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Effect of some fermentation factors on the fermented tiger stripe peanut production by *Rhizopus microsporus* fungus

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ABSTRACT

Tiger stripe peanut (*Arachis hypogaea* L.) (TSP) is a famous Thailand geographical indication (GI) product of the Mae Hong Son province, Thailand. However, the value-added products of this peanut have been required by many local entrepreneurs. Therefore, the production of fermented TSP, a Tempe-like product, by *Rhizopus microsporus* fungus was proposed in our research. The objectives of this study were to investigate the effects of inoculum size (10^5, 10^6, and 10^7 spores/g), incubation temperature (25, 30, 35, and 40°C), and fermentation time (1–7 days) on the soluble reducing sugar, available phosphate and soluble protein of fermented TSP. Subsequently, some chemical properties of the product were studied. The results showed optimal fermentation conditions included an inoculation size of 10^7 spores/g and incubation at 35°C for two days. Moreover, the results from the proximate analysis study demonstrated that the crude protein content of fermented TSP increased by approximately 17% (by DW). The fat, carbohydrate, and crude fiber contents decreased by 3.5, 5.9, and 4.2% (by DW) compared to unfermented TSP, while their calculated energy contents were between 538-539 kcal/100 g DM. Total phenolic content (gallic acid equivalent, mg/g DW) and ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) scavenging antioxidant capacity (Trolox equivalent, mg/g DW) of TSP were enhanced by approximately 2.6 and 1.9 folds, respectively, after fungal fermentation. Overall, the fermented TSP obtained from these optimized processes will be a new nutritious food product. However, further study on its health benefits will be able to apply this product as a novel functional Thai food.

Keywords: tiger stripe peanut, process optimization, functional food, fermentation

INTRODUCTION

Tiger stripe peanut (*Arachis hypogaea* L.) (TSP) or “Kalasin 2” and “Thua-Lai-Suer” in Thai is a local peanut Cultivar that was collected from the International Crops Research Institute for the Semi-Arid Tropics, India, in 1973. It had been selectively bred by the Kalasin Agricultural Research and Development Center since 1979. Nowadays, it is widely cultivated by farmers in several regions of Thailand. However, it is suitably cultivated in 4 districts of Mae Hong Son province in northern Thailand, i.e., Muang, Pang Ma Pa, Kun Yuam, and Pai, because of appropriate weather and soil conditions. The quality of TSP grown in Mae Hong Son is outstanding and is domestically accepted among consumers because it tastes sweet and creamy. Moreover, the texture is crunchy. After TSP and its relevant processing products became well known, a policy and guideline for quality control (including internal control and traceability) were issued by the Mae Hong Son Provincial Administrative Organization. This promotion policy led to the registration of the Mae Hong Son TSP as the Thailand geographical indication (GI) product in 2019 by the Department of Intellectual Property, Thailand. The GI sign can guarantee a specific geographical origin, quality, and reputation of products. Typically, peanuts are a source of oil, proteins, carbohydrates, and minerals. Arya et al. (2016) reported the data according to the USDA National Nutrient database that protein, carbohydrate, and fat content is about 25.8, 16.13 and 49.24% (by wt.), and they are also excellent sources of active compounds, such as resveratrol, phenolic acids, flavonoids, and phytosterols. Moreover, peanuts contain high unsaturated fatty acids, low saturated fatty acids, and cholesterol-free (Arya et al., 2016). Thus, the consumption of peanuts is expected to improve serum lipid profiles, decrease LDL oxidation, cardio-protective effect, and the risk reduction of cardiovascular disease, type 2 diabetes,

and cancers (Arya et al., 2016; Çiftçi and Suna, 2022).

In Thailand, many community enterprises and local small and medium enterprises (SMEs) commercially produce and process the TSP and relevant products. However, the variety of products is still low. A salted roasted TSP seems to be the leading and well-known product in the market. The research and development of novel products, especially functional foods, from TSP to consumers are of interest and required among those enterprises. In this study, we focused on the product development of TSP by using microbial fermentation technology, an effective and simple technique, and a low-cost process to enhance the nutritional quality and value of such agricultural raw materials. Fermentation allows microorganisms to make several biochemical changes in the substrates, improving the nutritional value, degrading anti-nutritional factors, and increasing nutrient bioavailability and bioactivities. (Mukherjee et al., 2016; Lo et al., 2022). Furthermore, the market demand for healthy foods, especially plant-based protein foods for vegetarians and vegans, has expanded. In this study, solid-stage fermentation by *Rhizopus microsporus* fungus was proposed. There are six varieties of *R. microsporus*, i.e. vars *microsporus*, *azygosporus*, *chinensis*, *oligosporus*, *rhizopodiformis* and *tuberosus*, based on their spore size and shape (Dolatabadi et al., 2013). This fungus has been used to produce oriental fermented foods for centuries. It is the predominant fermenting microbe found in Tempe, a local fermented soybean food of Indonesia, especially current Tempe products. The commercial Tempe starter has been widely used instead of natural starter (Sjamsuridzal et al., 2021). Therefore, the fermented TSP product was proposed and developed in our study to serve these recent demands. The objectives of this study were to study the effects of an inoculum size, incubation temperature, and fermentation time on the fermentation efficiency of TSP and to evaluate some nutritional quality, i.e., proximate analysis, phenolic content, and antioxidant activity, of the obtained product.

