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Improvement of antioxidant activities of okara, a soybean by-product, through fermentation with *Bacillus subtilis* TISTR001

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ABSTRACT: Health benefit of okara which is a by-product from soymilk production can be improved by fermentation. This study aimed to ferment okara by *Bacillus subtilis* TISTR001. The effect of okara fermented with *Bacillus subtilis* TISTR001 on enzyme activities including alkaline protease, α-amylase, and β-glucosidase was investigated. Fermented okara was analyzed for total phenolic content, 2,2-diphenyl-l-pricrylhydrazyl (DPPH) radical scavenging activity, and 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activity. After 96 h of fermentation, the highest alkaline protease (208.40 U/g), α-amylase (146.90 U/g), and β-glucosidase (10.81 U/g) activities were obtained from fermented okara by *B. subtilis* TISTR001 at the concentration of 7.0 Log CFU/g. Total phenolic compounds increased from 16.84 to 139.22 µg GAE/g, indicating higher antioxidant activities compared to unfermented okara. Meanwhile, the $DPPH^{\bullet}$ free radical and $ABTS^{\bullet+}$ cation radical scavenging activities of the free and bound phenolic fractions in okara became stronger after fermentation. This indicated that the changes of antioxidant activities of phenolic fractions might be closely related to the structural modification by microbial enzymes in the fermented okara by *B. subtilis* TISTR001 at the highest concentration of cell biomass. The fermentation of okara with *B. subtilis* TISTR001 has shown to have a health-beneficial effect by enhancing its antioxidant properties.

KEYWORDS: okara, *Bacillus subtilis*, fermentation, phenolic compounds, antioxidant activity

INTRODUCTION

Okara, also known as soy pulp, is a major agro-waste produced from the soymilk and tofu industries. Ground soybean residue or okara has remained after filtering the water-soluble fraction during production of soymilk or soybean curd (tofu). Considering the chemical constituents of okara, they primarily include dietary fibers (5.00 g/100 g wet basis), carbohydrates (6.01 g/100 g wet basis), and protein (8.08 g/100 g wet basis) [1]. Moreover, okara is also rich in isoflavones and other biologically active substances such as daidzein (33.0 mg/100 g) and genistein (57.9 mg/100 g). It contains total phenolic content which has antioxidant activity at a level of 122.57 mg gallic acid/100 g [1]. Nowadays, increasing the value of industrial by-products has been emphasized. The nutritive foods such as polysaccharides, proteins, fats, fibres, flavour compounds, phytochemicals, and bioactive compounds have been developed as alternative sources for functional food products [2], including lactic acid bacteria-fermented lemon juice [3]. Due to physicochemical properties of okara, several research has been conducted to confer the increment of nutritional and beneficial effect of okara [4].

The possibility of reintroducing okara through fermentation has already been confirmed in various studies on the solid-state fermentation (SSF) and submerged fermentation (SMF). The okara biological modification by microbial biotransformation influences the conversion of high molecular weight okara proteins to smaller bioactive peptides or amino acids. Noteworthy, with the advancement of fermentation processes, it is also reported that starter organisms effectively reduce the content of trypsin inhibitors through microbial enzymes, resulting in the improvement of nutritional quality and bioactivity of okara [5]. In recent years, many researches indicate the importance of successful digestion, assimilation, and metabolism in the organism [5]. Okara can also be used as a fermentation substrate to produce a variety of products (natto, fibrinolytic enzymes, α-glucosidase inhibitor, edible fungi, etc.) for human consumption and non-food production [6]. Studies have been admitted that okara can be used as an economical and nutrient-dense substrate for microbial fermentation as well as for the extraction of various bioactive metabolites after the pre-treatment [7]. *Bacillus subtilis* has been reported to be used in fermenting soybeans, as seen in foods such as natto, kinema, and Tungrymbai [8]. Furthermore, *B. subtilis* hydrolyzes substrates in fermented foods and produces enzymes such as protease, cellulase, amylase, and β-glucosidase [5]. These enzymes can enhance the accessibility of nutrients and health benefits of okara [9].

Mok et al [7] investigated the production of pro-

biotic drink by fermentation of okara with *B. subtilis*. Metabolomic analysis shows that the nutritional profile of the beverage is enhanced after fermentation including essential amino acids, short-chain fatty acids, total phenolic content, and antioxidant content (in terms of DPPH radical scavenging activity).

