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(Research Paper)

Improving antioxidant properties of fermented poultry slaughterhouse byproducts under proteolytic isolated bacteria

Reyhaneh Zakeri
Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
zreyhaneh70@gmail.com

Tayebeh Hadi toranposhti
Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
toranposhti.tayebeh.73@gmail.com

Fakhri Sadat Hosseini [1]
Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran

f.hosseini@alzahra.ac.ir

Abstract:
The disposal of slaughterhouse waste such as feather meal, blood meal, meat and bone meal and poultry meal poses biological problems. In addition, these wastes contain different amounts of proteins that can be used to improve the nutritional value of these low-value products through microbial processes. In this study, proteolytic isolates, slaughterhouse powder, blood meal, meat meal, and a mixture of meat and blood and bone meal were first isolated from slaughterhouse waste. Then, proteolytic isolates were identified and isolates with better protease properties than other isolates were used for fermentation of the waste. Immersion fermentation was performed at 30 °C and 150 rpm for 5 days by three isolates B4, C3 and A5 with better protease properties than other isolates. The protein content and degree of hydrolysis of the isolates were then measured in 4 sources of slaughterhouse powder, meat powder, blood meal and a mixture of meat and blood and bone meal from slaughterhouse waste. Antioxidant tests were performed based on DPPH radical scavenging and iron chelation methods. Finally, molecular techniques and phylogenetic trees were used to identify the selected strain. The results showed that all hydrolyzed protein wastes removed DPPH and chelated free radicals and had antioxidant properties, and hydrolysis of the wastes could be used for feeding.

Keywords: Antioxidant activity, Fermentation, Protein hydrolysis, Slaughterhouse waste

[1] Corresponding Author

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Introduction

The meat industry produces large amounts of inedible by-products such as offal and meat powder, skin, bones, venom, blood, etc. each year (1). These wastes are thrown away in the form of food, fertilizer, and animal food, processed or unused, sometimes causing environmental problems. The use of methods to produce valuable biological components from these wastes has been carried out on a small amount in various sections of the world. The presence of different types of proteins in different amounts makes these wastes suitable for protein hydrolysis. Researchers have paid much attention to these products due to the inherent biological activities of the peptides resulting from the hydrolysis of proteins (2). Bioactive peptides are defined as "derived (actually produced)" food compounds that have a physiological effect on the body in addition to their nutritional value (3). These peptides, with a molecular mass of less than 6 kDa and sizes ranging from 2 to 20 amino acids, can have different structures and amino acid sequences that affect their bioactive activity (4). These peptides, which are inactive in the parent protein sequence can be released in three different ways: a) proteolysis in the organism using digestive enzymes; b) proteolysis under laboratory conditions using commercial enzymes from plant, animal, or microbial sources;c) proteolysis by microbial fermentation (5). Microbial fermentation is a cheaper method of producing bioactive peptides than enzymatic hydrolysis due to the safety of the microorganisms, low cost of cultivation, short growth time and minimal nutritional requirements of the microorganisms (6). Antioxidant peptides are effective with different mechanisms such as inhibiting lipid peroxidation, eliminating free radicals, chelating metal ions and forming additional compounds. These antioxidant peptides, in addition to inhibiting oxidation, which increases the survival of body cells, are essential in food because they reduce the possibility of spoilage (7). Due to their better reaction with free radicals, low molecular weight peptides have greater antioxidant properties than high molecular weight peptides. This result has also been reported in various studies on peptides obtained from the hydrolysis of barley, meat and rapeseed proteins (8, 9, 10). A study of biomaterials derived from meat industry waste also showed that these biomaterials can remove heavy metals and have antibacterial properties. As they are not toxic to humans, they can be used in fish farming ponds (11). It has also been shown that hydrolyzed poultry by-products can be used to improve feed quality (12). Considering the antioxidant properties of bioactive peptides and their low-cost production, the aim of this study was to produce these bioactive peptides from poultry slaughterhouse waste and to investigate their antioxidant properties.