MATERIALS AND METHODS

Raw material

Fresh seeds of tiger stripe peanut (TSP) were collected from a product cultivated by the community enterprise in Pang Ma Pa district, Mae Hong Son province. Seeds were cleaned and de-hulled before being stored in a vacuum-sealed plastic bag at below 10°C.

Fungus

Rhizopus microsporus BG5 from the Agro-Industrial Biotechnology Laboratory, Faculty of Science, Maejo University, was used in this study. The fungal starter was prepared as a spore suspension in 0.85% (w/v) NaCl solution after being cultivated on potato dextrose agar (PDA) at 30°C. The spores were enumerated by counting them in a hemacytometer.

Effect of fermentation factors on the quality of fermented TSP

The dehulled TSP was soaked in distilled water containing 0.5% (w/v) lactic acid (KemAus™, Australia) for 12 h at 4°C, afterward drained, steamed until cooked, and cooled down at room temperature. Cooked TSP was used as the raw material in the production of fermented TSP. The fermentation was conducted in the 7x10 cm polyethylene Ziplock bag (without perforation) with 50 g of TSP (dry weight) and generally fermented with 10^6 spores/g, 30°C for 24 h. The optimal conditions to ferment TSP were investigated in this experiment according to the one-variable-at-a-time method. Three important factors involving the *Rhizopus* fermentation were optimized, i.e., inoculum size (10^5, 10^6, and 10^7 spores/g), incubation temperature (25, 30, 35, and 40°C), and fermentation time (1 to 7 days). Subsequently, the fermentation of all treatments was terminated by air drying at 70°C until constant weight and grinding into fine powders using a grinder machine (ARTC, UAE). The powders were stored in a sealed polypropylene bag at −20°C for further analysis. The content of soluble reducing sugar, protein, and phosphate of fermented TSP were the main criteria for consideration. Moreover, the finished product and raw material were subjected to analysis of their contents of macronutrients (proximate analysis), total phenolic content, and ABTS radical scavenging antioxidant capacity.

Chemical analysis of some fermented TSP nutrition

One gram of fermented TSP powder was mixed well with 9 ml DI water, sonicated without temperature control for 30 min, and centrifuged to collect the supernatant (water-soluble fraction) at 10,000 RPM and 10 min for the analysis of soluble reducing sugar, protein, and phosphate contents. To determine the soluble reducing sugar content, DNS (3,5-dinitrosalicylic acid method modified from the protocol of Miller (1959), was performed (Wongputtisin et al., 2015). The reducing sugar content was calculated from the calibration curve of standard D-glucose (Ajax-Finechem, Australia)

plotted versus the light absorbance at 540 nm, and the results were expressed as mg/ g DW. The soluble protein content was analyzed according to the spectrophotometry method described by Bradford (1976). The Coomassie Brilliant Blue G-250 - protein complex was measured by the light absorbance at 595 nm and estimated protein content by the standard curve of bovine serum albumin (BSA) (HiMedia, India). The soluble phosphate or available phosphate content was determined according to the principle of the heteropolyblue method with some modification (Wongputtisin et al., 2012). K_2PO_4 was the standard phosphate for calibration curve preparation and estimating soluble phosphate content from the light absorbance at 820 nm.

The proximate analysis of a finished product and raw material included moisture, total ash, crude protein, crude fiber, crude fat, and carbohydrate content, which were determined using the AOAC method (AOAC, 2012) and calculated energy values. Moreover, they were freeze-dried, ground into powder, and defatted by hexane. Three grams of dry powder was mixed with 40 ml of 80% (v/v) methanol and continuously stood for two hours with intermittent shaking. The extracts were recovered by centrifugation twice at 3,500 RPM and 10,000 RPM for 15 min without temperature control. Methanol in the extract was removed by vacuum evaporation and subsequently resubstituted in DMSO at the original concentration. These extracts were subsequently analyzed for the total phenolic content (TPC) and ABTS scavenging antioxidant capacity according to the method of Suguhara et al. (2015). The content of TPC was calculated by comparing it to a standard curve of gallic acid. ABTS scavenging activity was expressed as the equivalent capacity of Trolox (positive control), and the half-maximum effective concentration (EC50) value was estimated.

