Annals of Microbiology (2018) 68:473–484
https://doi.org/10.1007/s13213-018-1352-0

ORIGINAL ARTICLE

Low-cost culture medium for the production of proteases by *Bacillus mojavensis* SA and their potential use for the preparation of antioxidant protein hydrolysate from meat sausage by-products

Amal Hammami[1] · Ahmed Bayoudh[1] · Ola Abdelhedi[1] · Moncef Nasri[1]

Received: 12 February 2018 / Accepted: 27 June 2018 / Published online: 15 July 2018

Abstract

The present study aims to maximize proteases production by *Bacillus mojavensis* SA strain and their use to produce bioactive protein hydrolysates from a meat by-product. The production of SA bacteria proteases was maximized using a culture medium based on wheat bran, which offer an advantage in minimizing the production cost and enhancing the enzyme activity by using agro-industrial wastes. The composition of media and cultural conditions for optimal proteases production by *B. mojavensis* SA strain were investigated. A successful and significant improvement of the alkaline proteases production (four folds) by the SA strain was achieved using the medium composed of (g/l): wheat bran, 50.0; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $CaCl_2$, 2.0; pH 6.0, where the growth conditions were monitored at 37 °C with an agitation speed of 200 rpm. Interestingly, the enzyme preparation of *B. mojavensis* was applied for the preparation of protein hydrolysates from a meat by-product. Hydrolysis was carried out for 180 min at pH 12.0. The resulting hydrolysate displayed an important antioxidant activity as evaluated by the radical scavenging capacity, the reducing power, and the β-carotene bleaching inhibition. The present study showed the high proteases' producing level by *B. mojavensis* SA strain in a low-cost fermentation medium (wheat bran) and their potential use in the production of bioactive protein hydrolysate from meat by-products.

Keywords Low-cost medium · *Bacillus mojavensis* SA · Protease · Meat by-products · Protein hydrolysates · Antioxidant activity

Introduction

Alkaline proteases produced by microorganisms are of prime interest in biotechnological applications, including detergents, foods, pharmaceutical, tannery, and leather industries (Pandey et al. 2000; Gupta et al. 2002a, b). The use of microorganisms for the production of hydrolytic enzymes is in economical bulk, as microbes are easy to manipulate and to produce enzymes with desired characteristics (Ramesh and Lonsane 1990). However, the overall production process of extracellular industrial enzymes is expensive due to the high cost of substrates and media components used for bacterial growth (Sharma et al. 2017). This is the most critical factor limiting the use of alkaline proteases in industrial applications. Therefore, developing novel processes for enzymes' production, with respect to industrial requirements and low production cost, is commercially appreciable.

In general, no defined medium has been designed for the common production of alkaline proteases from different microorganisms. In fact, each strain has its specific conditions for maximum enzyme production (Gupta et al., 2002a, b). Many researchers have been attempted to improve enzyme production using glucose or starch, coupled with expensive nitrogen sources such as yeast extract, peptone, or casamino acids. Thus, many industrial endeavors have been made to induce enzymes' production using inexpensive carbon and nitrogen sources (Prakasham et al. 2006; Mhamdi et al. 2014). It is, therefore, important to search new low-cost substrates that are suitable for proteases activity enhancement.

Highlights
- Alkaline proteases production using agro-industrial wastes was enhanced.
- The best medium found to be advantageous for maximum proteases production was based on wheat bran.
- Successful use of *Bacillus mojavensis* proteases to digest meat by-product proteins and produce bioactive protein hydrolysates.

✉ Amal Hammami
amal.hammami1@gmail.com

[1] Laboratoire de Génie Enzymatique et de Microbiologie, Ecole Nationale d'Ingénieurs de Sfax, Université de Sfax, B.P. 1173-3038, Sfax, Tunisia

The growth and enzyme production of the microorganism are strongly influenced by medium components, like carbon and nitrogen sources. Besides the nutritional factors, the cultural parameters, such as temperature, pH, and incubation time, played a major role in enzymes' production (Mazzucotelli et al. 2014). Therfore, the optimization of media components and cultural parameters are the primary task in a biological process would be crucial.

Besides their role in industrial applications, microbial proteases have been widely used in the elaboration of bioactive peptide mixtures through fermentation (Jemil et al. 2014) or hydrolysis processes (Lassoued et al. 2015; Abdelhedi et al. 2016). A great deal of interest has been made for antioxidant peptides from food proteins, including fish (Abdelhedi et al. 2016), ham (Mora et al. 2014), and chicken (Centenaro et al. 2014) meats. Poultry meat sausage is one of the oldest and most consumed meat products in the world, for its sensory quality, nutritional value, and economic cost. Due to the important sausages production, the meat processing industries generate huge quantities of wastes and by-products. Therefore, meat sausage by-products (MSB) represent a potential source of proteins that can be used to generate protein hydrolysates following proteolysis action.

In the present work, different low-cost and agricultural by-products were screened in view of producing alkaline proteases from *Bacillus mojavensis* SA with the highest yield and the lowest cost fermentation media. A various number of parameters affecting the production of alkaline proteases are described. On the other hand, MSB were digested with *B. mojavensis* proteases in order to produce protein hydrolysate, with antioxidant potential.

Materials and methods

Chemicals and reagents

Casein, 1,1-diphenyl-2-picrylhydrazyl (DPPH•), butylated hydroxyanisole (BHA), β-carotene, and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), sodium hydroxide, and other chemicals were of analytical grade.

Microorganism

Bacillus mojavensis SA, producing stable alkaline proteases, was isolated from slaughterhouse waste water. It was identified on the basis of 16S rRNA gene sequencing and assigned by the accession number MF407277.1. The strain was conserved in 30% (*v*/*v*) glycerol/LB broth at − 80 °C and replicated in Luria-Bertani (LB) medium for routine using. The optimal proteases activity conditions have been previously studied in a previous work (Hammami et al. 2017).

Enzyme assay

Proteolytic activity was measured by the method of Kembhavi et al. (1993). An aliquot of 0.5 ml from the culture supernatant, suitably diluted, was mixed with 0.5 ml of 100 mM KCl-NaOH (pH 12.0) containing 1.0% (*w*/*v*) casein. The reaction mixture was then incubated for 15 min at 60 °C and stopped by the addition of 0.5 ml of TCA (20%, *w*/*v*). The reaction tubes were allowed to stand at room temperature for 15 min and centrifuged at 12,500 rpm for 15 min to remove the precipitate. The optical density of the soluble fraction was read at 280 nm. A standard curve was generated using solutions of 0–50 mg/l of tyrosine. One unit of protease activity against casein was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions. Protease activity represented the mean of at least two determinations carried out in duplicate.

Analysis of different carbon and nitrogen sources for proteases production

Initial medium (M1) consisted of the following composition (g/l): hulled grain of wheat, 10; yeast extract (YE), 2.0; $CaCl_2$, 2.0; K_2HPO_4, 0.1; and KH_2PO_4, 0.1. The initial pH of the culture was adjusted at pH = 9.0. The physicochemical composition of hulled grain of wheat is illustrated in Table 1.

Different carbon sources with low cost (at 10 g/l), instead of hulled grain of wheat, were screened to maximize proteases production. By-products from semolina factories such as hulled grain of wheat, spent grain of corn, soya meal and wheat bran, and others prepared in our laboratory, such as *Mirabilis jalaba* tuber powder, shrimp wastes powder, feather meal, waste octopus powder, crab flour, corn flour, poultry feathers, and cuttlefish's waste powder, were tested. After selecting the best carbon source, the effect of increasing concentration (10–50 g/l) on the protease activity was assessed. On the other hand, different nitrogen sources (used at 2 g/l) such as, casein peptone, soy peptone, ammonium sulfate $(NH_4)_2SO_4$, ammonium chloride (NH_4Cl), sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3), urea ($CO(NH_2)_2$) and soya meal were tested for enzyme production by *B. mojavensis* SA. All media were autoclaved at 120 °C for 20 min.

