis of the Complete Genome Sequence of Bacillus Atrophaeus GQJK17 Reveals Its Biocontrol Characteristics as a Plant Growth-Promoting Rhizobacterium. *Biomed Research International*. 2018; 2018.

24 Mohanta, T. K., and Bae, H. The Diversity of Fungal Genome. *Biological Procedures Online*. 2015; 17(8): 1–9

25 Boldrini-França, Santos, R.R., Santos-Silva, L.K., de Souza, D.L. Gomes, M.S., and Cologna, C.T., et al. Expression of a New Serine Protease from Crotalus Durissus Collilineatus Venom in Pichia Pastoris and Functional Comparison With the Native Enzyme. *Applied Microbiology and Biotechnology*. 2015; 99(23): 9971–9986

26 Tobe, S., Shimogaki, H., Ohdera, M., Asai, Y., Oba, K., Iwama, M., and Irie, M. Expression of Bacillus Protease (Protease BYA) from Bacillus sp . Y in Bacillus Subtilis and Enhancement of Its Specific Activity by Site-Directed Mutagenesis - Improvement in Productivity of Detergent Enzyme. *Biological and pharmaceutical bulletin*. 2006; 29(1): 26–33

27 Prakash, D., Verma, S., Bhatia, R., and Tiwary, B.N. Risks and Precautions of Genetically Modified Organisms. *ISRN Ecology*. 2011; 2011: 1–13.

28 Ruppen M.E., Van Alstine, G.L., and Band, L. Control of Intracellular Serine Protease Expression in Bacillus subtilis. *Journal of bacteriology*. 1988; 170(1): 235–244.

29 Popping, B. Identifying genetically modified organisms (GMOs). In Food Authenticity and Traceability. L Michele, eds., Woodhead Publishing. Cambridge. 2003. 406-416.

30 Asha, B., and Palaniswamy, M. Optimization of Alkaline Protease Production by Bacillus Cereus FT 1 Isolated from Soil. *Journal of applied Pharmaceutical Science*. 2018; 8(02): 119–127

31 Pant, G., Prakash, A., Pavani, J.V.P., Bera, S., Deviram, G.V.N.S., and Kumar, A. et al. Production, Optimization and Partial Purification of Protease from Bacillus Subtilis. *Journal of Taibah University for Science*. 2015;9(2015):50–55.

32 Beveridge TJ. Use of the Gram Stain in Microbiology. *Biotechnology and histochemistry*. 2001; 76(6): 111-118.

33 Taylor, W.I., and Achanzar, D. Catalase Test as an Aid to the Identification of Enterobacteriaceae. Applied microbiology. 1972;24(1):58–61.

34 Hamza, T.A. Isolation and Screening of Protease Producing Bacteria from Local Environment for Detergent Additive. *American Journal of Life Sciences*. 2017; 5(5): 116–124.

35 Josephine, F.S., Ramya, V.S., Neelam, D., Ganapa, S.B., Siddalingeshwara, K.G., and Venugipal, N. et al. Isolation, Production and Characterization of Protease from Bacillus Sp Isolated from Soil Sample. Journal of Microbiology and Biotechnology Research. 2012; 2(1): 163-168.

36 Robinson, P.K. Enzymes : Principles and Biotechnological Applications. Essays in biochemistry. 2015; 59: 1–41.

37 Hayat, R., Sheirdil, R.A., Iftikhar-ul-hassan, M., and Ahmed, I. Characterization and Identification of Compost Bacteria Based on 16S rRNA Gene Sequencing. *Annals of Microbiology*. 2013; 63(3): 905–912.

38 Janda, J.M., and Abbott, S.L. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *Journal of Clinical Microbiology*. 2007; 45(9): 2761–2764.

39 Hong, S.W., Kwon, S.W., Kimn, S.J., Kim, S.Y., Kim, J.J., and Lee, J.S., et al. Bacillus Oryzaecorticis sp. nov., a Moderately Halophilic Bacterium Isolated from Rice Husks. International *Journal of Systematic and Evolutionary Microbiology*. 2014; 64(8): 2786–279.1

40 Olson, D.G., Parrish, F.C.J.R., and Stromer, M.H. STROMER, Myofibril Fragmentation and Shear Resistance of Three Bovine Muscles during Postmortem Storage. *Journal of Food Science*. 1976; 41(5):1036–1041.

41 Aishe, I.N.A., and Sorensen, T.L., Effects of Papain and a Microbial Enzyme on Meat Proteins and Beef Tenderness. *Journal of Food Science*. 2002; 67(6): 2138–2142.

42 Arima, K., Iwasaki, S., and Tamura, G. Milk Clotting Enzyme from Microorganisms. Agriculture and Biological Chemistry. 1967; 31(5): 540-545..

43 Kaya, K., and Nikerel, E. Characterization of Highly Active Keratinase Procuder Bacillus Cereus KK69 for Biological Degradation of Feather Waste. *Journal of Environmental Treatment Techniques*. 2019; 4(3): 357–363.

44 Hosoi, T., Ametani, A., Kiuchi, K., Kaminogawa, S. Improved Growth and Viability of Lactobacilli in the Presence of Bacillus Subtilis (natto), Catalase , or Subtilisin. *Canadian Journal of Microbiology*. 2000; 46(10): 892–897.

45 Mamo, J., Assefa, F. The Role of Microbial Aspartic Protease Enzyme in Food and Beverage Industries. *Journal of Food Quality*. 2018; (2018).

46 Gomri, M.A., Rico-Díaz, A., Escuder-Rodríguez, J.J., El Moulouk, Khaldi, T., González-Siso, M.I., and Kharroub, K. Production and Characterization of an Extracellular Acid Protease from Thermophilic Brevibacillus sp. OA30 Isolated from an Algerian Hot Spring. *Microorganisms*, 2018; 6(2): 31.

47 Cho, S.J., OH, S.H., Pridmore, R.D., Juillerat, M.A., and Lee, Ch. Purification and Characterization of Proteases from Bacillus Amyloliquefaciens Isolated from Traditional Soybean Fermentation Starter. *Journal of Agriculture and Food Chemistry*. 2003;51(26):7664–7670.

48 Kelmendi, M., Sadiku, M., Kadriu, S., Dobroshi, F., Igrishta, L., Baruti, B. Research of Heavy Metals on the Agricultural Land in Bajgora Region, Kosovo. *Acta Chemica Iasi*. 2018; 26(1): 105–122.

49 Veselaj, T., Berisha, A., Mehmeti, M., Shala, A., Kastrati, B., Sahiti, F., and Buza, F. Kosovo Environmental Protection Agency. Report for water status in Kosovo. Kosovo. Accessed October 16 2019. https://www.ammkrks.net/repository/docs/raporti_ujrave%202010_shq.pdf, (2010).