Fermentation in a solid state of okara using the properties of *B. subtilis* was studied [10]. It was found that *B. subtilis* hydrolyzes the proteins contained in okara during the fermentation process. The protease and carboxypeptidase produced can effectively reduce bitterness. The amino acids consisting of phenylalanine, alanine, tyrosine, and leucine at the end C of peptides in hydrolysate rubbed okara were separated by carboxypeptidase. The hydrolysis of these amino acids resulted in complete bitterness reduction. Additionally, SSF has been recognized as a valuable tool to improve quality, usability, and value added to okara for developing better food products. The fermented okara has been used as food-producing components in order to enhance nutritional and healthful properties such as okara soy sauce, fermented okara-biscuits, and functional probiotic beverages [11, 12].

Therefore, the purpose of this study was to investigate the effect of okara fermentation by *B. subtilis* TISTR001 on hydrolytic enzyme production and the improvement of antioxidant activities in okara. A processing technology for enhancing the free phenol content in highly bioactive okara was presented. The upcycling of okara using microorganisms represents a valuable tool for improving quality and usability, aiding in the development of better functional food ingredients such as rice seasoning, porridge, premix powder, and peanut butter. This study delves into the mechanism of increasing antioxidant activity and the development of specific processing technology while previous reports [11, 12] focused only on improving the overall quality of okara.

MATERIALS AND METHODS

Materials and microorganisms

Fresh okara was kindly supplied by Lactasoy Co., Ltd. (Bangkok, Thailand) and was kept at −20 °C prior to use. *B. subtilis* TISTR001 obtained from the Thailand Institute of Scientific and Technological Research (TISTR) were employed. *B. subtilis* was activated in Trypticase soy broth (TSB) and incubated at 37 °C, 200 rpm for 18 h. The culture was kept in 15% glycerol at −20 °C for further use. To prepare starter cultures, a single loopful from a slant agar culture of *B. subtilis* TISTR001 was inoculated into 200 ml of TSB. The cultures were incubated at 35 °C for approximately 18 h. The turbidity of the cultures was checked against a McFarland standard No. 0.5 (The Grant bio DEN-1 Grant Instruments (Cambridge) Ltd., UK) using a spectrophotometer at 660 nm. The OD value should be between 0.08 and 0.10. If the culture was more turbid than the standard, it was diluted with TSB medium. Cultures with a turbidity matching the standard had an approximate concentration of 8.0 Log colony forming units (CFU)/ml. The cell suspension was then used for okara inoculation.

Fermentation

Okara (1 kg) was placed in a container and sterilized at 121 °C for 30 min. *B. subtilis* TISTR001 starter culture was initially prepared at a concentration of 8.0 Log CFU/ml. Subsequently, the concentration was diluted to 7.0 Log CFU/ml using 0.1% peptone water before being mixed with sterilized okara. The 100 g of inoculated okara was placed into perforated plastic bags (12×17 cm). SSF were conducted in the incubator (Memmert, 100-800, Germany) at 35 °C for 96 h. One hundred grams of samples were taken every 24 h and kept at −20 °C before being analyzed for cell concentration, enzyme activities, total phenolic compounds, and antioxidant activities.

Determination of viable cell and pH

A 25 g sample was mixed with 250 ml of 0.85% (w/v) NaCl solution. Then, 0.1 ml of serial (10-fold) diluted suspensions was spread onto tryptic soy agar (TSA) plates. Viable cells were counted after 24 h of incubation at 37 °C. For pH determination, 10 g of sample was dissolved in 90 ml of distilled water, and the pH values were measured using a pH meter (Inolab, Germany).

Extraction of crude enzyme

Crude enzyme solution from fermented okara was prepared according to the method of Verardo et al [13]. The samples were added with distilled water by the ratio of 1:1 and homogenized. The mixtures were centrifuged at 10,000×g at 4 °C for 15 min. The supernatants were used as the crude enzyme for analysis of alkaline protease and α-amylase activities.

Alkaline protease activity

According to the modified method of Kim et al [14], protease activity was assayed by using Hammerstein casein (Merck, Darmstadt, Germany) as the substrate. Firstly, 0.1 ml of crude enzyme was added to substrate solution which contained 0.9 ml of 0.5% (w/v) Hammerstein casein in 0.1 M sodium phosphate buffer (pH 9.5). The reaction mixture was then incubated at 45 °C for 20 min. To terminate the reaction, 2 ml of 10% (w/v) trichloroacetic acid (TCA) was added to the mixture. After removal of the undigested casein by filtration, the solution was determined for acid soluble products by spectrophotometer at 280 nm. One unit (U) of protease activity is defined as the amount of enzyme that liberates 1 µg of tyrosine per min at pH 9.5, 45 °C.