Materials and method

Isolation of the protease-producing bacterial strain

In order to isolate proteolytic isolates from poultry slaughterhouse powder (Sp), blood powder (Bp), meat powder (Mp), the mixture of meat powder and blood and bones of slaughterhouse waste (MBB), the following experimental steps were carried out**.** To isolate the microorganisms and transfer them to the liquid culture medium, a mixture of saline (0.9%) and Tween 80 (0.01%) was used for 30 h at 30 °C with a rotation of 150 rpm. Then a dilution series (10^{-1}-10^{-10}) was then prepared from the samples. From the dilutions, 100 µl were inoculated onto nutrient agar and incubated at 37 °C for 24 h. After the pure culture of the bacteria, 20 colonies were isolated. Then the isolates obtained after inoculation in 1% skim milk agar culture medium for 24 h at 37 °C were isolated on the basis of protease production. The selected strains were identified after studying the morphological characteristics and Gram staining.

Preparation of inoculum

The three isolates B4, C3 and A5 were grown in 50 ml nutrient broth for 18 h at 30 ± 2 °C in a shaking incubator at 150 rpm. Then, the initial culture medium suspension containing 1.5×10^{8} active bacteria, equivalent to half of McFarland was prepared for inoculation into the medium. For this purpose, after three washes in physiological serum, the turbidity was compared with the absorbance reading (OD: 0.08-0.13) at 620 nm at half McFarland.

Production of the enzyme

To investigate the culture conditions, growth was carried out in a basic medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$ (pH 7) and supplemented with 0.5-2% (w/v) of different carbon sources including Sp, Bp, Mp and MBB.

Immersion fermentation was carried out at 30 °C and 150 rpm for 5 days by three isolates B4, C3 and A5. After centrifugation (12,000 g, 4 °C, for 20 min), the supernatant was collected for measurement of antioxidant activity (13).

Evaluation of the degree of hydrolysis (DH)

The OPA **(**O-phthaldialdehyde**)** method was used to estimate the degree of hydrolysis of the hydrolyzed product (14, 15). A small sample (10 µl) was added to 1 ml of OPA solution and homogenized for 5 sec. After 2 min, the absorbance of the mixtures was measured at 340 nm. The standard curve of L-leucine (0.1 mg/ml) was used to determine the amount of α-amino acids**.** DH was calculated using equation 1 and expressed as a percentage:

$DH = h/ h_{tot} \times 100\%$ Eq.1

$h = (Serine\text{-}NH_2\text{-}\beta)/\alpha$

$Serine\text{-}NH_2 = Abs_{sample} - Abs_{control} / Abs_{serine} - Abs_{control} \times 0.9516 \times (0.1 \times 100/(W \times P))$

where Abs_{sample} is the absorbance of the fermented samples, $Abs_{control}$ is the absorbance of the control, Abs_{serine} is the absorbance of the serine standard, W is the weight in grams of the SPH sample in 100 ml and P is the protein content (%) of the sample. Nielsen, Petersen and Dombman (2001)(14) stated that for fish the constant values for the number of equivalent peptide bonds per gram of protein (h_{tot}), α and β are 8.6 meq g^{-1}, 1 and 0.4, respectively, so these values were used.

Antioxidant activities

Antioxidants prevent the spread of oxidation chain reactions by donating a hydrogen atom or transferring an electron to free radicals formed in the environment, thus neutralizing the free radicals (16). DPPH (2,2-diphenyl-1-picrylhydrazyl) and iron chelation were used to evaluate the antioxidant capacity of the samples.

DPPH radical-scavenging assay

At first, 150 µl of the sample was mixed with 37.5 µl of methanolic solution containing 0.75 mM DPPH radical. After incubation in the dark for 30 min, the absorbance was read at a wavelength of 517 nm. Ascorbic acid was used as a standard (13). The scavenging ability was calculated according to equation 2:

Scavenging ability (%) = [(A_{517} of control-A_{517} of sample)/ A_{517} of control] ×100

Iron (II) chelating activity assay

First, 3.7 ml of distilled water, 0.1 ml of $FeSO_4$ (Fe^{2+}) (2 mmol/l), and 0.2 ml of ferrozine (5 mmol/l) were mixed with 1 ml of the sample. After 10 minutes absorbance was read at 562 nm. EDTA (20 mg/ml) was used as standard (17). The results were expressed as chelating activity (%) using equation 3:

chelating activity (%) = [(A_0-A) /A_0]×100,

where A is the absorbance of the test and A_0 is the absorbance of the control.

