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Co-production of Proteases and Bioactive Protein Hydrolysates from Bioprocessing of Feather Meal

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HIGHLIGHTS

- Feather meal (FM) as a low-value organic substrate for microbial conversion.
- Bacterial proteolytic enzymes were produced during FM bioprocessing.
- FM protein hydrolysates displayed *in vitro* antioxidant and antidiabetic activities.
- Interesting approach to simultaneously obtain added-value products from FM.

Abstract: Feather meal conversion through submerged cultivations with *Bacillus* strains (CL33A, CL14) yielded proteases and protein hydrolysates. After 4-day (CL33A) and 10-day (CL14) cultivations, protease activities reached 461 U/mL; hydrolysates presented antioxidant (radicals-scavenging, 57-77%; Fe^{2+}-chelation, 14-28%; Fe^{3+}-reduction) and antidiabetic (dipeptidyl peptidase-IV inhibition, 49-52%) potentials. The obtained bioproducts present prospective commercial/industrial applications.

Keywords: *Bacillus*; bioprocess; enzyme; antioxidant; antidiabetic.

INTRODUCTION

Environmental and economic issues are essential aspects related to the management of organic wastes and by-products derived from agro-industrial activities. Recognition that such materials might be converted into multiple products through suitable methods,

representing an additional source of profit, established a major foundation of biorefineries within the principles of sustainable development [1,2].

Feathers, abundantly generated from poultry processing for meat production, are mainly constituted of keratins, fibrous and refractory proteins corresponding to 90% of the feather dry weight [3]. The recalcitrance of feathers, resulting from the compact packing and stabilization of keratin polypeptides, might be a drawback for its recycling and destination [4]. Hydrothermal conversion of feathers into feather meal (FM) is a major technological approach for feathers reclamation. FM is particularly intended for animal feed; however, such application is restricted due to its poor digestibility and low nutritional value [5,6].

Innovative processes could lead to further valorization of FM. In this sense, microbial technologies provide a vast array of eco-friendly possibilities to obtain valuable products from renewable biomass [7]. Bioconversion of FM by keratinolytic microorganisms might be exploited to obtain microbial enzymes with biotechnological significance, specially proteases and keratinases, and also microbial biomass and protein hydrolysates. FM presents a higher accessibility than raw feathers as substrates for microbial action, thus potentially hastening the processes intended to obtain such bioproducts [5,8].

Production of hydrolysates from protein-rich wastes and by-products is increasingly focused, since these hydrolysates might display biological activities, such as antioxidant and antidiabetic properties, with potential relevance for food, feed, and pharmaceutical industries [9,10]. Nevertheless, the bioactivities of feather and FM hydrolysates obtained through microbial conversion were only recently described, representing a promising field for research and development [11].

Therefore, the aim of this study was to utilize two feather-degrading bacteria for FM bioprocessing, as a strategy to obtain added-value products. From the proteinaceous composition of FM and the proteolytic potentials of the bacterial strains, it is hypothesized that the simultaneous production of bacterial proteases and bioactive feather meal hydrolysates (FMH) might be feasible.

MATERIAL AND METHODS

The previously isolated proteolytic bacteria, *Bacillus* sp. CL33A and *Bacillus* sp. CL14, were employed for FM bioconversion. Bacterial suspensions, used to inoculate feather meal broth (FMB), were prepared in saline (8.5 g/L NaCl). FMB consisted of (g/L): K_2HPO_4, 0.3; KH_2PO_4, 0.4; NaCl 0.5; and FM, 10.0 (Kabsa S.A., Brazil). Initial pH of FMB was adjusted to 7.5. Erlenmeyers (250 mL) containing 50 mL of FMB were inoculated with 1 mL of bacterial suspension, and cultivations were performed 30 °C, 125 rpm. At specific intervals, cultivations were filtered through filter paper, and the residual FM was determined gravimetrically after drying to constant weight (60 °C). Filtrates were centrifuged (10,000 *g*, 10 min) and supernatants were collected.

Protease production by the keratinolytic strains was evaluated on culture supernatants. Azocasein was used as substrate in assays performed for 30 min, at 55 °C, pH 8.0. One activity unit (U) was defined as the amount of enzyme resulting in an increase of 0.01 absorbance units at 420 nm [8].

Culture supernatants were heated (100 °C, 10 min) to inactivate microbial enzymes, and henceforth referred to FMHs. Soluble protein of FMHs was determined by the Folin-phenol method.