α-Amylase activity assay

α-Amylase activity was performed by spectrophotometry according to a modification of the Worthington assay procedure [15]. Briefly, 0.1 ml of crude enzyme solution was equilibrated at 35 °C for 4 min. Then, 0.1 ml of 1% (w/v) soluble starch in 20 mM sodium phosphate monobasic, 6 mM sodium chloride, pH 6.9 was added into the solution and allowed to incubate at 35 °C for 3 min. The reaction was immediately terminated by adding 1 ml of 3,5-dinitrosalicylic acid (DNS) solution. The mixture was boiled for 5 min. After cooling, the mixture was diluted with 10 ml of distillation water. The absorbance was measured at 540 nm. One unit of α-amylase activity was described as the amount of enzyme that hydrolyzes soluble starch to release 1 µg of maltose per min under the evaluation conditions.

β-Glucosidase activity assay

A modified method of Cho et al [16] was used to determine β-glucosidase activity. Fermented okara (10 g) was extracted by using 10 ml of 50 mM sodium phosphate buffer, pH 7.0 for 3 min and then centrifuged at 8000×g at 4 °C for 30 min. The supernatant was collected and filtered through a 0.45-µm filter before analysis. Substrate of β-glucosidase was prepared with 5 mM ρ-nitrophenol-β-D-glucopyranoside (ρ-NPG) in 50 mM sodium phosphate buffer, pH 7.0. For the enzymatic reaction, 250 µl of the crude extract was added to 250 µl of the substrate solution, then incubated at 37 °C for 30 min. Thereafter, the reaction was stopped by adding 500 µl of 0.2 M glycine-NaOH, pH 10.5, and the mixtures were immediately measured by a spectrophotometer at 405 nm. The blank solution was composed of 2.5 ml of 50 mM glycine-NaOH, 2.0 ml of substrate solution, and 0.5 ml of 50 mM citric buffer (pH 4.5) containing 0.1 M NaCl. The ρ-nitrophenol (ρ-NP) released by the action of the enzyme was determined by referring to a calibration curve prepared from the ρ-NP in concentrations that varied from 25 to 300 mM. One unit of β-glucosidase activity was defined as the amount of enzyme that liberated 1 µM of ρ-NPG.

Extraction of free and bound phenolic compounds

Following a modified method of Verardo et al [13], phenolic extractions were employed. In order to extract the free phenolic compound, 4 g of sample was extracted with 40 ml of 4:1 methanol:water by sonication for 30 min. The extracts were centrifuged at 1000×g for 10 min, and the supernatant was then separated. The residue was reextracted. The supernatants were collected and evaporated at 40 °C. The residue was reconstituted with 4 ml of 1:1 methanol:water.

Extraction of bound phenolic compounds was carried out by alkaline hydrolysis. The residue of free phenolic compound extraction was digested with 200 ml of 2 M NaOH at 35 °C for 20 h by shaking under nitrogen gas atmosphere. The mixture was then acidified to pH 2.3 with hydrochloric acid. The lipids were removed by extraction with 500 ml of hexane. After that, the aqueous solution was extracted 5 times with 100 ml of 1:1 diethyl ether:ethyl acetate. The organic fractions were pooled and evaporated to dryness. The bound phenolic compounds were reconstituted with 4 ml of 1:1 methanol:water.

Determination of total phenolic compound

Total soluble phenolic content (TPC) of fermented okara extracts was determined using Folin-Ciocalteu reagent with gallic acid as a standard phenolic compound [17]. Twenty µl of each diluted extract was added to 100 µl of Folin-Ciocalteu reagent and allowed to stand for 3 min. Then, 80 µl of 10% (w/v) sodium carbonate solution was added to the mixture. The solution was incubated at 35 °C for 1 h in dark condition, and the absorbance of the samples was measured at 765 nm. TPC was calculated as gallic acid equivalents (GAE) from the calibration cure obtained from the gallic acid standard solution and was expressed as µg GAE/g okara.