Amino acid analysis

After evaluation of the results, isolate C3 was selected as the selected strain and the remaining steps were performed with this isolate. In order to evaluate the amino acids in the hydrolyzed protein sample under optimal conditions, a high-performance liquid chromatography (HPLC) device manufactured by Waters was used. During the test, the column was exposed to 38 °C. The solvent system consisted of 60% acetonitrile and soluble buffer. Containing 0.14 M sodium acetate with 0.5 ml/l TEA (triethylamine) and was performed at pH 6.4 with acetic acid**.** A gradient elution was performed from 10% soluble buffer to 51% in 10 min using a convex curve. This was followed by a wash step with 100% soluble buffer to remove any residual sample components from the column. (18).

Identification of isolate C3 by 16S rRNA and phylogenetic tree drawing

The isolate C3 was selected for molecular identification and phylogenic tree drawing. The

stages of DNA extraction, quantitative and qualitative evaluation of DNA by electrophoresis and nanodrop, amplification of 16S rRNA gene with universal primers, and determination of its sequence were carried out by the company Geneiran. DNA extraction was performed according to the instructions of the kit manufacturer (Cinagene). Briefly, 1.5 ml of the overnight culture was centrifuged at 8000 rpm for 5 min and 100 µl of lysis solution was added to the resulting precipitate and vortexed for 15 sec. 300 µl of precipitation solution was added to the vial,vortexed, and centrifuged at 8000 rpm for 10 min. The supernatant was transferred to the column and then centrifuged at 12000 rpm for 1 min. Then, 400 µl of Wash Buffer I solution and 400 µl of Wash Buffer II solution were added to the column, respectively, and centrifuged at 12,000 rpm for 1 min in each step. The column was transferred to a new 1.5 ml vial, then 50 µl of Elution buffer was added and the vial was centrifuged at 12,000 rpm for 1 min. PCR was then performed using universal primers U1492R and B27F. The resulting sequences were integrated and edited using BioEdite software. The results obtained were compared with the 16S rRNA sequences of other similar bacteria in the NCBI database through the BlastN program, and the percentage of similarity and identity was checked. After checking the homologous sequences, some of them with high similarity to the desired sequence were selected. Then, the selected sequences were used by the MEGA 7 software to draw the phylogeny tree and determine the identity of unknown isolates.

Statistical analysis

Analysis of all data, including Shapiro-Wilk, Kolmogorov-Smirnov and histogram data for normality, was carried out using software (GraphPad Prism version 8). Finally, all normal data were analyzed using a t-test (and non-parametric tests) with a P-value <0.05. The results are presented as mean ± SEM.

Result

Identification of proteolytic isolates

Two isolates (B4 and B1) out of 4 isolates from the Bp source, one isolate (M3) out of 4 isolates from the Mp source, two isolates (C3 and C4) out of 8 isolates from the Sp source and three isolates (A1, A2 and A5) out of 5 isolates from the MBB source showed better protease activity than other isolates after culture on 1% skim milk agar. The results of microscopic examination and Gram staining are shown in Table 1. After the investigations, 3 isolates B4, C3 and A5 were selected as the best proteolytic isolates and used in the next steps (Fig. 1).

Determination of the degree of hydrolysis (DH)

Based on the Kjeldahl method, the initial protein content of the samples was Sp 58.8%, Bp 58.6%, Mp 56.4%, and MBB 55.9%. To investigate the amount of protein hydrolysis and its consumption as a nitrogen source by microorganisms, the degree of protein hydrolysis was calculated using the OPA reagent method and is shown in Fig. 2.

Table 1. Morphological characteristics of isolates with protease activity from Bp, Mp, Sp, and MBB sources.