Statistical analysis

The experimental results were analyzed using Minitab software (Minitab Version 21, Minitab Inc., USA). All data were expressed as the means ± standard deviations of triplicate measurements. A one-way analysis of variance (ANOVA) at the 95% significance level was used to determine significant differences ($P < 0.05$) between the means and obtain the optimized values.

RESULTS AND DISCUSSION

Effect of fermentation factors on the quality of fermented TSP

In the fermentation of TSP, many factors from nutritional, environmental and processing aspects influence the quality of the product. However, the scope of this study was to utilize TSP as the sole raw material. Thus, only three main factors, i.e., inoculum size, temperature, and fermentation time, were selected for initial investigation. In this regard, the enhancement of TSP bioavailability was one of our objectives; therefore, the resulting soluble sugar, protein, and phosphate content in the obtained products were criteria in this study. These criteria indirectly reflected the fungal growth and bioavailability of the product (solubility, digestibility, and absorbability). The results showed that soluble protein (Figure 1C) and phosphate (Figure 1C) increased along with the increase of inoculum size. At the same time, this correlation was not found in the resulting soluble reducing sugar (Figure 1A). Higher inoculation size generally shortens the lag phase of microbial growth during fermentation and also reduces the risk of microbial contamination. However, the cost of inoculum must be considered and compromised. *Rhizopus* sp. fungus can produce several enzymes responsible for polysaccharides, proteins, and phytic acid hydrolysis. Thus, a higher amount of the released protein and phosphate might correspond to the fermentation efficiency (fungal growth and nutrient bioavailability enhancement). On the other hand, the carbon source is the essential element for microbial mass production; therefore, a lowering of soluble sugar content could be observed in the treatments with higher inoculum size even though soluble protein contents of the treatments with 10^6 and 10^7 spore/g were not significantly different ($P > 0.05$) (70 – 77.32 mg/g DW). The soluble phosphate found in the treatment of 10^7 spore/g was significantly greater than in other treatments. *Rhizopus* sp. fungi are capable of phytase production (Sato et al., 2014). This enzyme hydrolyzes phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate), one of the antinutritional factors contained in leguminous seeds, to inositol and 6 molecules of phosphate. Thus, the higher soluble phosphate content might imply a decrease in phytic acid. According to these results, an inoculum size of 10^7 spores/g was chosen.

The effect of incubation temperature was studied at 25, 30, 35 and 40°C. The results are presented in Figure 2 (A to C). It was demonstrated that incubation at 35°C resulted in the highest content of soluble reducing sugar, available phosphate, and protein to fermented TSP. The soluble contents reached 53.82 and 272.24 mg/g DW, respectively. In 2000, Han and Nout reported that the optimal temperature for mycelium germination of *R. microsporus* on an agar medium was 40°C. However, a different growth rate of this fungus at 40°C was observed in our study. We found that the mycelium germination in the treatment of 40°C was lower than that of 35°C. It might be because of the difference in

substrate characteristics. Metabolic heat accumulation was present in the TSP substrate bed during fermentation. Thus, the actual temperature inside the TSP bed in the treatment of 35ºC might reach close to 40ºC. Furthermore, the incubation at 40ºC led to a higher evaporation rate of water, resulting in a lowering of the water activity value (a_w). The optimum a_w for *R. microsporus* is 0.995 (Han and Nout, 2000). From these results, the incubation temperature of 35°C was selected for further study.

