Valorization of tomato waste proteins through production of antioxidant and antibacterial hydrolysates by proteolytic *Bacillus subtilis*: optimization of fermentation conditions

Ali Moayed¹ · Maryam Hashemi² · Mohammad Safari^{1,3}

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Abstract In this study, protein-rich waste of tomato processing industries was fermented by *Bacillus subtilis* A14h to produce hydrolysates with antioxidant and antibacterial activities. The effects of different levels of initial pH, incubation temperature, fermentation time, protein concentration and inoculum size on proteolytic activity, release of amino acids and peptides, antioxidant and antibacterial activities of hydrolysates were evaluated and optimized by using response surface methodology (RSM). Results showed that all the evaluated variables significantly influenced the hydrolysis and bioactivities of hydrolysates in polynomial models. Hydrolysates showed remarkable 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (up to 70 %), ferric ion reducing power, and inhibitory activity against *B. cereus* (up to 69.8 %) and *E. coli* (up to 29.8 %). Overall, good correlation between the concentration of amino acids and peptides, and antioxidant as well as antibacterial activities (in particular for *B. cereus* inhibition activity) was observed. Finally, optimum conditions for fermentative conversion of tomato waste proteins to antioxidant and antibacterial hydrolysates were established. Results

of this study showed that tomato waste protein can be valorized to produce antioxidant and antibacterial hydrolysates in a fermentative system using *B. subtilis* A14h.

Keywords Bioactive peptide · Tomato seed protein · *B. subtilis* A14h · Antioxidant activity · Antibacterial activity

Introduction

During the last decades many researches have been performed to address the conversion of agricultural and food industries wastes to the new products with added value. In this case, protein-rich materials were of interest to be recovered or converted to more useful valuable products such as protein concentrates (Sari et al. 2013) and bioactive peptides. In general, bioactive peptides contain 3–20 amino acid residues with diverse potentials such as antioxidant, immunomodulatory, antimicrobial, antihypertensive and anticoagulant effects (Udenigwe and Aluko 2012). The type of activities depends on their amino acid composition and sequences which are affected by the source of protein and hydrolysis conditions (Shahidi and Zhong 2008).

Regard to the health-related concerns raised from application of synthetic antioxidants in foods during the recent years, antioxidant peptides are of great interest. Wide spread antibiotic resistance genes in pathogens, and the tolerance of some food deteriorating microorganisms and food-borne pathogens to the conditions applied in the preservation of foods (making acidic and salted conditions) are among the main reasons of searching for new antimicrobial compounds including antimicrobial peptides (Shahidi and Zhong 2008). Compared to conventional antibiotics and chemical food preservatives antimicrobial peptides have some advantages such as faster

✉ Maryam Hashemi
hashemim@abrii.ac.ir

✉ Mohammad Safari
msafari@ut.ac.ir

¹ Department of Food Science, Engineering and Technology, University of Tehran, Karaj, Iran

² Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology Research Institute of Iran (ABRII), AREEO, P.O. Box 3135933151, Karaj, Iran

³ Center of Excellence for Application of Modern Technology for Producing Functional Foods and Drinks, University of Tehran, Karaj, Iran P.O. Box 4111, 31587-77871

bactericidal effects, maintaining the organoleptic properties of foods and fewer adverse effects (Shahidi and Zhong 2008).

Production of antibacterial, antihypertensive and antioxidant peptides from muscle, skins and waste of fishes and shrimps (Dey and Dora 2014; Guerard et al. 2007; Lee et al. 2014; Naqash and Nazeer 2013; Sila et al. 2014), algae protein waste (Sheih et al. 2009), some legumes (Boschin et al. 2014; Siow and Gan 2013), and meat industry by-products (Bhaskar et al. 2007) are examples of some investigations carried out to produce bioactive peptides from food wastes and by products. However in most of such researches, commercial proteases or combination of several proteolytic enzymes have been employed to obtain reasonable hydrolysis. In addition to the specificity of many commercial and pure proteases, they are expensive and their industrial application to convert high amounts of protein substrates to new value added products would not be economically efficient. Among the strategies used to hydrolyze proteins to bioactive peptides, microbial fermentation seems to be more cost effective and environment-friendly. Jemil et al. (2014) studied the functionalities of protein hydrolysates from fish meat after fermentation process. Balakrishnan et al. (2011) compared the antioxidant and antimicrobial activities of the hydrolysates obtained from proteins of delimed tannery fleshing after lactic fermentation and acidic treatments and observed that those from fermentation had higher scavenging activities.