Evaluation of antioxidant activities

The DPPH free radical scavenging activity was determined using a modification from Chatatikun & Chiabchalard [18]. Briefly, 60 µl of sample extracts including free and bound phenolic compounds or standard solutions of trolox in absolute methanol was added to 140 µl of 0.16 mM DPPH reagent in 96-well plate. Absolute methanol was used for reagent blank. The reaction mixtures were mixed well and incubated for 30 min at 30 °C under dark condition. The absorbance was measured at 517 nm with a Microplate Reader (PerkinElmer, USA). Experiments were all done in triplicates. The percentages of the $DPPH^{\bullet}$ free radical scavenging activity were calculated as follows:

$$\% \text{ Scavenging activity} = \frac{Ab_{control} - Ab_{sample}}{Ab_{control}} \times 100.$$

The $ABTS^{\bullet+}$ free radical scavenging activity of the samples was determined by using a modification from Chatatikun & Chiabchalard [18]. $ABTS^{\bullet+}$ formation was generated by oxidation of ABTS reagent and potassium sulfate. Seven mM of ABTS solution was mixed with 2.45 mM potassium persulfate and incubated for 12–16 h in the dark at room temperature before use. To study the okara extracts, the $ABTS^{\bullet+}$ solution was freshly prepared by dilution with methanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Twenty µl of sample extracts (free and bound phenolic compounds) or standard solutions of trolox in distilled water was added to 180 µl of $ABTS^{\bullet+}$ working reagent in a 96-well plate. Distilled water was used as reagent blank. The plate was incubated for 45 min at room

temperature in a dark condition. The absorbance was measured at 734 nm with a Microplate Reader (PerkinElmer). Experiments were all done in triplicate. The percentages of the $ABTS^{\bullet+}$ scavenging activity were calculated as follows:

$$\% \text{ Scavenging activity} = \frac{Ab_{control} - Ab_{sample}}{Ab_{control}} \times 100.$$

Statistical analysis

All experiments in this study were performed in triplicate. Data were reported as mean ± standard deviation. All data were statistically analyzed by One-way analysis of variance (ANOVA) using SPSS Statistics 27.0 software (SPSS Inc., Chicago, USA). The difference among group were assessed by Duncan's post-hoc test (significance level $p < 0.05$) [16].

RESULTS AND DISCUSSION

Growth of *B. subtilis* TISTR001 and pH changes during okara fermentation

The growth of *B. subtilis* TISTR001 and pH changes during okara fermentation at different time intervals were presented in Fig. 1. The viable cell increased when fermentation time increased. The initial concentration of *B. subtilis* TISTR001 at 7.0 Log CFU/g yielded the highest cell concentration at the level of 8.9 Log CFU/g at 96 h of fermentation. Aligned with the growth of *B. subtilis* TISTR001, the activities of alkaline protease and α-amylase significantly escalated during fermentation as shown in Fig. 2 and Fig. 3, respectively. *B. subtilis* TISTR001 utilized their extracellular enzymes to degrade proteins and polysaccharides in okara into small molecules such as peptides, amino acids, and mono- and disaccharides, which were then used for their biomass growth and product formation. According to Ilyas et al [19], *B. subtilis* KCTC 13241, at the level of 7.4 Log CFU/g, was utilized for the fermentation of wheat grains. The fermented wheat exhibited significantly higher free radical scavenging potential than unfermented wheat. These results agreed with the previous research. *B. subtilis* could utilize some of saccharides in okara as carbon sources, resulting in many kinds of products such as cells, organic acids, and enzymes produced during the sugar metabolism [20].

The pH increased during okara fermentation as presented in Fig. 1. The pH value of okara fermented with *B. subtilis* TISTR001, with initial concentration of 7.0 Log CFU/g, increased from 6.84 to 8.25. The proteolysis by fermenting microorganism apparently resulted in the rise in pH. Protein was hydrolyzed into amino acids, ammonia, and amines, showing alkalinity and following the utilization of amino acids by the increase of *B. subtilis* population [16, 21]. In accordance with protease activity (Fig. 2), *B. subtilis* TISTR001 produced alkaline protease during fermentation.

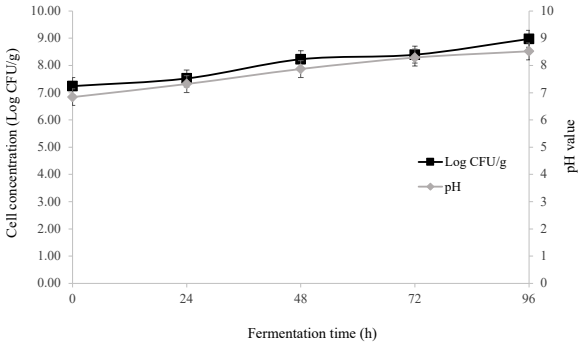

Fig. 1 Growth of *Bacillus subtilis* TISTR001 and profile of pH value during okara fermentation at 35 °C for 96 h.