Inocula were routinely grown in Luria-Bertani (LB) broth medium, composed of the following (g/l): peptone, 10.0; yeast extract, 5.0 and NaCl, 5.0 (Miller 1972). Then, the culture medium was inoculated with a level of 0.1% and cultivations were conducted in 250-ml Erlenmeyer flasks, with a working volume of 25 ml, and incubated at 37 °C on a rotatory shaker at 200 rpm for 24 h. Thereafter, cultures were centrifuged, and the cell-free supernatants were used for the proteolytic activity estimation. All experiments were carried out in duplicate and repeated at least twice.

Table 1 Chemical composition of hulled grain of wheat, wheat bran, soya meal, and meat by-product

	Hulled grain of wheat	Wheat bran	Soya meal	Meat sausage by-product
Proteins (%)	18.17 ± 0.1	21.13 ± 0.62	56.52 ± 0.06	41.69 ± 0.52
Fat (%)	4.52 ± 0.17	5.45 ± 0.13	4.39 ± 0.1	32.71 ± 1.50
Ash (%)	2.41 ± 0.07	11.66 ± 0.06	8.62 ± 0.021	15.57 ± 0.10
Carbohydrates (%)	72.63 ± 5.18	60.48 ± 2.71	32.65 ± 1.74	6.51 ± 0.08

Analytical results are reported, on dry matter basis

Effect of salts' concentrations

The effect of various salts (K_2HPO_4, KH_2PO_4, and $CaCl_2$) on enzyme activity and the biomass concentration (CFU) was studied by varying their concentrations. After 24 h of bacteria cell growth, the supernatant of the fermented substrate extract was used for the enzyme activity assay, as previously described.

Effect of additives on enzymes' production

To determine the effect of metal ions ($MnSO_4$, $MgSO_4$, $CuSO_4$, NaCl, $BaCl_2$ and $ZnCl_2$) on the enzyme activity production, different ions were individually added at a concentration of 5 mM to the media cell culture. Each culture was incubated at 37 °C for 24 h at 200 rpm and the supernatant was analyzed for protease activity.

Effect of experimental conditions on enzymes production

The effect of pH, temperature, and agitation rate on the enzymes production was assessed. Initially, the medium pH was adjusted at different values of 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, and then incubated at 37 °C for 24 h. Similarly, the ability of *B. mojavensis* SA to grow and produce enzymes, at different temperatures (30, 37 and 40 °C) was investigated. The optimal temperature for enzyme production was determined. On the other hand, to study the agitation rate effect, bacterial cultivation was carried out at different agitation rates (150 and 200 rpm) at 37 °C for 24 h. For each experiment, after 24 h of culture time, the supernatant was analyzed for protease activity. Three independent experiments were performed for each parameter.

Time course of enzymes production by *B. mojavensis* SA

To study the relation between enzymes production and the growth profile of the bacterium, a volume of 100 ml of the final production media was inoculated and the culture was monitored under the optimized conditions, using 1-l flasks. The growth was measured at a regular interval of time by total viable count (spread plate method) determination. In parallel, the proteases production at different time intervals was determined after removal of cells by centrifugation.

Determination of chemical composition

The moisture and ash content were determined according to the A.O.A.C. standard methods numbers 930.15 and 942.05 (A.O.A.C. 2000), respectively. The protein content was determined by estimating its total nitrogen content by the Kjeldahl method according to the A.O.A.C. method number 984.13 (A.O.A.C. 2000). A factor of 6.25 was used to convert the nitrogen value to protein. Fat was determined gravimetrically after Soxhlet extraction of dried samples with hexane. Carbohydrates content was estimated as described by Dubois et al. (1956). All measurements were performed in triplicate.

Preparation of protein hydrolysate from meat sausage by-product using SA proteases by the *in vitro* enzymatic hydrolysis technique

The meat sausage by-product (MSB), which corresponds to the product generated during the manufacture of poultry sausages with technological defects, was collected from a local processing industry (Chahia, Sfax, Tunisia) and minced into small pieces. The chemical composition of MSB (g/100 g MSB) is presented in Table 1.

First, the raw material (50 %, *w*/*v*) was cooked in distilled water at 95 °C for 15 min and then the mixture was homogenized in a Moulinex® blender for 5 min. Hydrolysis was carried out under optimal conditions of alkaline proteases activity (pH 12.0; 50 °C) (Hammami et al. 2017). Crude enzymes were added with an enzyme/substrate ratio of 3 units of enzyme per milligram of protein. During hydrolysis, the pH was maintained constant at the desired value by the addition of 4 N NaOH solution. After the achievement of the digestion process, the reaction was stopped by heating the solution at 95 °C for 20 min, for enzyme inactivation. The obtained product was centrifuged at 8000 rpm for 20 min and the supernatant was freeze-dried (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France). The resulting hydrolysate from meat by-product (MBH) was stored at − 20 °C for further use. The

undigested meat by-product (UMB) was treated under the same conditions without enzyme addition and serves as control.

The hydrolysis degree (HD), defined as the percent ratio of the number of peptide bonds cleaved to the total number of peptide bonds in the substrate per mass unit, was calculated from the amount of NaOH solution added to keep the pH constant during the hydrolysis (Adler-Nissan 1986).

The protein fraction contained in the hydrolysate was precipitated using ammonium sulfate (saturation up to 80%). Then, the precipitate was dissolved in distilled water and dialyzed for 48 h against water. Dialyzed proteins were freeze-dried to be further analyzed.

Determination of antioxidant activities

DPPH• free radical scavenging activity

The DPPH• (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of the different samples was determined as described by Bersuder et al. (1998). A volume of 500 μl of MBH and UMB at different concentrations (1 to 5 mg/ml) was added to 375 μl of absolute ethanol and 125 μl of 0.2‰ DPPH• solution. The mixtures were then kept for 60 min in dark at room temperature, and the reduction of DPPH• radical was measured at 517 nm using a UV-visible spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd., China). Lower absorbance of the reaction mixture indicated higher DPPH• radical scavenging activity. The control was conducted in the same manner, except that distilled water was used instead of sample. BHA was used as positive control. The DPPH• radical scavenging activity was calculated as follows:

$$\text{Scavenging activity } (\%) = [(A_C - A_S + A_B) \times 100]/A_C$$

where A_C, A_S, and A_B represent the absorbances of the control, the sample reaction, and the blank tubes, respectively. The test was carried out in triplicate.

Reducing power assay

The capacity to convert Fe^{3+} into Fe^{2+} was evaluated according to the method of Yildirim et al. (2001). A sample solution (0.5 ml) of each hydrolysate at different concentrations (from 1 to 5 mg/ml) was mixed with 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 1% (*w*/*v*) potassium ferricyanide solution. The reaction mixtures were incubated at 50 °C for 30 min. After incubation, 0.5 ml of 10% TCA was added and the reaction mixtures were then centrifuged at 3000 rpm for 10 min. Finally, 1.25 ml of the supernatant solution, from each sample mixture, was mixed with 1.25 ml of phosphate buffer and then 0.25 ml of 0.1% ferric chloride was added. The absorbance of the resulting solutions was measured at 700 nm after 10 min. Values presented are the mean of triplicate analyses.