Several bacteria and molds are known to have considerable proteolytic activities and may be employed to hydrolyze proteins. *Bacillus subtilis*, known as grass bacillus (Zhang et al. 2014) is traditionally used to process fermented soybean foods due to its potent proteolytic activity. *B. subtilis* is able to excrete different endopeptidases in fermentation media (Zhang et al. 2014). The activities of various endopeptidases in a medium containing proteins may result in the generation of different small peptides and free amino acids probably at a higher amounts than enzymatic hydrolysis. This may be one of the advantages of employing highly proteolytic bacillus species over pure enzyme.

In Iran, tomato is one of the main crops with wide applications in food industries, households and restaurants. According to the statistics of food and agriculture organization (FAO), 6,000,000 tones tomato was produced in Iran in 2012. It has been reported that tomato processing plants produce 7–7.5 % solid wastes, of which 70–72 % is pomace. Seeds are the main part of the pomace as they constitute more than 33 and 44 % of wet and dried pomace, respectively (Sogi et al. 2002). Tomato seed has been reported to contain 20–30 % protein (Sogi et al. 2002).

To the best of our knowledge, no study has been published on the hydrolysis of protein fraction of tomato seed to obtain bioactive hydrolysates. Therefore, in the current study, the fermentation of de-fatted tomato waste protein by means of *B. subtilis* A14h was optimized in a submerged fermentation system to produce bioactive hydrolysates. At first, we

performed a factorial test in order to evaluate the proteolytic activities, hydrolysis kinetic, choosing the proper temperature and pH level, as well as to determine the suitable ranges of variable factors for the optimization step. By using the response surface methodology (RSM), the effects of fermentation parameters (inoculum concentration, protein content and time) on trends of hydrolysis, antibacterial properties, and antioxidant capacities of the resulting hydrolysates were investigated.

Materials and methods

Materials

Tomato pomace was obtained from Golgashte Shirin plant (Ghazvin, Iran). The skin was removed by immersing pomace in water, and the remaining seeds were sun-dried. The dried seeds were ground in a Moulinex blender and defatted by the soxhlet method using n-hexane as the solvent. Then, the powder was dried and further ground to pass a 40-mesh sieve. The powdered tomato seed meal (TSM) was kept in the refrigerator until to be used. 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich (Darmstadt, Germany). All the chemicals and solvents used in this study were of analytical grade. The cells of *B. subtilis* A14h, *Escherichia coli* O157 and *B. cereus* were obtained from the microbial collection of Agricultural Biotechnology Research Institute of Iran (ABRII).

Chemical compositions of tomato seed

The moisture and ash contents were determined according to AOAC methods of 925.40 and 923.03, respectively (Helrich 1990). The Kejehldal method with the conversion factor of 6.25 (Al-Wandawi et al. 1985) was used for determination of protein content in the tomato seeds and TSM. Total oil content in the tomato seed was determined using a Soxhlet apparatus. Total Carbohydrate content was obtained by subtracting the percentages of other components (protein, moisture, lipid and ash) from 100.

Bacterial culture

The *B. subtilis* A.14 h showed high protease activity and was recommended in a previous screening study evaluating protease activities of *B. subtilis* strains isolated in Iran. Cultures of *B. subtilis* A14h were grown in the nutrient broth medium (containing 3 % peptone, 0.5 % beef extract, 0.5 % NaCl, and 0.1 % glucose) and incubated at 30 °C for 18 h under agitation conditions (160 rpm). Then, the cells of *B. subtilis* A14h was harvested by centrifugation at 3800 rpm (Suprema 25, TOMY, Japan) for 20 min at 15 °C and resuspended in

sterile physiological serum saline solution (to reach around 10^8 CFU/mL) before inoculating to the fermentation media.