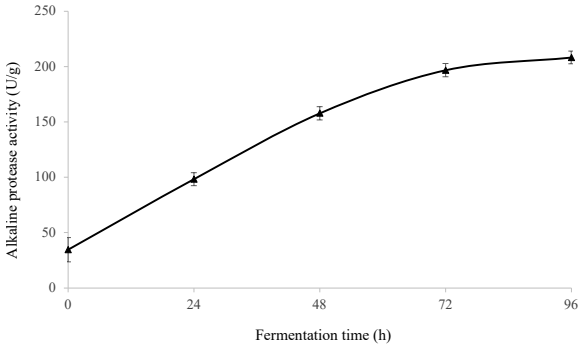

Fig. 2 Alkaline protease activity in *Bacillus subtilis* TISTR001 at 7.0 Log CFU/g initial cell concentration of okara fermentation at 35 °C for 96 h.

Changes in hydrolytic enzyme activities

In the okara fermentation, the changes in hydrolytic enzyme activities including alkaline protease, α-amylase, and β-glucosidase were observed. Alkaline protease activity of *B. subtilis* TISTR001 increased remarkably during fermentation, as shown in Fig. 2. The maximum activity of alkaline protease at 208.40 U/g

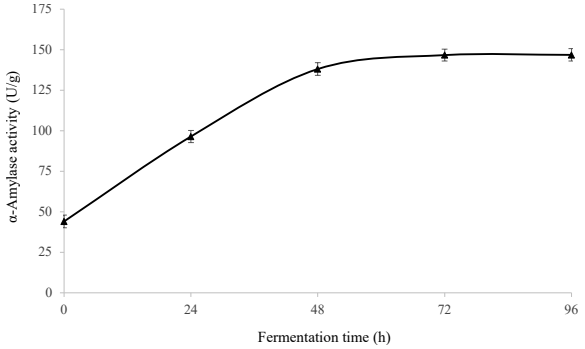

Fig. 3 α-Amylase activity in *Bacillus subtilis* TISTR001 at 7.0 Log CFU/g initial cell concentration of okara fermentation at 35 °C for 96 h.

was significantly noted in the enzyme extract harvested at 96 h of fermentation by 7.0 Log CFU/g of *B. subtilis* TISTR001. The result generally reflected the pH changes observed. The increase in pH during okara fermentation is believed to be due to the protease activities of *Bacillus* sp., causing hydrolysis in proteins and resulting in the release of ammonia. This release, in turn, causes a rise in pH [22].

Similarly, Li & Wang [21] demonstrated that the protease was produced during *B. subtilis* chickpea fermentation and protease activity increased with longer fermentation time. In our study, *B. subtilis* TISTR001 was able to produce extracellular protease by using okara as substrate. In concordance with Zhang et al [23], the study reported that okara and soybean protein are suitable support-substrates for production of alkaline protease by *Bacillus* sp. under SSF.

Throughout the fermentation process, the α-amylase production of *B. subtilis* TISTR001 exhibited growth trends, as illustrated in Fig. 2. As observed, α-amylase activity dramatically increased within the first 24 h of fermentation. This α-amylase production continued to be monitored until the end of the fermentation process. The peak α-amylase activity was measured at a level of 146.89 U/g.

In our study, α-amylase activity increased throughout 48 h of okara fermentation by *B. subtilis* TISTR001 and remained constant until 96 h of the fermentation. The presence of starch in substrates induced the production of α-amylase during fermentation. α-amylase was synthesized by *Bacillus* sp. to degrade the starch present in the medium and release glucose and maltose for growth [24].

The activity of β-glucosidase during fermentation is illustrated in Fig. 3. Across all experiments, β-glucosidase activity increased during fermentation. The β-glucosidase activity peak (10.81 U/g) was observed at 72 h of fermentation, while the activity slightly decreased at the end of fermentation (96 h). The reduction in β-glucosidase activity resulted from an increase in pH, which was approximately 7.02–8.25. β-Glucosidases from *Bacillus* strains exhibit optimal activity in slightly acidic or neutral pH at 37–55 °C, and the optimal pH was found to be 6.00 [25].