Antioxidant assay using β-carotene bleaching method

The ability of MBH and UMB to prevent β-carotene from bleaching was assessed as described by Koleva et al. (2002). First, the emulsion of β-carotene/linoleic acid was freshly prepared by dissolving 0.5 mg of β-carotene, 25 μl of linoleic acid and 200 μl of Tween 40 in 1 ml of chloroform. The chloroform was then completely evaporated under vacuum in a rotatory evaporator at 40 °C. Then, 100 ml of distilled water were added and the resulting mixture was vigorously stirred. Thereafter, 2.5 ml of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 ml of each sample (from 1 to 5 mg/ml). Control tube was prepared in the same conditions by adding 0.5 ml of H_2O to the emulsion instead of sample. The absorbance of each test tube was measured at 470 nm before and after incubation for 1 h at 50 °C. BHA was used as a positive standard. The antioxidant activity was evaluated using the following formula:

$$\text{Antioxidant activity } (\%) = \left[1-(A_0-A_t)/\left(A_0'-A_t'\right)\right] \times 100$$

where A_0 and A_t represent the absorbances of the test sample measured before and after incubation, respectively, and A_0' and A_t' represent the absorbances of the control measured before and after incubation, respectively. The test was carried out in triplicate.

Statistical analysis

All experiments were carried out at least in triplicate, and average values with standard deviation errors were reported. Significance between values was analyzed using the SPSS software package (SPSS, ver. 17.0 for windows professional edition). A one-way analysis of variance (ANOVA) was then performed and followed by Duncan's test to estimate the significance at the 5% probability level.

Results and discussion

Effect of different carbon sources on the proteases activity

Because carbon is considered the primary nutrient for bacteria growth, different low-cost complex carbon sources, added at 10 g/l in M1 medium, were analyzed. Data reported in Fig. 1 showed that the maximum proteases production (~ 400 U/ml) was obtained using wheat bran and soya meal, followed by corn flour (254.54 ± 0.01 U/ml). However, poultry feathers were ineffective for proteases production.

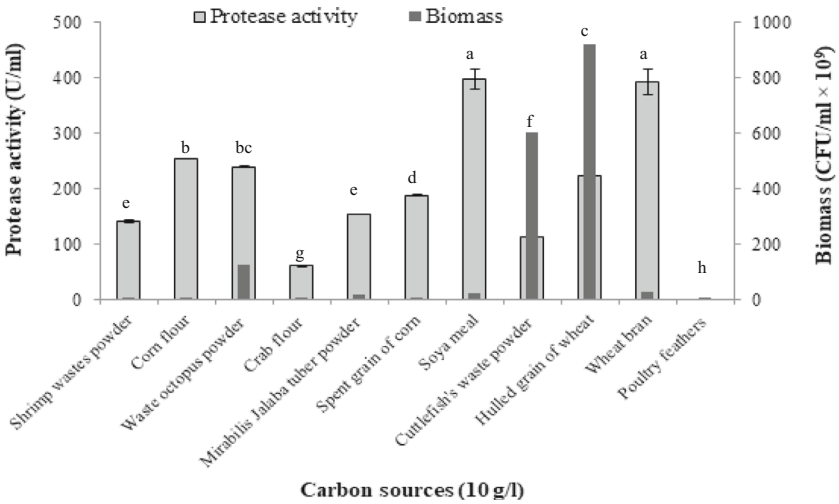
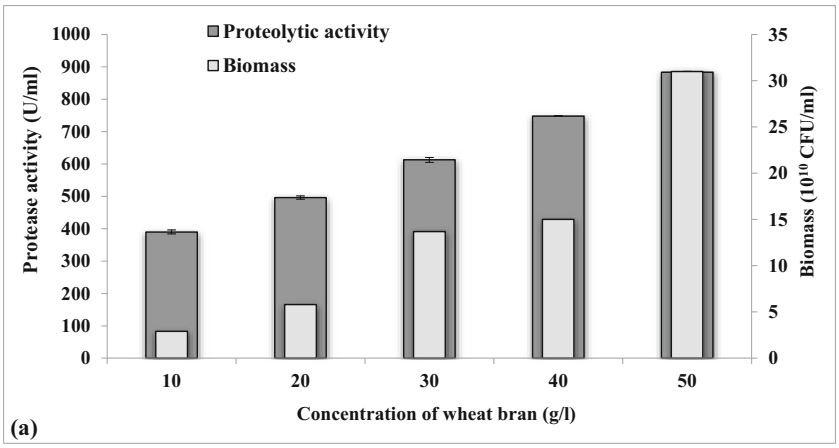


Fig. 1 Effect of different carbon sources on the biomass and the production of proteases by *B. mojavensis* SA. Cultivations were performed for 24 h at 37 °C in basal medium consisting of (g/l): carbon source 10, yeast extract 2; KH_2PO_4 0.1; K_2HPO_4 0.1, and $CaCl_2$ (pH 9.0). Data are expressed as mean ± SD. Different letters with different carbon sources indicate significant differences in the protease activity at $P < 0.05$

Wheat bran and soya meal, being the best carbon sources, were examined at different concentrations (10–50 g/l), as shown in Fig. 2. It was found that proteolytic activity was highly improved as the concentration of wheat bran increased and reached 745 and 883 U/ml at 40 and 50 g/l, respectively. It was further noted that bacterial growth increased with the increase of wheat bran concentration (Fig. 2a). However, the soya meal concentration correlated negatively with the proteolytic activity, which did not exceed 330 U/ml at 10 mg/ml ($P < 0.05$). Hence, wheat bran served as the better low-cost carbon source for the proteases' production. Previous study reported the use of wheat bran as a carbon source for enzyme



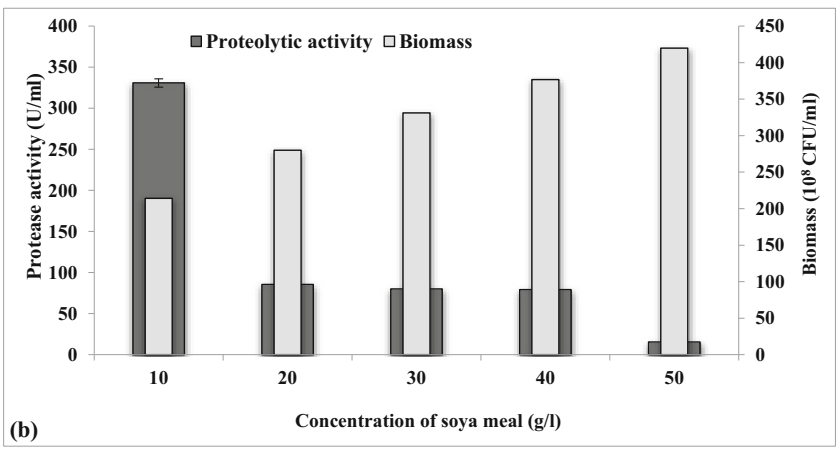


Fig. 2 **a**, **b** Effect of increasing concentration of carbon sources (wheat bran or soya meal) on proteases production by *B. mojavensis* SA. Different letters with different concentrations of carbon sources indicate significant differences at $P < 0.05$

production (Uyar 2004; Meena et al. 2013). Proteases' production from *Bacillus* species using various agricultural residues was widely described in literature, including the hulled grain of wheat (Kumar et al. 2014), soybean meal (Joo et al. 2002), wheat flower (Joo and Chang 2005) and rice bran (Naidu and Devi 2005). Such products constitute better substrates for enzyme production than simple sugars like glucose, maltose, and sucrose, which can induce catabolic repression mechanism and greatly decrease proteases' production (Mehta et al. 2006; Sinsuwan et al. 2015). In fact, in the absence of glucose, the protease synthesis could be repressed when the energy status of the cells would be high (Sharma et al. 2017). In this context, proteases production by *Pseudomonas aeruginosa* (MCM B-327) in soya bean and tryptone-based media was suppressed by 95 and 60%, following its supplementation with glucose and fructose, respectively (Zambare et al. 2011).