Fermentation and preparation of TSM hydrolysates

TSM was used as the only source of carbon and nitrogen in a submerged system. The submerged fermentation was carried out in 250 mL Erlenmeyer flasks with working volume of 25 mL. The initial medium used for the production of TSM hydrolysates contained TSM (5 %w/v for the first part of study, and different concentration in RSM section), K_2HPO_4 (0.05 %w/v), $MgSO_4$ (0.01 %w/v). The culture medium was autoclaved at 121 °C for 15 min. $CaCO_3$ was also separately autoclaved and added to the media (0.16 %w/v) after media was cooled. To evaluate the effects of fermentation conditions on the production and activities of hydrolysates, different levels of initial pH (7, 8 and 9) and incubation temperature (30, 37 and 40 °C) were chosen. Periodically (at times 8, 12, 20, 24 and 48 h), two aliquots of 1 ml were removed from each flask. One sample from each flask was centrifuged at 12,000 rpm (Suprema 25, TOMY, Japan) for 10 min at 4 °C to evaluate protease activity in the supernatant. The fermentation process was stopped in the other aliquot by heating the samples in boiling water for 15 min. Then, the samples were centrifuged at 12,000 rpm (Suprema 25, TOMY, Japan) for 10 min and the supernatants were collected and stored at freezing conditions until be used in the experiments.

Protease activity

The protease activity was measured according to the method previously described by Kambhavi et al. (1993) with slight alteration. Briefly, 250 µL of 1 % casein solution (w/v, in 50 mM sodium phosphate buffer with pH 7.5) was mixed with 250 µL diluted crude enzyme (fermentation supernatant) and incubated at 47.5 °C for 20 min. In order to stop the enzymatic reaction, 250 µL of 10 % (w/v) trichloroacetic acid (TCA) was added to the mixture and allowed to stand at ambient temperature for 20 min. Then, the mixture was centrifuged at 13,000 rpm (Biofugepico, Kendro, Germany) for 10 min and the absorbance of clear supernatant phase was read at 280 nm. A standard curve of different concentrations of L-tyrosine (0.0 to 0.2 mg/mL) was made for calculating protease activity which was reported as unite/mL (U/mL). One unite of enzyme activity was defined as the amount of enzyme needed to release one microgram of L-tyrosine per minute under the mentioned conditions.

Concentration of amino acids and peptides

The concentration of free amino acids and peptides (as L-leucine equivalent) was measured to evaluate the kinetic of hydrolysis during the fermentation time. Similar to the method

of Baer et al. (1996) a spectrophotometric method based on the reaction between Cd-ninhydrin reagent and free amino groups was applied for this purpose. A standard curve of L-leucine (concentrations ranged from 0.0 to 1.4 mg/mL) was also generated for quantifications.

Antioxidant activity

DPPH radical scavenging assay

Antioxidant activity based on scavenging free radical DPPH was carried out according to the method described by Jemil et al. (2014) with a little modification. For each treatment, 70 µL of supernatant was mixed with 350 µL of absolute ethanol followed by the addition of 300 µL DPPH solution (2 %w/v in absolute ethanol). The mixture was kept in a dark place for 30 min and after that the absorbance was measured at 517 nm. A lower absorbance value indicated higher DPPH scavenging activity. A control solution was prepared in which distilled water was used instead of the sample. The degree of DPPH scavenging activity was calculated according to following equation:

$$\%DPPH \text{ scavenging activity} = \left(1 - \frac{A_s}{A_c}\right) \times 100$$

Where, A_s is the absorbance of sample and A_c is the absorbance of the control.

Reducing power

The reducing power (on iron (III)) of the supernatant was measured similar to method of Jemil et al. (2014). Briefly, 100 µL of sample was mixed with 250 µL of 0.2 M potassium phosphate buffer (pH 6.6) and 250 µL of 1 % (w/v) potassium ferricyanide and incubated at 50 °C for 30 min. After that, 250 µL of 10 % TCA was added to the mixture and centrifuged at 12,000 rpm (Biofugepico, Kendro, Germany) for 10 min. Then, 250 µL of the supernatant was diluted by 250 µL distilled water followed by the addition of 50 µL of 0.1 % (w/v) ferrous chloride. After standing at the room temperature for 10 min, the absorbance of the resulting mixture was measured at 700 nm. The reducing power was expressed as the absorbance value at 700 nm.