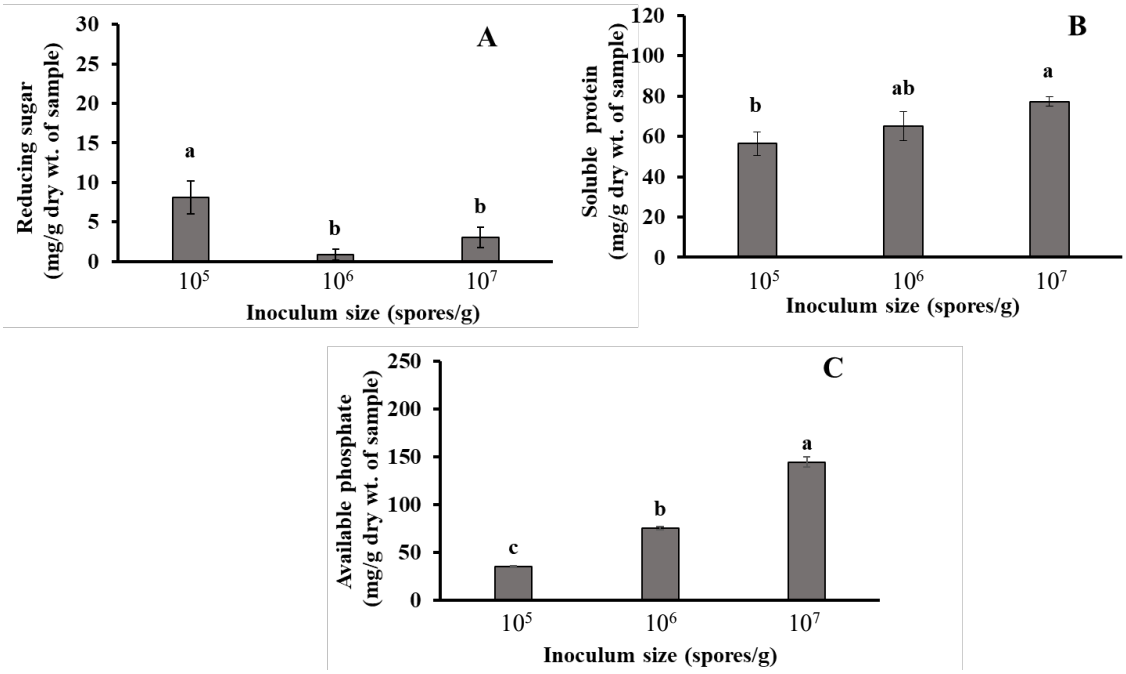

Figure 1. Effects of the inoculum size of *R. microsporus* BG5 on (A) soluble reducing sugar, (B) available phosphate, and (C) soluble protein of fermented TSP. Different letters in each experiment indicate significant differences at $P < 0.05$.

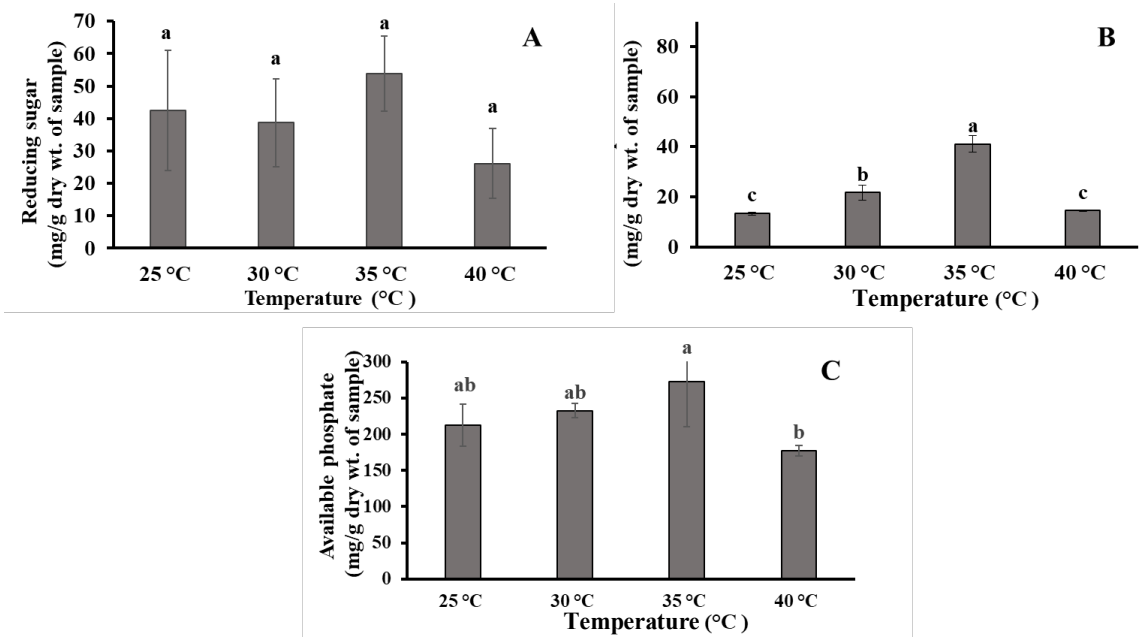

Figure 2. Effects of the incubation temperature of *R. microsporus* BG5 on (A) soluble reducing sugar, (B) available phosphate, and (C) soluble protein of fermented TSP. Different letters in each experiment indicate significant differences at $P < 0.05$.