Effect of nitrogen sources

Besides the carbon source, nitrogen serves as an important nutrient for the proteases production. The requirement of a specific nitrogen supplement differs from a microorganism to another. To this end, casein peptone, soya peptone, ammonium sulfate, ammonium chloride, sodium nitrate, potassium nitrate, urea and soya meal were investigated in M1 medium containing wheat bran at 50 g/l, as a carbon source (Fig. 3). Data show that all the tested nitrogen sources allowed an important proteases production, but they still lower than the yeast extract. On the contrary, ammonium chloride and ammonium sulfate have been reported to suppress alkaline proteases production by *Bacillus* sp. 2–5 (Darani et al. 2008). More interestingly, proteases production in the absence of yeast extract in the culture medium, based on the wheat bran alone, gave the best proteases activity level with an activity close to 1000 U/ml and a biomass of 320.10^9 CFU/ml. These findings showed that wheat bran could play the role of, both, carbon and nitrogen source.

The chemical composition of wheat bran is given in Table 1. Data showed that wheat bran contained high level of protein (21.13%) and sugar (60.48%). High ash (11.66%) and low lipid content (5.45%) were further noted. Thus, wheat bran could be considered as a complete medium containing the essential substances required for a microbial growth, and thereby it could play the role of carbon and nitrogen source. Hence, wheat bran may be considered as a cost-effective substrate for the production of proteases.

In the same context, Chu (2007), Tari et al. (2006) and Jaswal et al. (2008) have reported, respectively, that *Bacillus* sp. APP1, *Bacillus* sp. L21 and *Bacillus circulans* can considerably grow and produce proteases, when using soya bean meal as an organic nitrogen source. Zambare et al. (2011) similarly proved that maximum proteases production by *P. aeruginosa* MCM B-327 was obtained with a combination of soybean meal and tryptone. In another study, protein hydrolysates from different fish species such as tuna, cod, salmon, and unspecified fish have been used as nitrogenous sources for microbial growth (Dufossé et al. 2001).

Effect of oligo-elements addition

The influence of K_2HPO_4 and KH_2PO_4 concentration on the cell growth and alkaline protease production by SA strain was investigated (Fig. 4). After 24 h of incubation, the maximum alkaline protease production (1259 U/ml) was observed in the medium containing 0.5 g/l of both K_2HPO_4 and KH_2PO_4. Increasing K_2HPO_4 and KH_2PO_4 concentrations to 2 g/l led to a drastic reduction in the proteases production, while the bacterial growth increased. Similarly, Shirato and Nagatsu (1965) found that 0.8 g/l of KH_2PO_4 have the greatest effect on the production of alkaline proteases by *Streptomyces griseus*.

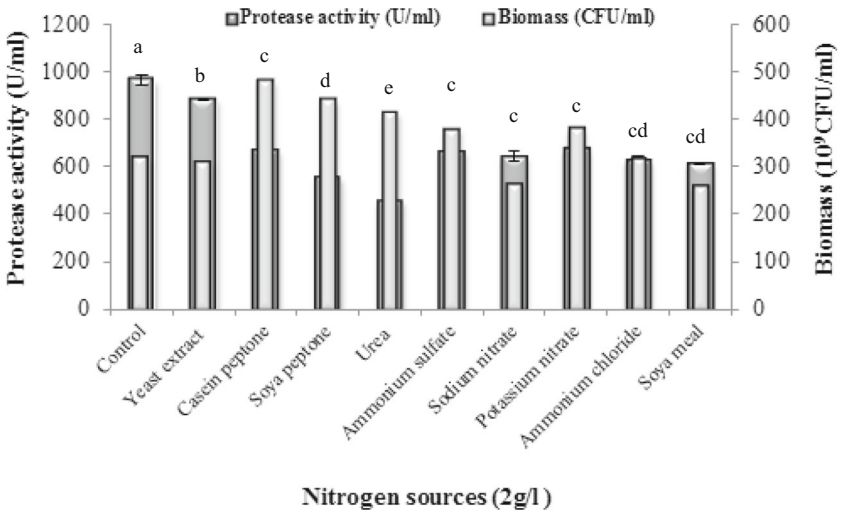


Fig. 3 Effect of different nitrogen sources on the proteases production by *B. mojavensis* SA. Cultures were conducted at 37 °C in the basal medium containing wheat burn at 50 g/l and supplemented or not with one of nitrogen sources (2 g/l). Different letters with different nitrogen sources indicate significant differences at $P < 0.05$

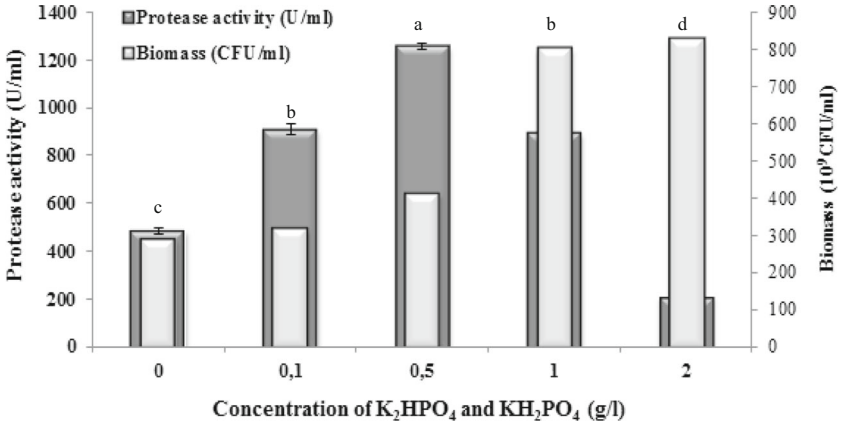


Fig. 4 Proteases production by *B. mojavensis* SA on medium containing wheat bran at 50 g/l supplemented with various concentrations of K_2HPO_4 and KH_2PO_4. Different letters with different concentrations of K_2HPO_4 and KH_2PO_4 indicate correlated negatively differences at $P < 0.05$

Moreover, the effect of $CaCl_2$ concentration was investigated (Table 2). An addition of 2 g/l of $CaCl_2$ was found to give the greatest effect for the production of alkaline proteases, whereas, the activity was gradually reduced when the salt concentration increased above 2.5 g/l. A slight activity reduction occurred in the absence of $CaCl_2$. It has been reported that an increased salt concentration creates changes in the lipid composition of cell membranes, leading to the growth rate decrease along with the enzyme production (Sandhya et al. 2005).

Metal ion requirements for proteases production

The results illustrated in Table 3 show the effect of different ions, supplemented at 0.5 g/l to the culture media, on the proteases production and cell growth. The activity depended on the nature of the element used, and the maximum protease yield, of 1356 U/ml, was obtained using the medium culture devoid of ions, as compared to all the other mixtures. However, the presence of Zn^{2+} and Cu^{2+} totally inhibited bacterial growth and, thus, the proteases production. Ibrahim et al. (2015) reported that the supplementation of the culture medium with Zn^{2+}, Cu^{2+}, Fe^{2+}, and Co^{2+} caused severe inhibition of proteases production by *Bacillus* sp. NPST-AK15, particularly at high cation concentrations. Various metal ions have been reported to affect the activity of proteases. In fact, Ca^{+2} and Mg^{+2} are known by their positive effect on the alkaline protease activity from *B. circulans* (Rao et al. 2009) and *Bacillus licheniformis* MP1 (Jellouli et al. 2011). Basu et al. (2008) reported the activation of protease from *Aspergillus niger* AB_{100} by metal ions, such as Ca^{+2}, Fe^{+2}, Zn^{+2}, and Mg^{+2}.