Antibacterial assay

The capacity of antibacterial activities of the hydrolysates was carried out using micro dilution method in 96-wells micro plates similar to the method previously reported by Salami et al. (2010). The cells of both *E. coli* and *B. cereus*, previously activated on nutrient agar, were cultured in nutrient broth and allowed to grow overnight. Into each well, 50 µL of 16 h-old

bacterial culture ($1\text{--}2 \times 10^7$ CFU/mL), 100 μL of sterile nutrient broth and 100 μL of the hydrolysate were loaded. Then, the plates were incubated at 37 °C for 16 h under the sterile condition and the optical density was measured at 600 nm in 2 h intervals. The antimicrobial effects were represented as the percentage of reduction in specific growth rate of the treatments in comparison with that of the control. In case of control, 100 μL of distilled water was used instead of hydrolysate. Specific growth rate was measured using the following equation: ($\mu = \frac{\Delta \ln OD_{600}}{\Delta t}$), where t is time (maximum 16 h). Finally, relative decline in specific growth rate of treatments in comparison to that of control was measured and reported as antimicrobial activity.

Experimental design

The RSM was used to determine optimum levels of time, TSM, and inoculum on the proteolysis, antioxidant and antibacterial activities of hydrolysates obtained from TSM fermented by *B. subtilis* A14h. Central composite design (CCD) with six replications in center point was used to set the model. Design-Expert (8.0.7.1) software was used for designing the RSM design space. The variable responses were: concentration of amino acids and peptides, DPPH scavenging activity, reducing power and antibacterial activities against *B. cereus* and *E. coli*. The ranges of independent variables and also the suitable pH and temperature were determined according to the previous full factorial test. The factors contributed to factorial test were: initial pH level (at three levels), incubation temperature (at three levels) and fermentation time (at six levels). Data from full factorial test were statistically analyzed by Statistical Analysis System (SAS) release 9.1 (SAS Institute, Inc., Cary, NC). The mean values were compared with each other using the Least Significant Difference (LSD) at 95 % confidence level.

Results and discussion

Proximate analysis

Chemical compositions of tomato seed and tomato seed meal (TSM) used as the protein substrate are given in Table 1. Ash, lipid, protein and carbohydrate contents of tomato seed were 9.7, 22.1, 28.1 and 40.1 % (w/w, based on dry weight),

respectively. Protein content of TSM was also 39 % (w/w) indicating that tomato seed is a suitable protein rich substrate to be hydrolyzed to more valuable products. Sogi et al. (2002) also reported that tomato seed contained 20–30 % of protein with different fractions out of which globulin was major.

Protease activity and concentration of amino acids and peptides

Incubation temperature and pH of the media are important factors that have been shown to influence on protease production and activity during fermentation. The results of bacterial protease activity and hydrolysis trend (as evaluated by measuring the concentrations of amino acids and peptides) as function of incubation temperature, initial pH level, and time are presented in Fig. 1a–c.

The main trend of proteolytic activity (Fig. 1a–c) reflects the fact that *B. subtilis* A14h was able to gain its nutritional requirements from TSM and grew well in the medium composed of TSM as the only source of carbon and nitrogen. In this sense, it can be observed that by conducting fermentation at 30 °C, no protease activities were found until 12 h. However, after 12 h fermentation, protease activity increased sharply and this trend continued to the end of fermentation period. Meanwhile, in cultivations performed at 37 and 40 °C, after 8 h fermentation, protease production was found at considerable level. At 40 °C, the protease activity reached to its maximum level at time 20 h of fermentation, afterwards decreased significantly. Proteolytic enzyme production in the culture media with initial pH 9 (141 U/mL in average) was found to be significantly lower than those in media with initial pH 7 and 8 (159 and 155 U/mL, respectively). Overall, the highest protease activity (308 U/mL) was found under the following conditions: Incubation temperature 40 °C, initial pH 7 and fermentation time 20 h.