The names of the isolates	A5	A2	A1	C4	C3	M3	B4	B1
Source	MBB	MBB	MBB	Sp	Sp	Mp	Bp	Bp
Shape	Bacillus	Bacillus	Cocci	Bacillus	Bacillus	Bacillus	Cocci	Bacillus
Gram	+	+	+	+	+	+	+	+
Spore	p	p	n	p	n	p	n	p

Gram-positive isolates are marked with a + sign and gram-negative isolates with a - sign. The P (positive) sign indicates the presence of spores and the n (negative) sign indicates the absence of isolated spores.

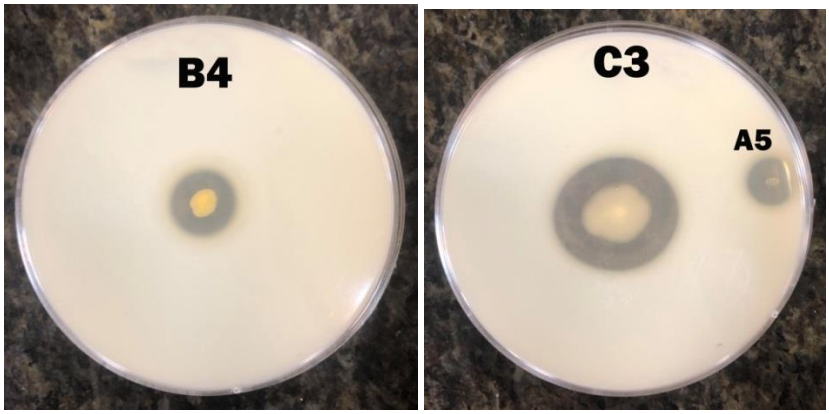

Fig. 1. Selective proteolytic isolates A5, B4 and C3 were cultured on 1% skim milk agar medium obtained from three sources, MBB, Bp, and Sp, respectively.

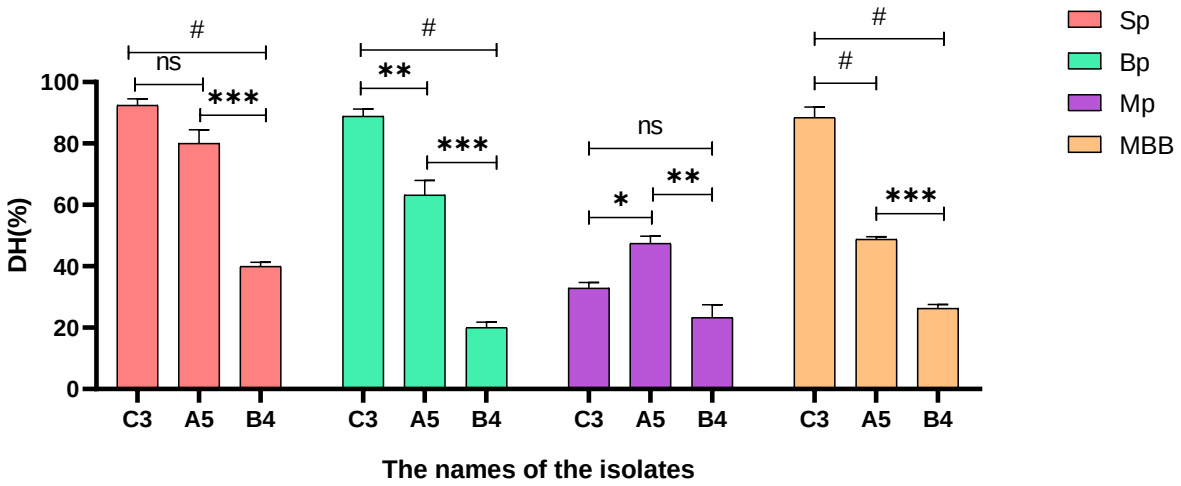

Fig. 2. It shows the amount of protein hydrolysis in Sp, Bp, Mp, and MBB with isolates B4, A5, and C3. * indicates a significant difference (P<0.05), ** indicates a significant difference (P<0.01), *** indicates a significant difference (P<0.001), # means a difference indicates a difference (P<0.0001) and ns indicates that there is no significant difference. Symbols (*,**,***,#, and ns) indicate significant differences between isolates in a protein source.