Table 2 Effect of $CaCl_2$ level on the proteases activity and biomass

$[Ca^{2+}]$ (g/l)	Activity (U/ml)	Biomass (CFU/ml × 10^9)	pH_f
0.0	1028.00 ± 0.51^c	342	8.70
1.0	1129.45 ± 22.62^b	404	8.51
2.0	1259.71 ± 12.85^a	412	8.32
2.5	1191.09 ± 0.00^d	387	8.76
3.0	728.25 ± 25.71^e	733	8.61

The initial pH of the culture was adjusted at pH = 9.0. pHf is the final pH value of the medium measured at the end of the reaction. Data are expressed as mean ± SD. Different letters in the same column indicate significant differences at $P < 0.05$

Factors affecting the production of proteases by SA

Temperature, agitation rate, and initial pH value of culture medium were investigated as the most important physical factors affecting the bacterial growth and enzyme production (Srividya and Mala 2011; Pathak and Deshmukh 2012) and results are summarized in Table 4.

Proteases production by *B. mojavensis* SA was studied at three different temperatures 30, 37, and 40 °C. Culture performed at 37 °C showed the maximum production level. However, at 40 °C, the activity lost more than 83% after 24 h of incubation, compared to the maximum level. Hence, *B. mojavensis* SA strain could be classified as a mesophilic bacterium. Similarly, the optimum temperature for protease production by a number of *Bacillus* species such as *Bacillus subtilis* strain (Abusham et al. 2009), *Bacillus aquimaris*

Table 3 Effect of metal ions, added at 0.5 g/l, on the proteases activity and biomass

Metal ion	Activity (U/ml)	Biomass (CFU/ml × 10^9)	pH_f
Control	1356.70 ± 44.90^a	412	8.32
$MgSO_4$	1175.66 ± 28.40^c	496	9.10
NaCl	953.87 ± 0.02^d	479	9.13
$MnSO_4$	649.39 ± 3.81^e	338	9.09
$BaCl_2$	1269.03 ± 11.60^b	367	9.01
$ZnCl_2$	0.00^f	130	7.96
$CuSO_4$	0.00^f	40	7.60

The initial pH of the culture was adjusted at pH = 9.0. pH_f is the final pH value of the medium measured at the end of the reaction. Data are expressed as mean ± SD. Different letters in the same column indicate significant differences at $P < 0.05$

Table 4 Effect of temperature, agitation rate and pH on the proteases activity and biomass after 24 h of incubation time

Parameters	rpm/ °C	Activity (U/ml)	Biomass (CFU/ml × 10^9)	pH_f
Temperature (°C)	30	1198.18 ± 12.85[b]	550	7.37
	37	1467.27 ± 95.13a	420	8.32
	40	240.00 ± 10.28c	80	8.97
Agitation rate (rpm)	150	989.27 ± 15.42[b]	460	8.71
	200	1388.38 ± 23.14[a]	420	8.32
pH_i	6	834.54 ± 2.57[c]	220	8.11
	7	850.90 ± 15.42[c]	250	8.43
	8	1067.27 ± 23.14[b]	390	8.6
	9	1463.63 ± 17.99[a]	420	8.32
	10	1432.72 ± 10.28[a]	370	8.62
	11	836.36 ± 5.14[c]	200	8.77
	12	705.45 ± 0.10[d]	190	8.9

For the effect of temperature and the agitation rate, the initial pH of the culture was adjusted at pH = 9.0. PHi and pH_f represent the initial and the final pH values of the medium, respectively. Data are expressed as mean ± SD. Different letters in the same column indicate significant differences at $P < 0.05$

VITP4 (Shivanand and Jayaraman 2009), *Bacillus proteolyticus* CFR3001 (Bhaskar et al. 2007) and *Bacillus amovivorus* (Sharmin et al. 2005) was found to be at 37 °C. These findings may be explained by the effect of temperature on monitoring enzyme synthesis at mRNA transcription (Votruba et al. 1991) and, thus, regulating the production of intracellular and extracellular enzymes. For extracellular enzymes, temperature influences their secretion, possibly by changing the physical properties of the cell membrane. Similarly, temperature strongly affects the secretion of proteases, influencing the rates of biochemical reactions.

Moreover, the effect of the agitation rate on the proteases production of *B. mojavensis* SA was studied (Table 4). Maximum protease production (around 1400 U/ml) was obtained after SA culture incubation at 37 °C and 200 rpm during 24 h. This activity was, however, markedly decreased at 150 rpm of agitation speed. These results indicated the importance of high aeration level for alkaline proteases' production by the studied isolate. This finding was in agreement with that reported by Ibrahim et al. (2015) and Joshi et al. (2008) for *Bacillus* sp. NPST-AK15 and halophilic bacterium MBIC3303, respectively. On the contrary, Nadeem et al. (2008) found that maximum alkaline protease production by *B. licheniformis* was obtained with agitation culture speed of 140 rpm.

The pH of culture is furthermore an important parameter to be optimized. In fact, pH affects all enzymatic processes and transportation of various components across the cell membrane (Sharma et al. 2017). As proton motive force in chemiosmosis is affected by the medium pH value, it is possible that under optimum pH range, the relative metabolic efficiency will be high (Singh et al. 2010). With respect to pH, *B. mojavensis* SA could grow and produce alkaline proteases over a wide pH range from 6.0 to 12.0, with optimum production obtained at pH 9.0, confirming the alkali nature of the SA proteases (Table 4). Under both acidic and alkaline pH

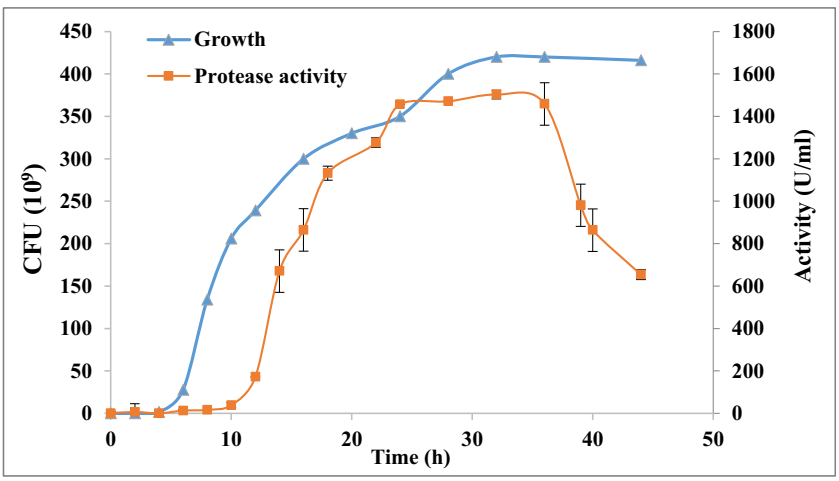


Fig. 5 Time course of proteases production and growth of *B. mojavensis* SA in the final medium. Shaking cultivation was carried at 37 °C. Proteolytic activity was determined in culture filtrate obtained after removal of cells by centrifugation