Concentration of amino acids and peptides (L-leucine equivalent) was affected by initial pH, incubation temperature as well as fermentation time (Fig. 1a–c). Some amino acids or peptides was observed in the media before the inoculation might be released during the sterilization treatment. However, as shown in Fig. 1a–c, in the cultivations performed at 30 °C, a reduction in amino acids and peptides content was

Table 1 Proximate analysis (dry weight basis) of tomato seed and tomato seed meal (TSM) used in this study

Compound	Ash	Fat	Protein (TS)	Protein (TSM)	Carbohydrate
Concentration (% w/w)	9.7±0.7	22.1±1.2	28.1±0.8	39±1.3	40.1

Values are the averages of two replications±standard deviation

Fig. 1 Protease activities (—) and free amino acid and peptide contents (---) of hydrolysates as affected by initial pH levels of 7 (a), 8 (b), and 9 (c) and different levels of cultivation temperatures 30 °C (□ or ■), 37 °C (△ or ▲) and 40 °C (○ or ●). Data are mean values of two replications

found during the first 8 h of fermentation. In this case, as the time increased to 24 h, amino acids and peptides content increased constantly followed by a significant decline during the rest of fermentation. Mean values measured for amino acids and peptides contents in the culture media with initial pH 7 and 8 were significantly higher than the values found in the media with initial pH 9. Regardless the pH, in the media cultivated at temperatures 37 and 40 °C, concentration of amino acids and peptides tended to increase as fermentation progressed to 20 h, and then significantly decreased by progressing fermentation from 20 to 48 h.

Increase in the concentration of amino acids and peptides reflects the susceptibility of TSM proteins to microbial proteases. Apart from the traditional application of some *B. subtilis* strains to produce soybean-based foods such as Natto, Tufo and Okara (Hu et al. 2010;

Huang et al. 2011), the ability of *B. subtilis* strains to hydrolyze plant origin proteins such as rapeseed proteins (He et al. 2012), peanut meal (Zhang et al. 2014) and walnut meal (Wu et al. 2014) has been shown in several researches. In the current study, in all cases, concentration of amino acids and peptides decreased after 20 or 24 h fermentation which might be due to the utilization of amino acids and peptides by the microorganisms. This finding was in agreement with the results of Limón et al. (2015) who reported that concentration of peptides and amino acids decreased when the lactic fermentation of kidney bean progressed after 48 h. The kinetic of release of amino acids and peptides indicated that the maximum fermentation time needed to obtain the maximum hydrolysis under the conditions applied in this study was 20 or 24 h.

Antioxidant activities of the hydrolysates

It has been reported that bioactive peptides in protein hydrolysates exert their antioxidant activities through different mechanisms including lipid peroxidation inhibition, free radical scavenging and metal ion chelating (Corrêa et al. 2014). Therefore, evaluation of antioxidant capacities of hydrolysates by performing more than one method would give better description on this activity. In this study, antioxidant activity was measured by evaluating the reducing power (ferric ion) and DPPH radical scavenging activities which are shown in Fig. 2a–c. Similar to protease activity and free amino acids and peptides content, in vitro antioxidant activities of hydrolysates depended on initial pH, incubation temperature and time. Reducing power activity which was evaluated by measuring absorbance at 700 nm, ranged from 0.21 at time 0 to 0.77 after 24 h fermentation. In the cultivations performed at 30 °C, no considerable change was observed in reducing power activities during the first 12 h, but it started to increase markedly after 20 h reaching to the maximum level at 24 h. At higher incubation temperatures, the lag phases in changing reducing power were shorter than those at 30 °C. In all treatments reducing power decreased significantly as fermentation time increased from 24 to 48 h.

In case of DPPH scavenging activity, interestingly the trends showed high similarity with those of reducing power and free amino acids and peptides content. DPPH scavenging activity was found to be in the range of 23.5 % (at time 0) to 68.5 % after 20 h fermentation. Moreover, DPPH scavenging activity decreased after 24 h fermentation up to 65 % of its maximum level. Hydrolysates produced in the media with initial pH level of 8 showed higher reducing power and DPPH

Fig. 2 DPPH scavenging activities (—) and reducing power (---) of hydrolysates as affected by initial pH levels of 7 (a), 8 (b), and 9 (c) and different levels of cultivation temperatures 30 °C (□ or ■), 37 °C (△ or ▲) and 40 °C (○ or ●). RP: Reducing power as represented by absorbance intensity at 700 nm. Data are mean values of two replications

scavenging activity than those generated in the media with initial pH levels of 7 and 9.