Antioxidant activities of FMHs were assessed through four *in vitro* methods. Scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was evaluated by adding FMH (1 mL) to 3.9 mL of DPPH solution (60 µM, prepared in methanol). Reactions were carried out for 60 min in the absence of light, at room temperature, and DPPH scavenging was assessed at 517 nm [12]. Distilled water was used as control. DPPH scavenging was expressed as: Scavenging (%) = $[1 - (A_s / B_c)] \times 100$, where A_s and B_c represents the absorbance with FMH or with distilled water, respectively.

Scavenging of the 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical was also assessed [13]. ABTS radical solution was diluted with phosphate-buffered saline (5 mM, pH 7.4) to reach 0.70 ± 0.02 absorbance units at 734 nm. A 10 µL aliquot of

FMH was added to 1 mL of ABTS radical solution and, after 6 min, the absorbance was measured at 734 nm. Controls were performed with distilled water. ABTS radical scavenging was expressed as: Scavenging (%) = [1 - (A_s / B_c)] × 100, where A_s and B_c indicates absorbance with FMH or distilled water, respectively.

Fe^{2+}-chelating ability of FMHs was assessed by adding 100 µL FMH to 3.7 mL of distilled water, 100 µL of 2 mM $FeSO_4$ and 200 µL of 5 mM ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine). After 10 min, absorbances were measured at 562 nm [14]. Control assays were performed using 100 µL of distilled water. Results were obtained as: Fe^{2+}-chelating activity (%) = [1 - (A_s / B_c)] × 100, where A_s and B_c represents the absorbance with FMH or with distilled water, respectively.

Reducing power of FMHs was evaluated by mixing 1 mL of FMH with 2.5 mL of 200 mM phosphate buffer (pH 6.6) and 2.5 mL of 10 g/L potassium ferricyanide. After incubation at 50 °C for 30 min, 2.5 mL of 100 g/L trichloroacetic acid were added. This mixture was centrifuged (3,000 *g*, 10 min), and 2.5 mL of the collected supernatant were mixed with 2.5 mL distilled water and 500 µL of 1 g/L $FeCl_3$. Controls were performed using 1 mL of distilled water instead of FMH. Absorbances were measured at 700 nm, and results expressed as absorbance units at 700 nm (A_{700}), as described elsewhere [14].

In vitro antidiabetic capacity of FMH was evaluated by the ability to inhibit dipeptidyl peptidase-IV (DPP IV) activity towards Gly-Pro-*p*-nitroanilide [15]. Briefly, 25 µL of FMH was mixed with 25 µL of 2.0 mM Gly-Pro-p-nitroanilide (prepared in 50 mM Tris-HCl buffer, pH 7.6), and this mixture was heated at 37 °C for 10 min. Reactions were initiated by adding 50 µL of DPP IV (0.01 U/mL, prepared in Tris-HCl buffer) and, after incubation (37 °C, 60 min), reactions were finished with 100 µL of 1 M acetate buffer (pH 4.0). Absorbance was measured at 405 nm. DPP IV inhibition were calculated as: Inhibition (%) = {1 - [(A - B) / (C - D)]} × 100, where A is the absorbance in reactions containing DPP IV, substrate and FMH; B is the absorbance in reactions containing substrate, FMH, and buffer; C is the absorbance in reactions containing DPP IV, substrate, and distilled water; D is the absorbance in reactions containing substrate, with buffer and distilled water instead of DPP IV and FMH, respectively.

All described assays were conducted in triplicates. Data were presented as average values ± standard deviations, which were subjected to analysis of variance, and the means were compared by Tukey's test at 95% confidence level.

RESULTS AND DISCUSSION

Bacterial conversion of FM was evaluated by the residual mass of FM during cultivations. *Bacillus* sp. CL33A degraded 62% and 92% of FM after 3 and 7 days of cultivation, respectively (Fig. 1a), whereas 76% and 98% of FM was degraded by *Bacillus* sp. CL14 after 7 and 13 days (Fig. 1b). Son et al. [16] reported 96% of FM degradation by *B. pumilis* F3-4 after 7 days of submerged cultivations in FM medium. In an investigation with *Bacillus* sp. P7, 87% of FM was degraded after 4 days [8], and *B. subtilis* 1270, *B. subtilis* 1273, and *B. licheniformis* 1274 degraded 63%, 70%, and 76% of FM after 7 days [17].