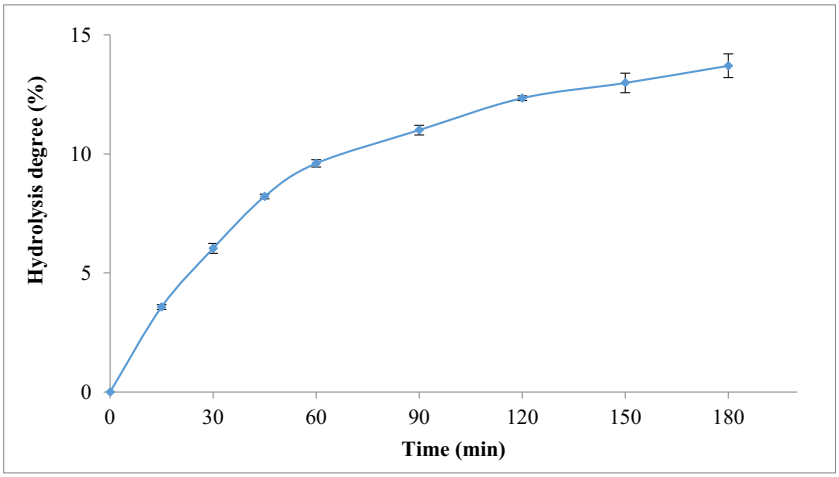


Fig. 6 Kinetic hydrolysis curve of MBH elaborated by the action of SA proteases

conditions, the bacterial growth and proteases production were significantly reduced, compared to those obtained at pH values, ranged between 8.0 and 10.0 ($P < 0.05$). Similar results have been reported by Chu (2007), Thumar and Singh (2007), and Bhaskar et al. (2007), who found that the alkaline proteases from *Bacillus* sp. strain APP1, *Streptomyces clavuligerus* strain Mit-1, and *B. proteolyticus* CFR3001, respectively, were produced at pH 9.0. Higher initial pH values, about 10.0 for *B. licheniformis* TISTR 1010 (Vaithanomsat et al. 2008), 10.5 for *B. circulans* (Jaswal et al. 2008), and 10.7 for *Bacillus* sp. 2–5 (Darani et al. 2008), have been reported for maximum proteases production.

Time course of proteases production by *B. mojavensis* SA and cell growth

Generally, the time required for the optimum proteases production by bacteria is comprised between 48 h to 9 days (Sharma et al. 2017). The effect of incubation time on proteases production and cell growth of *B. mojavensis* SA under the optimal conditions (pH 9.0, 37 °C and 200 rpm) is displayed in Fig. 5. As it can be seen, the proteolytic activity started to appear after 10 h of incubation, then, it was gradually increased during the exponential phase of bacterial growth and reached its maximum after 24 h (1456 U/ml). After 32 h of culture period, the activity gradually decreased to 653 U/ml at 44 h, during the stationary phase. In fact, the proteases production can be both growth and non-growth dependent. First, the protease production profile was found to be associated with growth, then the enzyme production peak occurred at the mid exponential phase (9 h), and thereafter the enzyme denaturation occurred, while the biomass reached its maximum level (Soares et al. 2005). Accordingly, Thumar and Singh (2007) and Lazim et al. (2009) showed that the maximal protease production started in early stationary phase of bacterial growth and then decreased with increasing incubation time.

Preparation of protein hydrolysate from MSB

Hydrolysate (MBH) from MSB was prepared using the crude enzymes of *B. mojavensis* SA under optimal conditions of protease activity (pH 12.0, 50 °C). The kinetic curve of hydrolysis is presented in Fig. 6. Hydrolysis profile was characterized by a high initial rate during the first minutes, which was subsequently decreased with reaction time and then the enzymatic reaction reached a steady-state phase after 120 min. The shape of hydrolysis curve could be explained by the action of inhibitory peptides, which were continuously solubilized during the hydrolysis and they may inhibit the enzyme action. The obtained HD value of 12% after 180 min of hydrolysis time reflected the presence of short peptides in MBH, which could serve as natural bioactive compounds (antioxidant, antibacterial, etc.). Microbial proteases have been widely used to obtain protein hydrolysates from many sources (Nasri et al. 2013; Lassoued et al. 2015).

Antioxidant potential of MBH

Peptides contained in the MBH were concentrated by the addition of ammonium sulfate (80%) followed by a dialyze step for 48 h to remove salts. The same treatment was applied for the undigested meat by-product (UMB).

DPPH• radical scavenging assay

The results presented in Fig. 7a indicated that MBH exhibited a significant radical scavenging activity that increased with increasing the concentration to reach 96% at 5 mg/ml, while the UMB did not show any antioxidant effect ($P < 0.05$). These results suggest that MBH contained some peptides that were hydrogen donors, which could react with free radicals to convert them to more stable products and terminate the radical chain reaction (Khantaphant and Benjakul 2008). Previous studies have reported that DPPH• radical scavenging activity

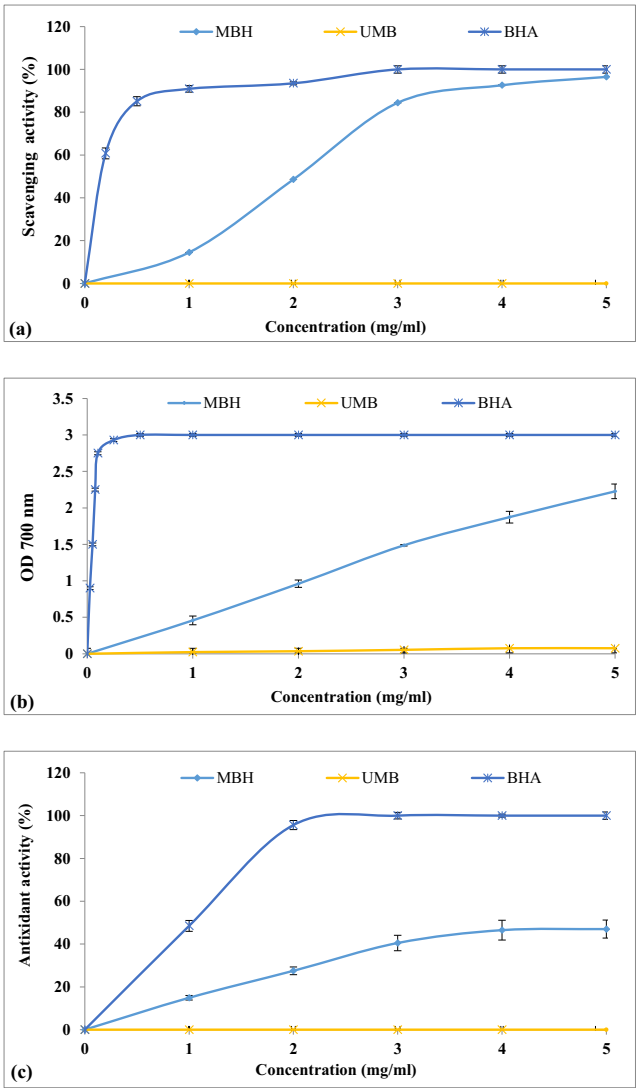


Fig. 7 DPPH• free radical scavenging effect (**a**), reducing power (**b**) and antioxidant activity using the β-carotene bleaching method (**c**) of MBH and UMB used at different concentrations. All values are given in mean ± standard deviation (SD) of three determinations. MBH and UMB represent meat sausage by-product hydrolysate and undigested meat by-product, respectively. BHA (butylated hydroxyanisole) was used as positive control

increased with the smallest peptides content present in the protein hydrolysate (Nasri et al. 2014).

Reducing power activity

The reducing power assay is often used to evaluate the ability of antioxidant to donate an electron Fe^{3+} ion (Khantaphant and Benjakul 2008). The reducing power of MBH and UMB as well as BHA, at different concentrations is shown in Fig. 7b. As expected, the reducing power values of MBH increased with increasing its concentration. However, the hydrolysate showed lower reducing power activity than did BHA at the tested concentrations. The obtained results indicate that MBH, with an HD of 12%, contained biopeptides that could serve as electron donors. It has been reported by Abdelhedi et al. (2016) that the highest reducing power was observed for the hydrolysate having a medium HD of 13.7%.