Many factors including microbial species, pH, temperature, fermentation time, substrate type and aerobic conditions have been reported to have effect on the antioxidant activities of plants-based fermented foods (Hur et al. 2014). In our study, the significant effects of pH, temperature and time on proteolytic activity and antioxidant activities of resulting hydrolysates were shown. pH is an important factor that influences on microbial fermentation as it has close relationships with microbial growth and stability of proteases or cell wall degrading enzymes (Hur et al. 2014). In the study of Fontoura et al. (2014) on fermentative hydrolysis of feather, initial pH levels had no effects on protease activity and final soluble protein levels, but affected the antioxidant activities of resulting hydrolysates. While, Fakhfakh et al. (2013) reported significant effect of initial pH level on the protease activity, free amino acid and peptide production and antioxidant activities of hydrolysates obtained from wool waste fermented by *Bacillus pumilus*A1. In the current study, no positive influence of a high pH level (9) on the release of amino acids and peptide and also antioxidant activity was found. Accordingly, pH 8

was chosen as the suitable pH for the next step of the study, optimization of fermentation conditions.

The effect of temperature on the antioxidant activities of hydrolysates in this study is in consistent with the results obtained by Huang et al. (2011), who reported that Sufu fermented at higher temperature showed higher antioxidant activity. Thavasi et al. (2009) reported that temperature was the most important factor affecting antioxidant activity during fermentation.

Antioxidant activities of the hydrolysates might be attributed to the hydrolysis in TSM protein that resulted in release of peptides and amino acids with antioxidant activity. Decline in DPPH scavenging activity and reducing power after 24 h fermentation may result from the further degradation of antioxidant peptides by the microbial proteases or utilization by the bacterial cells as the nitrogen source.

Optimization of fermentation conditions by using RSM

According to the results obtained from the factorial test and the previous one factor at a time tests by the authors (data not shown), the ranges of independent variables including time, TSM concentration, and initial inoculum concentration in the medium were chosen to modeling the effects of these factors on hydrolysis and bioactivities of resulting hydrolysates. The results corresponding the concentration of amino acid and peptides (as L-leucin equivalent), reducing power and DPPH scavenging activities as well as antibacterial properties of hydrolysates against *B. cereus* and *E. coli* as dependent variables are given in Table 2. The maximum value for amino acids and peptides content (2.77 mg/mL) was found in design points 4 and 8, and the highest DPPH scavenging activity (70 %) and reducing power (0.82) were observed at design points 6 and 4, respectively. From the antibacterial activity points of view, the hydrolysates obtained by running design point 8 showed the highest inhibition against *B. cereus* and the hydrolysates from design point 9 showed the highest antibacterial activity toward *E. coli* (29.8 %). More reducing effect of TSM hydrolysates on specific growth rate of *B. cereus* in comparison to that of *E. coli* in the current study is in consistent with the results of some previous studies (Jemil et al. 2014) in which Gram-positive bacteria had more sensitivity against bioactive peptides. *B. cereus* has been found to produce diarrheal and vomiting toxins and also has been reported to have high resistance to antimicrobial phytochemicals (Jemil et al. 2014). Thus, the considerable inhibitory capacity of TSM hydrolysates against *B. cereus* is of great importance. Generally, antibacterial activities of fermented products may be attributed to both antibacterial compounds produced by the bacteria and peptides. However, as

Table 2 Central composite design matrix and responses of dependent variables for free amino acid contents, DPPH scavenging, reducing power and antibacterial activities against *B. cereus* and *E. coli*

Design point	Independent variables			Responses				
	TSM ^a (%w/v) X1	Time (h) X2	Inoculum (%v/v) X3	AAP ^b (mg/ml)	DPPH scavenging (%)	Reducing power	<i>B. cereus</i> inhibition (%)	<i>E. coli</i> inhibition (%)
1	4	12	1	1.00	63.0	0.64	6.0	8.6
2	7	12	1	1.03	61.4	0.67	5.0	7.4
3	4	24	1	1.13	27.0	0.55	32.0	17.5
4	7	24	1	2.77	48.9	0.82	60.0	26.3
5	4	12	3	0.93	46.2	0.52	23.4	0.0
6	7	12	3	1.02	70.0	0.63	1.8	9.1
7	4	24	3	0.66	23.6	0.54	53.6	29.0
8	7	24	3	2.77	50.6	0.78	69.8	10.1
9	3.0	18	2	0.50	20.9	0.43	40.9	29.8
10	8.0	18	2	2.27	66.9	0.65	20.5	12.3
11	5.5	7.9	2	0.64	50.3	0.46	0.0	0.6
12	5.5	28.1	2	1.71	35.1	0.52	34.1	6.0
13	5.5	18	0.3	1.71	47.4	0.56	34.3	0.8
14	5.5	18	3.7	1.62	42.2	0.51	10.1	25.7
15	5.5	18	2	1.46	41.8	0.63	48.9	2.3
16	5.5	18	2	1.73	37.1	0.60	51.1	9.5
17	5.5	18	2	1.60	39.8	0.52	46.0	5.5
18	5.5	18	2	1.72	37.1	0.62	45.5	7.9
19	5.5	18	2	1.42	35.7	0.54	53.0	12.4
20	5.5	18	2	1.42	28.2	0.57	48.0	10.4