Antioxidant activities

DPPH radical-scavenging assay

The free radical DPPH, when confronted with a hydrogen-donating substrate such as an antioxidant, causes radical scavenging and a decrease in absorbance (19). The decrease in absorbance is used as a measure of radical-scavenging activity. Fig.3 shows the DPPH radical-scavenging activity of hydrolyzed protein waste. The results show that the 3 selected isolates in 4 hydrolyzed protein sources have antioxidant activity and show a significant difference from the blank (no bacteria**)**.

Iron (II) chelating activity assay

The ability of protein hydrolysates to chelate metals was assessed by Fe^{2+} chelation. Ferrozine quantitatively forms complexes with Fe^{2+} ions. In the presence of chelating agents, complex formation is disrupted, resulting in a decrease in color formation (20). The ferrous chelating activity of hydrolyzed protein waste and EDTA used as a reference chelating agent, is shown in Fig. 4. According to the results, hydrolyzed protein waste can chelate Fe^{2+} ions.

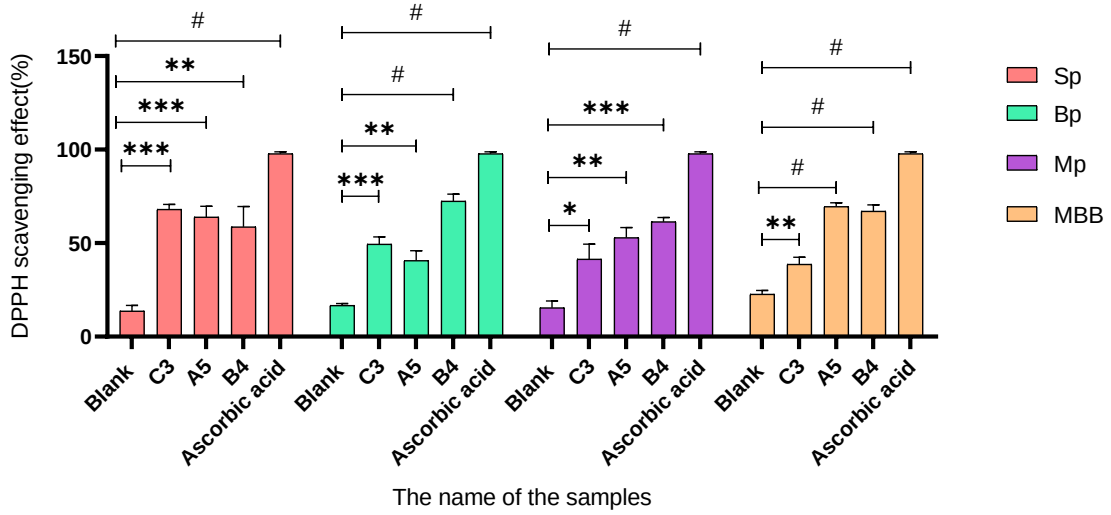

Fig. 3. Comparison of antioxidant activity with DPPH test in protein hydrolyzed from fermented Sp, Bp, Mp and MBB with isolates B4, A5 and C3. * indicates a significant difference (P<0.05), ** indicates a significant difference (P<0.01), *** indicates a significant difference (P<0.001), # means a difference indicates a difference (P<0.0001). Symbols (*,**,***,#) indicate significant differences between isolates in a protein source.