Antioxidant activity measured by the β-carotene bleaching method

In an oil-water emulsion-based system, linoleic acid acts as a free radical producer that generates peroxyl radicals under thermally induced oxidation. The preservation of the β-carotene color can be hindered by the presence of a stronger free radical scavenger. The antioxidant activity of MBH and UMB measured by β-carotene bleaching inhibition were reported in Fig. 7c. Except UMB, the MBH and BHA inhibited the oxidation of β-carotene in a dose-dependent way (47 and 99% at 5 mg/ml, respectively). These results demonstrated that MBH may contain peptides with hydrophobic character that can donate hydrogen atoms to peroxyl radicals of linoleic acid in an emulsified mixture.

The overall results proved that MBH contains peptides that exhibited interesting antioxidant activities, in terms of its radical scavenging activity, reducing power and β-carotene bleaching protection.

Conclusion

Due to the increasing economic relevance of enzymes, a part of this study was conducted in an attempt to screen a variety of fermentation parameters, including medium composition and culture conditions in order to maximize alkaline proteases production from *B. mojavensis* SA. In addition, the use of agro-industrial wastes (wheat bran and soya meal) as components in the bacterial growth medium offers an advantage in minimizing the production cost and maximizing the unities of proteases. The highest production level was obtained after 24 h under mesophilic conditions. This result would facilitate the economic design of large-scale fermentation operation system in bioreactor in batch/fed batch process. The second part of this study revealed the successful use of SA strain to produce bioactive protein hydrolysate from a meat sausage by-product. The resulting hydrolysate exhibited strong antioxidant activities. Therefore, this study provides a possible biotechnological application of *B. mojavensis* in food processing technologies.

Funding This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia.

Compliance with ethical standards

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

Informed consent Informed consent was obtained from all individual participants included in the study.

References

Abdelhedi O, Jridi M, Jemil I, Mora L, Toldrá F, Aristoy MC, Boualga A, Nasri M, Nasri R (2016) Combined biocatalytic conversion of smooth hound viscera: protein hydrolysates elaboration and assessment of their antioxidant, anti-ACE and antibacterial activities. Food Res Int 86:9–23. https://doi.org/10.1016/j.foodres.2016.05.013

Abusham RA, Rahman RNRA, Salleh AB, Basri M (2009) Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant *Bacillus subtilis* strain Rand. Microb Cell Factories 8:20. https://doi.org/10.1186/1475-2859-8-20

Adler-Nissen J (1986) A review of food hydrolysis specific areas. In J. Adler-Nissen (Ed.) Enzymic hydrolysis of food proteins. Elsevier Applied Science Publishers, Copenhagen, Denmark. pp 157-109

AOAC (2000). *Official methods of analysis* (17th ed.): Association of Official Analytical Chemists. Washington, DC

Basu BR, Banik AK, Das M (2008) Production and characterization of extracellular protease of mutant *Aspergillus niger* AB_{100} grown on fish scale. World J Microbiol Biotechnol 24:449–455. https://doi.org/10.1007/s11274-007-9492-6

Bersuder P, Hole M, Smith G (1998) Antioxidants from a heated histidine–glucose model system. I: investigation of the antioxidant role of histidine and isolation of antioxidants by high-performance liquid chromatography. J Am Oil Chem Soc 75:181–187. https://doi.org/10.1007/s11746-998-0030-y

Bhaskar N, Sudeepa ES, Rashmi HN, Selvi AT (2007) Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresour Technol 98:2758–2764. https://doi.org/10.1016/j.biortech.2006.09.033

Centenaro GS, Salas-Mellado M, Pires C, Batista I, Nunes ML, Prentice C (2014) Fractionation of protein hydrolysates of fish and chicken using membrane ultrafiltration: investigation of antioxidant activity. Appl Microbiol Biotechnol 172:2877–2893. https://doi.org/10.1007/s12010-014-0732-6

Chu WH (2007) Optimization of extracellular alkaline protease production from species of *Bacillus*. J Ind Microbiol Biotechnol 34:241–245. https://doi.org/10.1007/s10295-006-0192-2

Darani KK, Falahatpishe HR, Jalali M (2008) Alkaline protease production on date waste by an alkalophilic Bacillus sp. 2-5 isolated from soil. Afr J Biotechnol 7:1536–1542

DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356. https://doi.org/10.1021/ac60111a017

Dufossé L, De La Broise D, Guerard F (2001) Evaluation of nitrogenous substrates such as peptones from fish: a new method based on Gompertz modeling of microbial growth. Curr Microbiol 42:32–38. https://doi.org/10.1007/s002840010174

Gupta R, Beg QK, Khan S, Chauhan B (2002a) An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 60:381–395. https://doi.org/10.1007/s00253-002-1142-1

Gupta R, Beg QK, Lorenz P (2002b) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15–32. https://doi.org/10.1007/s00253-002-0975-y

Hammami A, Fakhfakh N, Abdelhedi O, Nasri M, Bayoudh A (2017) Proteolytic and amylolytic enzymes from a newly isolated *Bacillus mojavensis* SA: characterization and applications as laundry detergent additive and in leather processing. Int J Biol Macromol 24:56–68

Ibrahim ASS, Al-Salamah AA, Elbadawi YB, El-Tayeb MA, Ibrahim SSS (2015) Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes. Electron. J. Biotechnol 18:236–243. https://doi.org/10.1016/j.jbiotec.2015.06.026

Jaswal RK, Kocher GS, Virk MS (2008) Production of alkaline protease by *Bacillus circulans* using agricultural residues: a statistical approach. Indian J Biotechnol 7:356–360

Jellouli K, Ghorbel-Bellaaj O, Ben Ayed H, Manni L, Agrebi R, Nasri M (2011) Alkaline-protease from *Bacillus licheniformis* MP1: purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization. Process Biochem 46:1248–1256. https://doi.org/10.1016/j.nbt.2010.10.002

Jemil I, Jridi M, Nasri R, Ktari N, Ben Slama-Ben Salem R, Mehiri M, Hajji M, Nasri M (2014) Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from fish meat fermented by *Bacillus subtilis* A26. Process Biochem 49:963–972. https://doi.org/10.1016/j.procbio.2014.03.00

Joo HS, Chang CS (2005) Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. Process Biochem 40:1263–1270. https://doi.org/10.1016/j.procbio.2004.05.010

Joo HS, Kumar CG, Park GC, Kim KT, Paik SR, Chang CS (2002) Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. Process Biochem 38:155–159. https://doi.org/10.1016/S0032-9592(02)00061-4

Joshi RH, Dodia MS, Singh SP (2008) Production and optimization of a commercially viable alkaline protease from a haloalkaliphilic bacterium. Biotechnol Bioprocess Eng 13:552–559. https://doi.org/10.1007/s12257-007-0211-9

Kembhavi AA, Kulkarni A, Pant A (1993) Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No. 64. Appl Biochem Biotechnol 38:83–92. https://doi.org/10.1007/BF02916414

Khantaphant S, Benjakul S (2008) Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity. Physiol B, Biochem Mol 151:410–419. https://doi.org/10.1016/j.cbpb.2008.08.011

Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva LN (2002) Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem Anal 13:8–17. https://doi.org/10.1002/pca.611

Kumar RS, Ananthan G, Prabhu AS (2014) Optimization of medium composition for alkaline protease production by *Marinobacter* sp. GA CAS9 using response surface methodology—a statistical approach. Biocatal Agric Biotechnol 3:191–197. https://doi.org/10.1016/j.bcab.2013.11.005