^aTomato seed meal (powdered)^bAmino acids and peptides as L-Leucin equivalent**Table 3** Analysis of variance for different responses in the hydrolysates

Source	DPPH scavenging		Free amino acids and peptides		Reducing power		<i>B. cereus</i> inhibition		<i>E. coli</i> inhibition	
	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value
Model	7.95	0.0016***	46.78	<0.0001***	5.81	0.007***	4.54	0.0135**	10.94	0.0039***
A-TSM	35.08	0.0001***	194.95	<0.0001***	14.76	0.0014***	0.07	0.7972	13.57	0.0103**
B-Time	21.5	0.0009***	110.02	<0.0001***	1.55	0.2315	24.7	0.0006***	1.27	0.3033
C-Inoculum	0.54	0.4781	2.08	0.1803	1.12	0.3056	0.011	0.9192	27.47	0.0019***
AB	1.94	0.1935	93.15	<0.0001***			3.36	0.0967*	3.55	0.1084
AC	2.54	0.1423	2	0.1876			0.79	0.3959	3.39	0.1151
BC	0.12	0.7411	1.05	0.33			0.22	0.647	0.057	0.8196
A ²	3.95	0.0751*	2.75	0.1282			1.63	0.23	29.21	0.0017***
B ²	3.06	0.1107	14.15	0.0037***			7.31	0.0222**	2.92	0.1383
C ²	4.69	0.0555*	1.33	0.2752			4.7	0.0554*	5.2	0.0628*
ABC									16.07	0.0071***
A ² B									9.34	0.0223**
A ² C									23.1	0.003***
AB ²									7.11	0.0372**

*Significant at 10 % level; **significant at 5 % level; ***significant at 1 % level. R² for DPPH scavenging, free amino acids and peptides, reducing power, *B. cereus* inhibition and *E. coli* inhibition were 0.8773, 0.9768, 0.5213, 0.8032 and 0.9595 respectively

mentioned earlier and can be found in Table 2, the highest inhibitory activity against *B. cereus* was observed in design point with the highest amount of amino acid and peptides.

Therefore, antibacterial properties of hydrolysates obtained in this study could be related to the bioactive peptides produced during fermentation.

Fig. 3 Contour graphs indicating interactions of independent factors (time, inoculum and TSM concentrations) on different responses investigated in the current study

Statistical analysis of linear, quadratic or cubic and interaction effects of three independent variables on different responses are given in Table 3. The quadratic polynomial regression models for the generation of amino acids and peptides, DPPH scavenging activity and *B. cereus* inhibition effects were:

$$\begin{aligned} \text{Concentration of amino acids and peptides} = & +1.56 + 0.50A \\ & + 0.3B - 0.052C + 0.45AB + 0.066AC - 0.048BC - 0.058A^2 \\ & - 0.13B^2 + 0.040C^2 \end{aligned} \quad (1)$$

$$\begin{aligned} \text{DPPH scavenging} = & +36.45 + 10.87A - 8.51B - 1.35C \\ & + 3.34AB + 3.82AC + 0.81BC + 3.55A^2 + 3.13B^2 + 3.87C^2 \end{aligned} \quad (2)$$

$$\begin{aligned} \text{B. cereus inhibition} = & +48.44 - 0.92A + 17.31B + 0.36C \\ & + 8.34AB - 4.04AC + 2.15BC - 4.33A^2 - 9.17B^2 - 7.35C^2 \end{aligned} \quad (3)$$

The proposed linear regression model for reducing power was:

$$\text{Reducing power} = +0.59 + 0.076A + 0.024B - 0.021C \quad (4)$$

And the reduced cubic polynomial regression model for antimicrobial effects toward to *E. coli* was:

$$\begin{aligned} \text{E. coli inhibition} = & +7.95 - 5.20A + 1.59B + 7.40C \\ & - 2.24AB - 2.19AC + 0.28BC + 4.78A^2 - 1.51B^2 + 2.02C^2 \\ & - 4.76ABC + 5.64A^2B - 8.87A^2C + 4.92AB^2 \end{aligned} \quad (5)$$

Where A, B and C are independent variables for TSM concentration, time, and inoculum concentration, respectively.