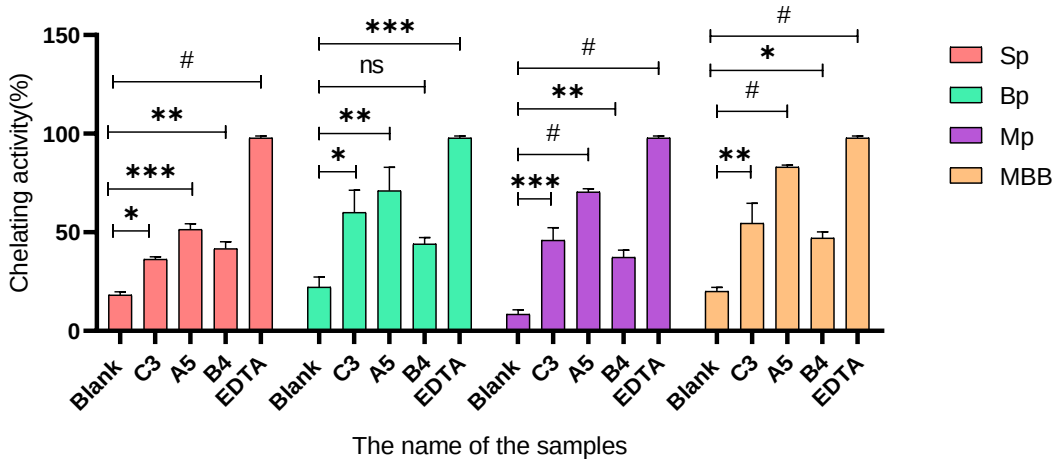

Fig. 4. Comparison of antioxidant activity with iron (II) chelating activity in protein hydrolyzed fermented in Sp, Bp, Mp, and MBB with isolates B4, A5, and C3. * indicates a significant difference (P<0.05), ** indicates a significant difference (P<0.01), *** indicates a significant difference (P<0.001), # means a difference indicates a difference (P<0.0001) and ns indicates that there is no significant difference. Symbols (*,**,***,#, and ns) indicate significant differences between isolates in a protein source.

Amino acid analysis

The total amino acid and free amino acid analysis results are shown in Tables 2 and 3.

Identification of isolate C3 and phylogenetic tree

After examining the results and the better results of C3, this isolate was analyzed for molecular identification by 16S rRNA. After BLAST search, the sequence of the C3 isolate showed that the C3 isolate was 100% similar to the *Bacillus cereus* strain. The phylogenetic tree of this isolate is shown in Fig. 5.

Table 2. Amino acids in protein hydrolyzed from fermented Bp of livestock waste with C3 isolate (mg AA / g sample)

Amino acid name	Total amino acid	Free amino acid
Asp	0.32	0.07
Glu	0.43	0.16
Ser	0.08	<0.01
Gly	0.36	0.17
His	0.03	<0.01
Arg	0.16	0.07
Thr	<0.01	<0.1
Ala	0.25	0.11
Pro	0.28	0.09
Tyr	0.33	0.08
Val	0.24	0.06
Met	0.04	<0.01
Cys	<0.01	<0.01
Ile	0.1	0.02
Leu	0.11	0.02
Phe	0.19	0.03
Lys	0.21	0.04
Total	0.31	0.09

Table 3. Amino acids in protein hydrolyzed from fermented poultry Sp with C3 isolate (mg AA / g sample)

Amino acid name	Total amino acid	Free amino acid
Asp	0.31	0.14
Glu	0.35	0.17
Ser	0.11	0.07
Gly	0.27	0.14
His	0.07	<0.01
Arg	0.13	0.07
Thr	0.07	<0.01
Ala	0.21	0.03
Pro	0.23	0.04
Tyr	0.34	0.06
Val	0.11	0.04
Met	0.09	0.02
Cys	<0.01	<0.01
Ile	0.07	0.01
Leu	0.08	0.02
Phe	0.08	0.03
Lys	0.11	0.04
Total	0.26	0.08