Increased protease production by CL33A was detected after four days on FMB (461 U/mL; Fig. 1a). Proteolytic activity was observed to decrease after day 4, possibly due to product negative feedback or autolysis [18]. Higher protease production by CL14 (462 U/mL; Fig. 1b) was similar to that detected for CL33A; however, this was observed only after 10 days of cultivation with *Bacillus* sp. CL14 (Fig. 1b). Production of keratinolytic proteases by 5 bacterial strains peaked after 3-6 days of cultivation on FMB [19], 4-day cultivations resulted in higher protease production by five *Bacillus* strains [20], and increased enzyme production was achieved by *B. subtilis* FDS15 in FM medium after 3 days [21].

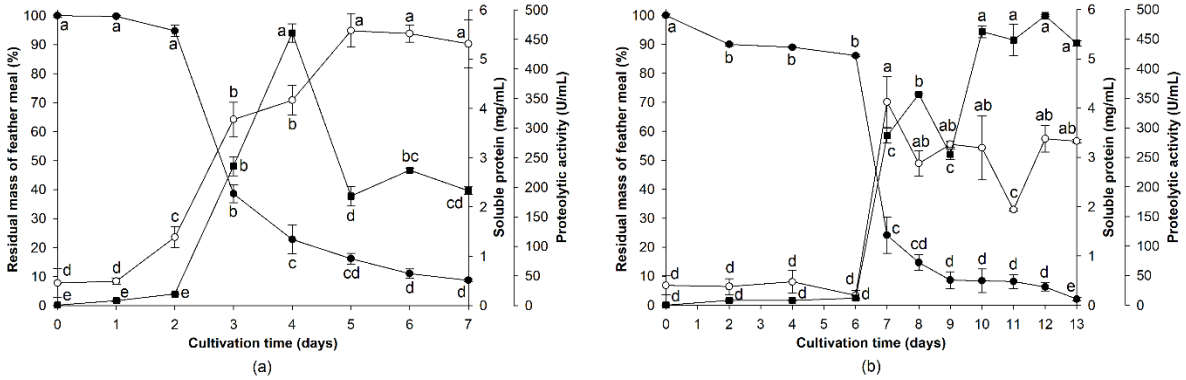

Figure 1. Feather meal degradation (●, %), protease production (■, U/mL), and soluble protein concentration (○, mg/mL) during submerged cultivations with *Bacillus* sp. CL33A (**a**) and *Bacillus* sp. CL14 (**b**) on feather meal broth. Data points are means ± standard deviation of experiments performed in triplicates. Different lowercase letters indicate significant differences ($P < 0.05$) within each evaluated parameter.

Considering that FM was the only organic substrate in FMB, and that FM is composed mainly of proteins (keratins), production of proteolytic enzymes is required to hydrolyze FM into absorbable peptides and amino acids that will sustain bacterial growth and energy production [5]. Beyond the microbial perspective, proteases from *Bacillus* are important commercial biocatalysts, employed by the detergent, food, feed, leather, pharmaceutical, and other industries [22]. Hence, the utilization of alternative low-price substrates, such as FM, is increasingly acknowledged as a strategy to diminish the costs of enzyme production [5].

Degradation of FM resulted, as expected, in increases on soluble protein contents assessed in culture supernatants [20]. For *Bacillus* sp. CL33A, soluble protein peaked at 5 days of cultivation (5.5 mg/mL), with similar values at increasing cultivation times (Fig. 1a). Soluble protein during cultivations with CL14 peaked at day 7 (4.1 mg/mL), and variations on soluble protein were observed afterwards (Fig. 1b). The buildup of soluble proteins during cultivations indicate its release in higher amounts than needed for the bacteria, and a decreased content at longer cultivations suggests the decreased availability of FM.

Cultivations with three *Bacillus* strains on FM medium resulted in soluble protein contents of 3.9-4.4 mg/mL after 7 days [17], whereas growth of *B. pumilis* F3-4 in FM-based medium for 7 days resulted in soluble protein concentration of 1.8 mg/mL [16]. The increased time required by *Bacillus* sp. CL14 for effective FM degradation, resulting from a lag period for increased protease production, with noticeable effects on soluble protein release, indicates the higher keratinolytic efficiency of *Bacillus* sp. CL33A [19].