β-glucosidase produced by bacteria was considered a liable source for the biotransformation of isoflavone glycosides to aglycones. In the fermentation process of natto, *B. subtilis* produced a high activity of β-glucosidase. Thus, the content of active isoflavones in natto was significantly improved [26]. The release of aglycones from isoflavone glucosides by the enzymatic hydrolysis of β-glucosidase during the fermentation process has been suggested to be responsible for the increased aglycone content of the products. In fact, the composition of isoflavone conjugates in soybean products such as natto or tempeh curds can also be changed during manufacturing [27]. In general, most isoflavones in soybean are presented in glycoside forms and converted into aglycones during fermentation by microbial β-glucosidase activity. Shon et al [28] also reported that content of isoflavone aglycones such as daidzein and genistein increased in cheonggukjang (fermented soybean) when fermentation time increased.

Table 1 Total phenolic contents (μg GAE/g) of free and bound phenolic fractions in okara fermentation with *Bacillus subtilis* TISTR001 at an initial cell concentration of about 7.0 Log CFU/g.

Fermentation time (h)	Phenolic fraction (μg GAE/g)		
	Free	Bound	Total
0	11.28 ± 0.81^{b}	5.59 ± 0.71^{b}	16.86 ± 1.52^{b}
72	125.15 ± 0.47^{a}	14.83 ± 0.86^{a}	139.98 ± 1.34^{a}
96	105.62 ± 0.34^{ab}	18.04 ± 0.28^{ab}	123.66 ± 0.62^{ab}

Results are expressed as mean ± standard deviation; fermented time ($n = 3$). Different lowercase letters (difference between fermentation time) are significantly different ($p < 0.05$), One-way ANOVA followed the Duncan's post-hoc test.

The specific hydrolytic enzymes, including protease, α-amylase, and β-glucosidases produced by *B. subtilis*, play a vital role in enhancing the functional properties of soybean [9]. Protease, produced by *B. subtilis*, cleaves protein and bound isoflavones into bioactive peptides and aglycone isoflavone [29]. Lemes et al [30] reported the simultaneous production of protease and antioxidant compounds during agro-industrial by-product fermentation with *Bacillus* sp. P45, suggesting that amino acids and peptides may contribute to antioxidant properties. Carbohydrate-hydrolyzing enzymes such as α-amylase and β-glucosidases produced by *B. subtilis* are associated with phenolic mobilization and antioxidant activity in soybean products [31]. α-Amylase catalyzes α-1,4-glucan links in polysaccharides, releasing maltose and glucose as energy sources, thereby supporting and promoting the liberation of phenolic compounds [32]. β-Glucosidase plays an essential role in hydrolyzing phenolic glucosides and releasing free phenolics such as aglycones, which possess high antioxidant activity [16]. Moreover, the inoculum concentration of *B. subtilis* may enrich the production of these enzymes, catalyzing further hydrolysis processes and increasing both TPC content and antioxidant activity [32].

Total phenolic content (TPC)

As previous results of hydrolytic enzyme activities, the okara fermented with 7.0 Log CFU/g of *B. subtilis* TISTR001 was further evaluated for TPC and antioxidant activities. TPC in fermented okara was presented in Table 1. Fermentation of okara significantly ($p <$

0.05) caused an increase in TPC. Free phenolic content was greater than bound phenolic content in fermentation of okara with *B. subtilis*.

Phenolic compounds in plant exist in soluble (free and conjugated phenolic acids) and insoluble-bound forms. According to Abd Razak et al [33], fermentation of okara can increase the content of free phenolic compounds in cereal products through the breakdown of cereal cell walls by microbial enzymes. Additionally, the study of Somdee et al [34] demonstrated that SSF of red rice bran using *Aspergillus oryzae* significantly enhanced antioxidant activity as well as phenolic and flavonoid compound levels, attributed to enzymatic hydrolysis. The increase in the free phenolic content and the decrease in the bound phenolic content after fermentation may result from microbial-secreted hydrolytic enzymes which hydrolyze glycosidic bonds of bound phenolics [35]. Therefore, the increase in free phenolic contents of fermented okara might be attributed to the production of hydrolytic enzymes, mainly β-glucosidase, by *B. subtilis*. These enzymes destroy the okara cell walls and the crystalline structure of starch, weakening the bonding linkage between the phenolic compounds and the cell walls as well as other macromolecules in okara [33]. Kuo et al [36] reported an increase in TPC during the fermentation of black soybean due to the β-glucosidase activity, which hydrolyzes phenolic glycosides to release their aglycones. As shown in Table 1, a reduction in TPC in fermented okara at 96 h was observed, which is related to the decrease in β-glucosidase activity, as presented in Fig. 4. In a recent study, *B. subtilis* was found to produce a large number of β-glucosidase during natto fermentation [26]. Consequently, the content of active isoflavones in natto was improved. This finding implies that the concentration of TPC was intimately associated with β-glucosidase during okara fermentation.