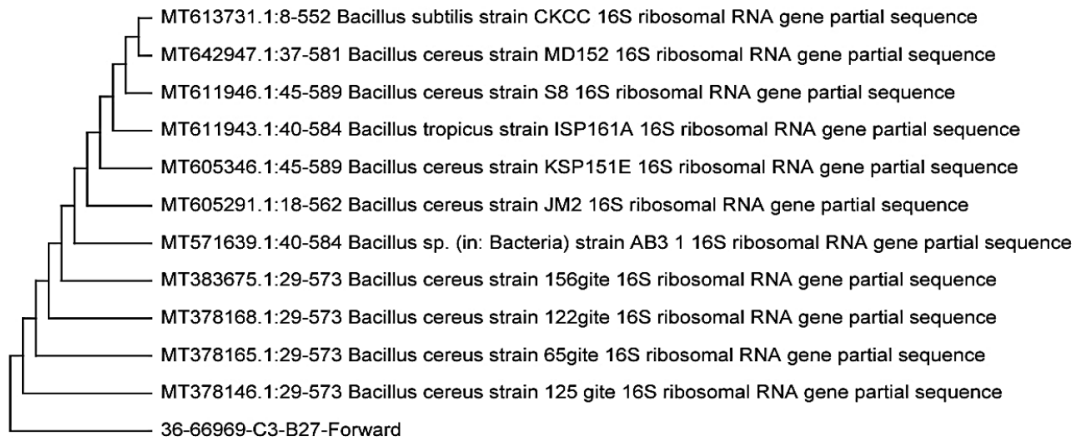

Fig. 5. A phylogenetic tree of a bacterial C3 isolate based on the nucleotide sequences of the 16S rRNA gene.

Discussion

Enzymes (such as proteases) are secreted by certain bacteria species in the extracellular growth medium that is the basis of this method; therefore, proteolysis and peptide production are possible after inoculating protein material with this type of bacterial cell (21). In fermentation processes, microorganisms grow well in a growing environment that meets their nutritional needs. The fermentation medium consists of a series of minimum and maximum materials carbon sources are among the maximum sources and will provide growth as long as they are available to the microorganisms. Laboratory carbon sources are generally pure, which is costly to use on an industrial scale. Other materials for microbial growth in culture medium include nitrogen, oxygen (if aerobic), and hydrogen in organic and inorganic forms. Trace elements such as iron and phosphorus are also required for growth (22). Waste can also be used as a carbon source, but in this study, in order for the bacteria to grow well at the start of fermentation and produce a protease enzyme for protein hydrolysis, 2% glucose was used, which is a good carbon source for all microorganisms. With a suitable and easily accessible carbon source, the bacteria grow well until the glucose runs out and the bacteria have to adapt to the new carbon source. Different sources have used different carbon sources for the growth of microorganisms during fermentation. Kherrati *et al*. (1997) used molasses as a carbon source to hydrolyze a mixture of slaughterhouse and poultry waste. This fermentation is a solid substrate and was carried out by *Lactobacillus plantarum* (23). Jemil *et al*. (2016) in the fermentation of fish meat, the meat was prepared in powder form and used as a source of carbon and nitrogen by *Bacillus* (24). Swarnalee Dutta *et al*. (2016) also used the bacterium *Bacillus leuciformis* to fermentation and hydrolysis of pork waste proteins (25). It was found that the degree of protein hydrolysis in Bp was much higher during fermentation with A5 bacteria than during fermentation with B4 bacteria. In Mp, the highest degree of hydrolysis is associated with A5 bacteria and has a significant increase compared to other bacteria. MBB also has the highest percentage of hydrolysis by A5 bacteria and is significantly different from other bacteria. Of all the substrates, the lowest amount of hydrolysis is associated with B4 bacteria, in other words, the lowest amount of peptide production is seen in the luminescence results of fermentation of these bacteria.

It is evident from the results presented that all the fermented protein hydrolysates tested exhibited high antioxidant activity in response to DPPH. Examination of the antioxidant activity of collagen and creatine samples from various animal sources (female beef, fishbone, chicken feathers and horn of female cows) showed that all sections had antioxidant activity for DPPH radical scavenging (26). All hydrolysates contain peptides that can transform them into more stable products by donating electrons and reacting with free radicals and terminating the radical chain reaction, as shown by the results obtained (27). Due to the difference in peptide length and also the difference in amino acid composition of peptides in protein hydrolysates, there is a difference in radical scavenging ability. Previous studies have found that high DPPH radical scavenging activity for protein hydrolysates or peptides is usually associated with highly hydrophobic amino acids (28). Similar

results have been reported for protein hydrolysates from mackerel (*Scomber austriasicus*) (29) and round scad (*Decapterus maruadsi*) (30), which have also been reported to possess DPPH radical scavenging activity. This result was similar to the results of studies by San-Lang Wang *et al*. (2009), who showed that after fermentation of fish and shrimp waste for 5 days by *Bacillus cereus*, the ability to radically inhibit DPPH is about 65% (31). Umayaparvathi *et al*. (2014) also showed that oyster peptides hydrolyzed by *Bacillus cereus* protease have the radical scavenging properties of DPPH (32).