Although FM and feathers are employed as substrates for keratinolytic bacteria, the main purpose is to produce proteolytic enzymes [5]. More recent studies also draw attention to applications of protein hydrolysates resulting from the bioconversion of keratinous materials. These are claimed as nitrogen-rich fertilizers and ingredients in animal feed [6,23]. An additional innovative approach for biomass valorization resides on prospecting the bioactivities of microbially-produced keratin hydrolysates, which depend on peptides and amino acids released upon hydrolysis [11,24].

The antioxidant and antidiabetic activities of FMHs were then evaluated *in vitro*. FMHs produced by *Bacillus* sp. CL33A and *Bacillus* sp. CL14 were able to scavenge the DPPH and ABTS radicals (Fig. 2). For strain CL33A, FMH obtained after 4 days of cultivation scavenged 63% and 76% of DPPH and ABTS radicals, respectively, and these values were maintained for FMHs recovered at longer cultivation periods (Fig. 2a). Maximal DPPH scavenging of FMHs produced by CL14 was observed after 7 days (61%); the same FMH displayed ABTS-radical scavenging of 67%, but FMHs obtained after 12 days were able to scavenge 85% of the ABTS radical (Fig. 2b). These results indicate the ability of FMHs to transfer hydrogen and electrons to DPPH and ABTS radicals, respectively, stabilizing them [25].

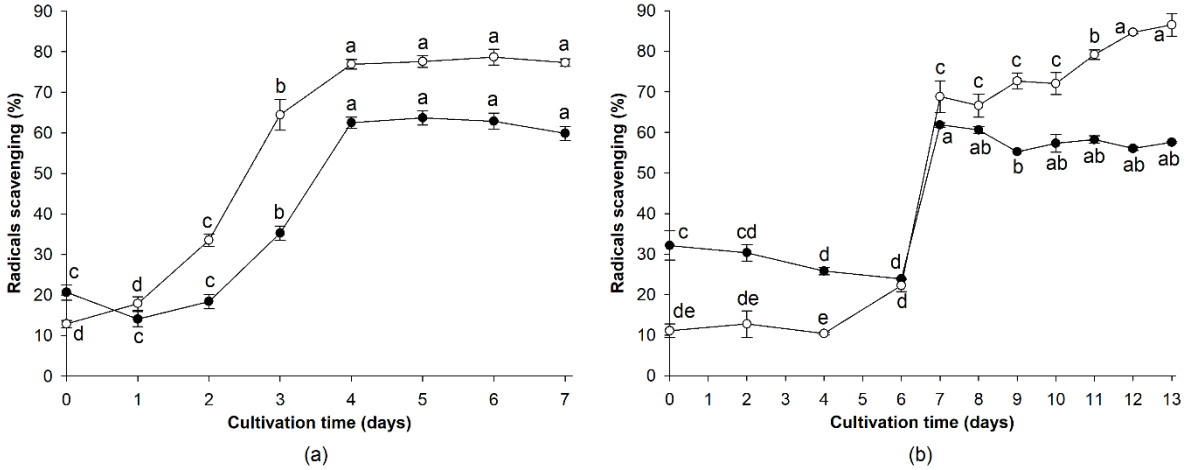

Figure 2. Antioxidant potential, as assessed through the scavenging of DPPH radicals (●, %) and ABTS radicals (○, %), of feather meal hydrolysates produced by *Bacillus* sp. CL33A (**a**) and *Bacillus* sp. CL14 (**b**) during submerged cultivations on feather meal broth. Data points are means ± standard deviation of experiments performed in triplicates. Different lowercase letters indicate significant differences ($P < 0.05$) within each evaluated parameter.

Bacillus sp. P45 was employed for FM bioconversion, and hydrolysates showed radical-scavenging abilities [26]. The scavenging of DPPH (68%) and ABTS (75%) was reported for feather hydrolysates produced by *Chryseobacterium* sp. kr6 [27]. Protein hydrolysates with radical-scavenging abilities were also obtained from fermentation of fish visceral waste and fish head waste by lactic acid bacteria [28,29]. Hydrolysates of squid pen waste and shrimp shell waste powders produced by *Serratia* sp. TKU016 exhibited higher DPPH-scavenging after 3 days (50%) and 2 days (76%) of cultivation, respectively [30].