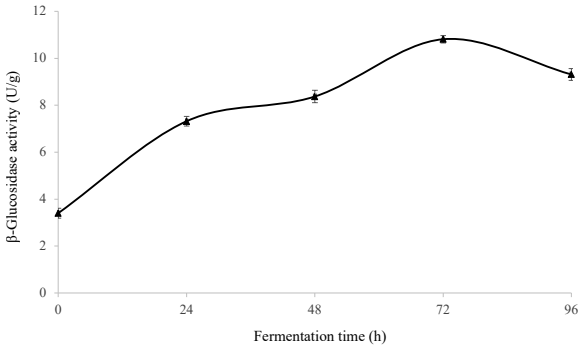

Fig. 4 β-Glucosidase activity in *B. subtilis* TISTR001 at 7.0 Log CFU/g initial cell concentration of okara fermentation at 35 °C for 96 h.

Antioxidant activities of the phenolic fractions

The efficacy of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging was used to assess the antioxidant activities of phenolic fractions from okara fermentation by *B. subtilis* TISTR001 at an initial cell concentration of 7.0 Log CFU/g. The results of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities were shown in Table 2 and Table 3. The antioxidant activities against the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ of fermented okara at 72 and 96 h were significantly higher than those of unfermented okara (0 h) as presented in Table 2. The fractions of free phenolic compounds obtained from fermented okara at 72 and 96 h had the highest $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities ($p < 0.05$), respectively.

The antioxidant activity of fermented okara slightly decreased at 96 h due to the reduction of β-glucosidase and TPC content. From our study, the findings indicated that *B. subtilis* TISTR001 hydrolyzes the substrate in okara and produces enzymes such as alkaline protease, α-amylase, and β-glucosidase. These enzymes can promote the accessibility of health benefits of okara. Alkaline protease and α-amylase activities showed positive correlations with the growth *B. subtilis* TISTR001. Strong positive correlations were also observed between β-glucosidase activity and TPC/antioxidant activities, suggesting that β-glucosidase significantly contributes to the release of phenolic compounds. The results from this study align with the previous research indicating that phenolic compounds demonstrate antioxidant properties through scavenging of radicals, exhibiting reducing power, and displaying chelating ability [16]. Similar findings were reported in *B. subtilis* fermented soybeans (cheonggukjang), where both total phenolic content and DPPH radical scavenging activity increased during fermentation [16].

The IC_{50} value was calculated to determine the concentration of the samples required to inhibit 50% of radical. The lower IC_{50} value led to the higher antioxidant activity of the samples. The IC_{50} values of fermented okara by *B. subtilis* TISTR001 were shown in Table 3. It was found that the IC_{50} values for $DPPH^{\bullet}$ scavenging activity of free phenolic compounds obtained from fermented okara at 72 h showed the highest antioxidant activity at 71.01 mg/g. For the $ABTS^{\bullet+}$ scavenging activity, free phenolic compounds obtained from fermented okara at 96 h had the highest scavenging activity at 18.90 mg/g. Moreover, the free phenolic compounds of fermented okara exhibited significantly higher antioxidant activities than those from bound phenolic compounds for scavenging $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (Table 2). After 72 h of fermentation, the DPPH radical scavenging capacity of free phenolic compounds significantly increased 22.14 times. The scavenging capacity ranged from 0.014 mg Trolox equivalent/g to 0.308 mg Trolox equivalent/g. Meanwhile, an increase of 6.19 times was found in the bound phenolic compounds at 72 h (0.138 mg Trolox

Table 2 Antioxidant activity (DPPH and ABTS free radical scavenging activity expressed as (mg Trolox Equivalent/g) of the phenolic fractions from okara fermentation by *B. subtilis* TISTR001 with an initial cell concentration of 7.0 Log CFU/g for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging.