Regarding amino acids and peptides contents, the high coefficient of determination (R^2) of 0.9768 was satisfactory indicating that proposed model can explain 97.68 % of variations in the concentrations of amino acids and peptides. Statistically, in the regression equations, the larger coefficient of a factor, the higher effect on the response. Thus, the highest coefficient for TSM percentage in the model proposed for the

concentration of amino acids and peptides indicated that TSM concentration had the most potent effect on concentration of amino acids and peptides. In case of DPPH scavenging activity, although the model was significant at 1 % probability, R^2 (0.8773) was lower than that obtained for amino acids and peptides content model. R^2 of the model (0.8032) fitted to the antibacterial activities of hydrolysates against *B. cereus* indicated that more than 80.32 % of variations in this activity can be explained by this model. However, as shown in Eq. 3, the highest regression coefficient belongs to the linear effect of time indicating that the inhibitory activity of hydrolysates against *B. cereus* is mostly depended on time as confirmed by its significance at 1 % probability level ($p < 0.01$). In case of reducing power, only the linear effect of TSM concentration was significant and relatively poor R^2 (0.5213) was obtained for the proposed model. In case of the effects of independent variables on the antibacterial activity toward to *E. coli*, a reduced cubic model was proposed by the software which was significant at 1 % probability. R^2 of 0.9595 for this reduced cubic model showed that the proposed model was a powerful model which can explain more than 95 % of variations in antibacterial activities of hydrolysates against *E. coli*. Considering the p values of the terms for the reduced cubic model and their coefficients in the proposed regression equation (Eq. 5), it can be concluded that TSM and inoculum concentrations had higher significant effects on the inhibitory effects of hydrolysates against *E. coli*.

In order to show interactions of the significant factors on hydrolysis and bioactivities of the resulting hydrolysates and to show the optimum locations, some contour graphs were made using Design-Expert software (Fig. 3). For each response, an individual optimum point was predicted regardless to the other responses. The optimum conditions to gaining different goals are given in Table 4. For example, the optimum condition to obtain the highest antioxidant activity was TSM 7 %, time 12 h and inoculum concentration 1 %, and to reaching to the maximum inhibitory effect against *B. cereus* was TSM 6.83 %, time 24 h, inoculum concentration 1.93 %.

Confirmation of the models was done by performing the assays under the optimum conditions. As shown in Table 4, the experimental values were in the range of lower limit and

Table 4 Optimum processing conditions to obtain the maximum responses and confirmation of validity of statistical models

Goal	Response	Optimum conditions			Predicted value	Experimental value
		TSM concentration	Time (h)	Inoculum		
Antioxidant activity	DPPH scavenging activity (%)	7	12	1	61.4	51
	Reducing power	7	12	1	0.66	0.51
Antibacterial activity	<i>B. cereus</i> inhibition (%)	6.83	24	1.93	59.8	39.1
	<i>E. coli</i> inhibition (%)	4	24	3	28.7	19.2
Hydrolysis	Amino acids and peptide content (mg/ml)	7	24	1.03	2.77	2.92

upper limit (not shown in the Table 4) and close to the predicted values indicating that proposed models were powerful models to navigate and predict design space.

Conclusion

In this study, protein waste of tomato processing plants was converted to antioxidant and antibacterial hydrolysates through fermentation by *B. subtilis* A14h. Results showed that *B. subtilis* A14h, was a potent proteolytic microorganism able to hydrolyze vegetable proteins to bioactive hydrolysates. Hydrolysates from fermented TSM were found to be antimicrobial and antioxidant compounds which may be considered as food preservatives and pharmaceutical agents. However, further purification steps are required for the identification of the most active peptide fractions being performed by the authors at current.

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