FMHs were observed to chelate Fe^{2+} (Fig. 3). This metal ion is involved in the production of highly oxidant hydroxyl radicals that leads to radical chain reactions [31]. The chelating ability of hydrolysates produced by strain CL33A reached 28% and 47% after 4 and 7 days of cultivation, respectively (Fig. 3a), whereas 14% and 41% of Fe^{2+} ions were chelated by FMHs obtained after 10 and 13 days with CL14 (Fig. 3b). FMHs were also assessed for their capability to donate electrons to Fe^{3+}, reducing it to Fe^{2+} [31]. Increased reducing power was measured for FMHs obtained at 4-6 days of cultivation with CL33A (Fig. 3a). Similar reducing power (0.42-0.46 A_{700}) was observed for FMHs produced by strain CL14 after 7-10 days; however, superior reducing power (0.58 A_{700}) was detected after 11 days of cultivation (Fig. 3b).

Protein hydrolysates obtained by submerged cultivations with *Serratia marcescens*, *Pseudomonas aeruginosa*, or *Bacillus pumilus* on shrimp shell waste displayed reducing power, and hydrolysates with higher activity were produced by *S. marcescens* [32]. Salmon viscera hydrolysates, produced through lactic acid fermentation, displayed slight increases on Fe^{2+}-chelation after 6 h of cultivation, which then decreased at longer periods (up to 8 days), including values lower than the starting material. Also, microbial hydrolysis was detrimental to the reducing power of salmon viscera [33].

Feather hydrolysates produced by *B. pumilus* A1 displayed Fe^{2+}-chelating ability and reducing power [34,35]. However, feather hydrolysates obtained with *Chryseobacterium* sp. kr6 exhibited reducing power, but not Fe^{2+}-chelation [36]. Reducing power and radical scavenging were also displayed by feather hydrolysates produced by *Bacillus* sp. MPTK6 [37], and *Kocuria rhizophila* p3-3 [38]. Such potentials were also verified for hydrolysates of delimed tannery fleshings obtained by 4-day cultivations with *Enterococcus faecium* NCIM5335 [39], for shrimp shell waste hydrolysates produced by *P. aeruginosa* A2 [40]. In addition, multiple antioxidant activities, such as radical scavenging, reducing power and the ability to chelate ferrous ions, were demonstrated for fish (*Acanthogobius hasta*) processing

by-product protein hydrolysates, produced through solid-state fermentations with *Aspergillus oryzae* [10].

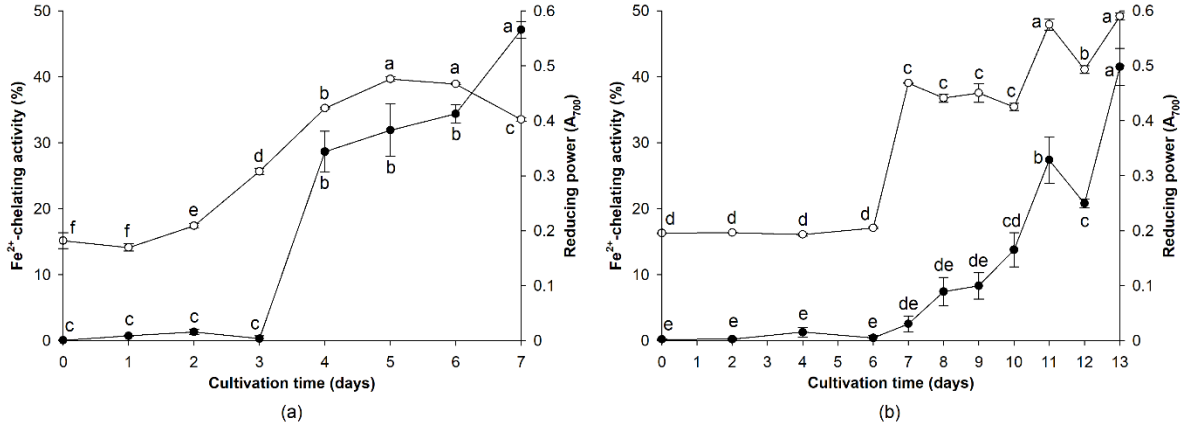

Figure 3. Antioxidant potential, as assessed through reducing power (○, A_{700}) and Fe^{2+}-chelating ability (●, %), of feather meal hydrolysates produced by *Bacillus* sp. CL33A (**a**) and *Bacillus* sp. CL14 (**b**) during submerged cultivations on feather meal broth. Data points are means ± standard deviation of experiments performed in triplicates. Different lowercase letters indicate significant differences ($P < 0.05$) within each evaluated parameter.