Antioxidant activity (mg Trolox Equivalent/g)			
Phenolic fraction	Fermentation time (h)	$DPPH^{\bullet}$	$ABTS^{\bullet+}$
Free phenolic compound	0	0.014 ± 0.007^{c}	ND^{c}
	72	0.308 ± 0.032^{a}	2.232 ± 0.05^{b}
	96	0.262 ± 0.008^{b}	2.320 ± 0.097^{a}
Bound phenolic compound	0	0.022 ± 0.014^{c}	0.452 ± 0.029^{c}
	72	0.138 ± 0.021^{a}	0.922 ± 0.053^{b}
	96	0.069 ± 0.028^{b}	0.960 ± 0.044^{a}

Results were expressed as mean ± standard deviation; fermentation time ($n = 3$). Different lowercase letters (difference between fermentation time of each phenolic fractions) are significantly different ($p < 0.05$) by Duncan's post-hoc test. ND = non detected.

Table 3 IC_{50} of the phenolic fractions from okara fermentation by *B. subtilis* TISTR001 with an initial cell concentration of 7.0 Log CFU/g for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging.

Phenolic fraction	Fermentation time (h)	IC_{50}	
		$DPPH^{\bullet}$ (mg/g)	$ABTS^{\bullet+}$ (mg/g)
Trolox	-	0.0265 ± 0.0005	0.0677 ± 0.0039
Free phenolic compound	0	ND	ND
	72	71.01 ± 5.59^{a}	19.71 ± 4.44^{b}
	96	121.54 ± 5.34^{b}	18.90 ± 2.32^{a}
Bound phenolic compound	0	ND	ND
	72	262.63 ± 12.76^{a}	67.69 ± 0.90^{b}
	96	545.24 ± 37.66^{b}	64.71 ± 2.46^{a}

Results were expressed as mean ± standard deviation; fermentation time ($n = 3$). Different lowercase letters (difference between fermentation time of each phenolic fractions) are significantly different ($p < 0.05$) by Duncan's post-hoc test. ND = non detected.

equivalent/g) in relation to the unfermented sample (0 h) (0.022 mg Trolox equivalent/g). The antioxidant potential of the samples evaluated by ABTS radical cation scavenging capacity was shown in Table 2. The results also revealed that fermentation of the okara sample contributed to an increase in its antioxidant potential. Antioxidant activity of free phenolic compounds obtained from the fermented okara after 96 h varied from 0 to 2.320 mg Trolox equivalent/g. An increase of antioxidant activity ranging from 0.452 to 0.960 mg Trolox equivalent/g was also observed in bound phenolic compounds obtained from the fermented okara after 96 h. According to Santos et al [37], the antioxidant capacity of soybean products significantly related to levels of total phenolic compounds and the nature of the chemical structure of the aglycones and the different types of phenolic compounds. This study also observed that due to the increase of TPC, antioxidant activities of okara increased after fermentation. The fermentation by *B. subtilis* could promote the nutritional and health benefits of this soybean by-product.

Our results were in agreement with previous studies in that the TPC has been closely proportional to the antioxidant power of plant food. Oboh et al [35] reported that fermentation of legumes caused a significant increase in the antioxidant capacity of soluble free phenolics extracted from fermented legumes. Microbial enzymes catalyzed the modification of galloylated phenolic compounds to phenolic acid, especially gallic acid with higher antioxidant activities [38]. Therefore, the changes of antioxidant activities of phenolic fractions might be related to the structural modification of phenolics by microbial enzymes during the fermentation process with *B. subtilis*.

Different phenol forms including free and bound fractions present clear differences in biological availability and activity. According to the study of Vattem et al [39], SSF of cranberry pomace which is a by-product of the cranberry processing industry with food grade fungus *Lentinus edodes* can improve phenolic profile and antioxidant activity. A possible explanation for the improved antioxidant activity of soluble free phenolics extracted from fermented legumes is that microbial enzymes may hydrolyze glycosidic bonds to release free phenolics with more available functional

groups for antioxidant activity [32].

These results were also in line with the study of Oboh & Rocha [40], showing the reducing power of free and bound phenols from some varieties of hot pepper. The study reported that the higher free-radical scavenging capacity of the free polyphenol compared to the bound phenol may be caused by the reduction of glycoside moiety in the bound polyphenol. Thus, the functional group (OH) required for the free-radical scavenging capacity was exposed.

CONCLUSION

SSF with *Bacillus subtilis* TISTR001 has proven to be an effective method for significantly enhancing the health benefits of okara. This fermentation process increased the activities of protease, α-amylase, and β-glucosidase, leading to a notable rise in TPC and free phenolic compounds, with a slight improvement in the bound phenolic fraction. These changes resulted in a marked increase in antioxidant activities in the fermented okara. Additionally, the cost-effectiveness, sustainability, and nutritional health improvements offered by okara fermentation make it a promising candidate for further application in the healthy food and nutraceutical industries.

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