In general, the radical scavenging activity of DPPH does not follow a specific pattern in relation to the degree of protein hydrolysis, because antioxidant compounds such as linoleic acid, conjugates, pigments, etc. are also present in meat and waste, or because fermentation releases other antioxidant pigments that are not necessarily subject to protein hydrolysis. However, most studies have reported that as the degree of hydrolysis and production of bioactive peptides increases, so do the antioxidant properties. To provide more accurate evidence, it is necessary to purify, separate, and sequence the peptides produced during fermentation in order to more confidently attribute these effects to the peptides produced during fermentation.

Transition metal ions are known to be involved in many oxidation reactions in the body. Iron ions (Fe^{2+}) can cause the formation of hydroxyl radicals by catalyzing the Haber-Weiss reaction. Then the hydroxyl radicals can cause severe damage by reacting with nearby biomolecules. Ferrous ion is one of the products formed in the Fenton reaction, in which hydrogen superoxide produces hydroxyl radicals (33). Antioxidants are reported to scavenge hydroxyl radicals mainly by chelating metal ions. The peroxidative process could be affected by a compound that interferes with the catalytic activity of metal ions, and to evaluate the free radical scavenging activity of a compound, it is important to measure its chelating ability (34, 35).

To determine the metal chelating activities in this study, the rate of reduction of red color was measured, which was quantitatively formed by ferrozine with ferrous ions. All the fermented protein hydrolysates tested exhibited high antioxidant activity against ferrous ions, as indicated by the results presented. However, its metal chelating activity was significantly lower than that of EDTA, which had the strongest chelating capacity, reaching 97.82% at 0.1 mg/ml. The ability to chelate metals can play a role in antioxidant activity and impact other functions that contribute to antioxidant activity (36, 29). Accordingly, it is suggested that the chelating effect of all fermented protein hydrolysates on ferrous ions might be the mechanism of free radical scavenging and will be somewhat beneficial in exerting protection against oxidative damage.

Tables 2 and 3 present the amino acid profiles of fermented Bp from livestock waste and fermented poultry Sp with C3 isolate. Some amino acids consisting of His, Tyr, Met and Cys were reported to have antioxidant activity. Particularly, histidine exhibited robust radical scavenging activity because of the degradation of its imidazole ring (37). Amino acids also exhibit a wide range of biological activities, including the ability to activate nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that regulates endogenous antioxidant responses (38). In general, hydroxyl radicals produced during the oxidation of unsaturated fatty acids are neutralized by hydrophobic amino acids such as leucine, valine, alanine and proline. Amino acids such as histidine, leucine, tyrosine, methionine and cysteine inactivate free radicals by donating protons. Aromatic amino acids (phenylalanine, tryptophan and tyrosine) convert free radicals into stable molecules by donating electrons (5). Therefore, the antioxidant activities of fermented Bp of livestock waste and fermented poultry slaughterhouse powder with C3 isolate appeared to be due to these amino acids within the peptides. Furthermore, the antioxidant activity of the fermented Bp of livestock waste and the fermented poultry slaughterhouse powder with C3 isolate depended on the amino acid sequence of the peptides.

Conclusions

Given the importance of environmental protection and the challenges associated with the disposal of slaughterhouse waste, utilizing these by-products can be a key solution to address such issues. The study demonstrated that slaughterhouse waste, often considered to be of low value, contains

bacteria with proteolytic properties that can effectively hydrolyze protein substrates after fermentation. Additionally, these wastes contain varying amounts of proteins which, through enzymatic hydrolysis, have high antioxidant properties and can potentially be used as animal feed.

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