The antidiabetic activity of protein hydrolysates is usually evaluated, *in vitro*, by the inhibition of DPP IV, since this enzyme is responsible for the degradation of insulinotropic incretin hormones. Therefore, protein hydrolysates might act as natural DPP IV-inhibitors, potentially contributing to glycemic control in type 2 diabetes [41]. The inhibitory activity of FMHs produced by strain CL33A peaked after 3 days of cultivation (58%), with lower percentages of inhibition at increasing cultivation times (42-48%; Fig. 4a). Considering strain CL14, FMHs obtained at days 7-9 inhibited DPP IV by 33-39%; nevertheless, FMHs produced after 10 and 12 days of cultivation exhibited 52% and 55% of DPP IV inhibition (Fig. 4b).

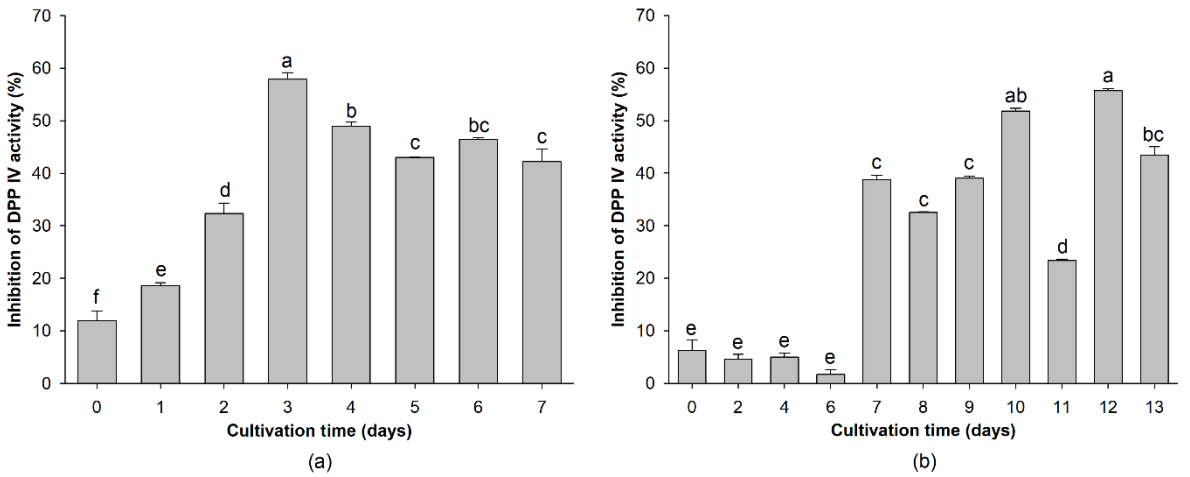

Figure 4. Inhibition of DPP IV activity (%) by feather meal hydrolysates produced by *Bacillus* sp. CL33A (**a**) and *Bacillus* sp. CL14 (**b**) during submerged cultivations on feather meal broth. Each column represents the mean ± standard deviation of experiments performed in triplicates. Different lowercase letters indicate significant differences ($P < 0.05$).

Current research is devoted to describe the antidiabetic capacity of protein hydrolysates obtained from industrial by-products. Feathers bioprocessing by *Chryseobacterium* sp. kr6 resulted in hydrolysates that inhibited DPP IV activity by 43-45% [27]. DPP IV inhibition was

also reported for enzymatic hydrolysates of salmon skin and trimmings [42], cuttlefish viscera [43], and brewers' spent grain protein-enriched isolate [44].

In conclusion, FM was successfully employed for the co-production of proteolytic enzymes and bioactive protein hydrolysates by *Bacillus* sp. CL33A and *Bacillus* sp. CL14. The former strain was more efficient for FM bioprocessing. From the culture conditions employed, 4-day cultivations with *Bacillus* sp. CL33A on FMB are regarded as appropriate aiming to obtain proteases and bioactive hydrolysates.

Bacterial proteases might be of significance for biocatalytic processes, deserving further investigations, especially regarding the production of bioactive hydrolysates from food and non-food proteins. The antioxidant properties of FMHs are relevant considering the deleterious action of free radicals and prooxidant metals on biological systems, and also food and feed products. The ability of FMHs to inhibit DPP IV activity indicates the possibility of obtaining antidiabetic peptides from an abundant and low-cost biomass. From the current and limited uses of FM, bioprocessing is a suitable approach for its valorization.

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