Lassoued I, Mora L, Barkia A, Aristoy AC, Nasri N, Toldrà F (2015) Bioactive peptides identified in thornback ray skin's gelatin hydrolysates by proteases from *Bacillus subtilis* and *Bacillus amyloliquefaciens*. J Proteome 128:8–17. https://doi.org/10.1016/j.jprot.2015.06.016

Lazim H, Mankai H, Slama N, Barkallah I, Limam F (2009) Production and optimization of thermophilic alkaline protease in solid-state fermentation by *Streptomyces* sp. CN902. J Ind Microbiol 36:531–537. https://doi.org/10.1007/s10295-008-0523-6

Mazzucotelli CA, Durruty I, Kotlar CE, Moreira MR, Ponce AG, Roura SI (2014) Development of a microbial consortium for dairy wastewater treatment. Biotechnol Bioprocess Eng 19:221–230. https://doi.org/10.1007/s12257-013-0517-8

Meena P, Tripathi AD, Srivastava SK, Jha A (2013) Utilization of agro-industrial waste (wheat bran) for alkaline protease production by *Pseudomonas aeruginosa* in SSF using Taguchi (DOE)

methodology. Biocatal Agric Biotechnol 2:210–216. https://doi.org/10.1016/j.bcab.2013.05.003

Mehta VJ, Thumar JT, Singh SP (2006) Production of alkaline protease from an alkaliphilic actinomycete. Bioresour Technol 97:1650–1654. https://doi.org/10.1016/j.biortech.2005.07.023

Mhamdi S, Haddar A, Hamza Mnif I, Frikha F, Nasri M, Sellami Kamoun A (2014) Optimization of protease production by *Bacillus mojavensis* A21 on chickpea and faba bean. Adv Biosci Biotechnol 5:1049–1060. https://doi.org/10.4236/abb.2014.514120

Miller JH (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Mora L, Escudero E, Fraser PD, Aristoy MC, Toldrá F (2014) Proteomic identification of antioxidant peptides from 400 to 2500 Da generated in Spanish dry-cured ham contained in a size-exclusion chromatography fraction. Food Res Int 56:68–76. https://doi.org/10.1016/j.foodres.2013.12.001

Nadeem M, Qazi JI, Syed Q, Baig S (2008) Optimization of process parameters for alkaline protease production by *Bacillus licheniformis* N-2 and kinetics studies in batch fermentation. Turkish J Biol 32:243–251

Naidu KSB, Devi KL (2005) Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. Afr J Biotechnol 4:724–726

Nasri R, Chataigné G, Bougatef A, Chaâbouni MK, Dhulster P, Nasri M, Nedjar-Arroume N (2013) Novel angiotensin I-converting enzyme inhibitory peptides from enzymatic hydrolysates of goby (*Zosterisessor ophiocephalus*) muscle proteins. J Proteome 91:444–452. https://doi.org/10.1016/j.jprot.2013.07.029

Nasri R, Jridi M, Lassoued I, Jemil I, Ben Slama-Ben Salem R, Nasri M, Karra-Châaboun M (2014) The influence of the extent of enzymatic hydrolysis on antioxidative properties and ACE-inhibitory activities of protein hydrolysates from goby (*Zosterisessor ophiocephalus*) muscle. Appl Microbiol Biotechnol 173:1121–1134. https://doi.org/10.1007/s12010-014-0905-3

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000) Advances in microbial amylases. Biotechnol Appl Biochem 31:135–152

Pathak AP, Deshmukh KB (2012) Alkaline protease production, extraction and characterization from alkaliphilic *Bacillus licheniformis* KBDL4: a Lonar soda lake isolate. Indian J Exp Biol 50:569–576

Prakasham RS, Rao CS, Sarma PN (2006) Green gram husk an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation. Bioresour Technol 97:1449–1454. https://doi.org/10.1016/j.biortech.2005.07.015

Ramesh MV, Lonsane BK (1990) Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus licheniformis* M27 in a solid-state fermentation system. Appl Microbiol Biotechnol 33:501–505. https://doi.org/10.1007/BF00172541

Rao CS, Sathish RP, Prakasham RS (2009) Production of alkaline protease by *Bacillus circulans* using agricultural residues: a statistical approach. Process Biochem 44:262–268

Sandhya C, Nampoothiri KM, Pandey A (2005) Microbial proteases. In: Barredo JL (ed) Microbial enzymes and biotransformations, methods in biotechnology. Humana Press, León

Sharma KM, Kumar R, Panwar S, Kumar A (2017) Microbial alkaline proteases: optimization of production parameters and their properties. Journal of Genetic Engineering and Biotechnology 15:115–126. https://doi.org/10.1016/j.jgeb.2017.02.001

Sharmin S, Hossain MT, Anwar MN (2005) Isolation and characterization of a protease producing bacteria *Bacillus amovivorus* and optimization of some factors of culture conditions for protease production. J Biol Sci 5:358–362

Shirato S, Nagatsu C (1965) Fermentation studies with *Streptomyces griseus* I. Carbohydrate sources for the production of protease and streptomycin. Appl Microbiol 13:669–672

Shivanand P, Jayaraman G (2009) Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. Process Biochem 44:1088–1094. https://doi.org/10.1016/j.procbio.2009.05.010

Singh SK, Tripathi VK, Jain RK, Vikram S, Garg SK (2010) An antibiotic, heavy metal resistant and halotolerant *Bacillus cereus* SIU1 and its thermoalkaline protease. Microb Cell Factories 9:59. https://doi.org/10.1186/1475-2859-9-59

Sinsuwan S, Jangchud A, Rodtong S, Roytrakul S, Yongsawatdigul J (2015) Statistical optimization of the production of NaCl-tolerant proteases by a moderate halophile, Virgibacillus sp. SK37. Food Technol Biotechnol 53:136–145. https://doi.org/10.17113/ftb.53.02.15.4015

Soares V F, Castilho LR, Bon EPS, Freire DMG (2005) High-yield *Bacillus subtilis* protease production by solid-state fermentation. In: Davison B.H., Evans B.R., Finkelstein M., McMillan J.D. (eds) Twenty-sixth symposium on biotechnology for fuels and chemicals. ABAB Symposium. Humana Press

Srividya S, Mala M (2011) Influence of process parameters on the production of detergent compatible alkaline protease by a newly isolated *Bacillus* sp. Y Turkish J Biol 3:177–182. https://doi.org/10.3906/biy-0906-47

Tari C, Genckal H, Tokatli F (2006) Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Process Biochem 41:659–665. https://doi.org/10.1016/j.procbio.2005.08.012

Thumar JT, Singh SP (2007) Secretion of an alkaline protease from a salt-tolerant and alkaliphilic, *Streptomyces clavuligerus* strain MIT-1. Braz J Microbiol 38:766–772. https://doi.org/10.1590/S1517-83822007000400033

Uyar F (2004) Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. Process Biochem 39:1893–1898. https://doi.org/10.1016/j.procbio.2003.09.016

Vaithanomsat P, Malapant T, Apiwattanapiwat W (2008) Silk degumming solution as substrate for microbial protease production. Kasetsart J 42:543–551

Votruba J, Pazlarova J, Dvorakova M, Vachora L, Strnadova M, Kucerova H, Vinter V, Zourabian R, Chaloupka J (1991) External factors involved in the regulation of synthesis of an extracellular proteinase in *Bacillus megaterium*: effect of temperature. Appl. Microbiol Biotechnol 35:352–357. https://doi.org/10.1007/BF00172725

Yildirim A, Mavi A, Kara AA (2001) Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. J Agric Food Chem 49:4083–4089. https://doi.org/10.1021/jf0103572

Zambare V, Nilegaonkar S, Kanekar P (2011) A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. New Biotechnol 28:173–181. https://doi.org/10.1016/j.nbt.2010.10.002