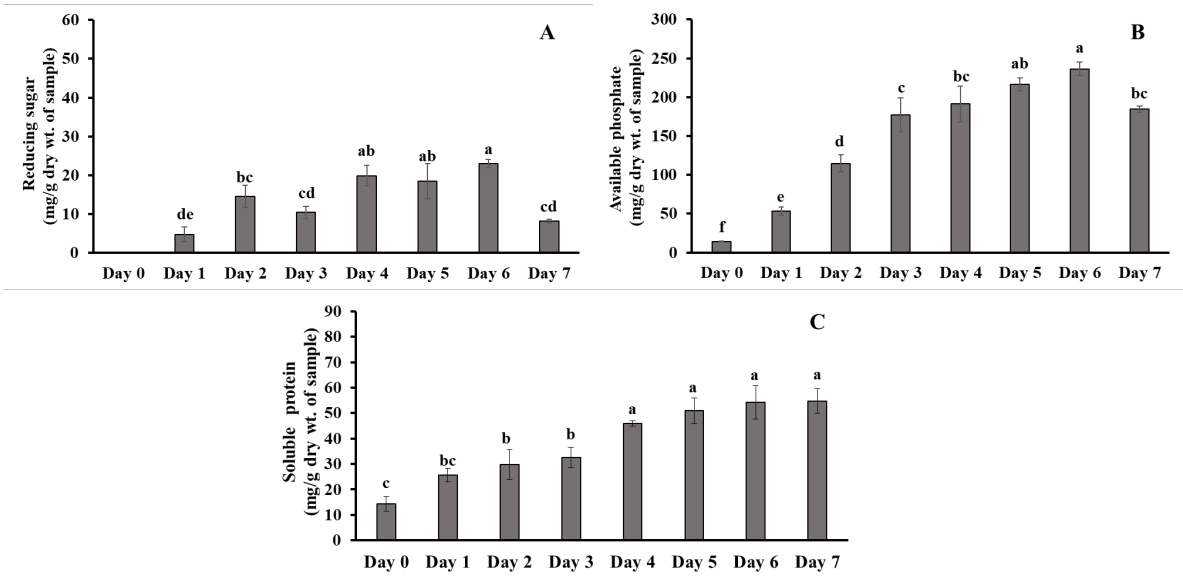

Figure 3. Effects of the fermentation time of *R. microsporus* BG5 on (A) soluble reducing sugar, (B) available phosphate, and (C) soluble protein of fermented TSP. Different letters in each experiment indicate significant differences at $P < 0.05$.

The effect of fermentation time on the soluble sugar, available phosphate, and soluble protein was investigated within the 1–7 days range; these results are shown in Figure 3. The results showed that all soluble contents increased relating to fermentation time. Soluble protein content gradually increased until day 5 and did not significantly change. The same trends were found in soluble reducing sugar and phosphate content. These contents were higher from day 0 to day 6 ($P < 0.05$) but decreased on day 7. Moreover, the capacity of ABTS radical scavenger was enhanced by approximately 10–12 folds after 5 –7 days of fermentation, referring to our preliminary test (data not shown). This finding could also indicate that TSP was a nutrient-rich substrate for microbial cultivation. However, the appearance of fermented TSP was also changed when extended fermentation time as shown in Figure 4. Color, smell, and texture were the obvious attributes that had been negatively changed and were unacceptable to consumers. The appeared caramel-like color was expected from the enzymatic browning reaction (Liu et al., 2023), while the pungent smell occurred was mainly an ammonia from protein metabolism. The softening texture could be caused by the degradation of cell structural polysaccharides and proteins. From day 0 until day 2, *R. microsporus* mycelium rapidly spread, covered, and tightly attached to the entire TSP substrate bed. This fermentation pattern was similar to that reported by Liu et al. (2023) who studied fermented dehulled soybean and found that the white mycelium of *R. oligosporus* fully covered the substrate after two days. The fermented TSP obtained in this study exhibited satisfactory attributes that met Tempe's acceptable quality standards. A good Tempe includes its compactness, with the whole surface covered by white mycelium, devoid of black spores, non-slimy, easily sliced, non-rotten, and free from ammonia odor (Nabilah et al., 2021). For the above reasons, two days of fermentation were decided to meet both nutritional and sensory acceptance. The optimal process conditions for fermented TSP production were an inoculum size of 10^7 spores/g DW of TSP, with incubation temperature at 35°C and two days of fermentation time.

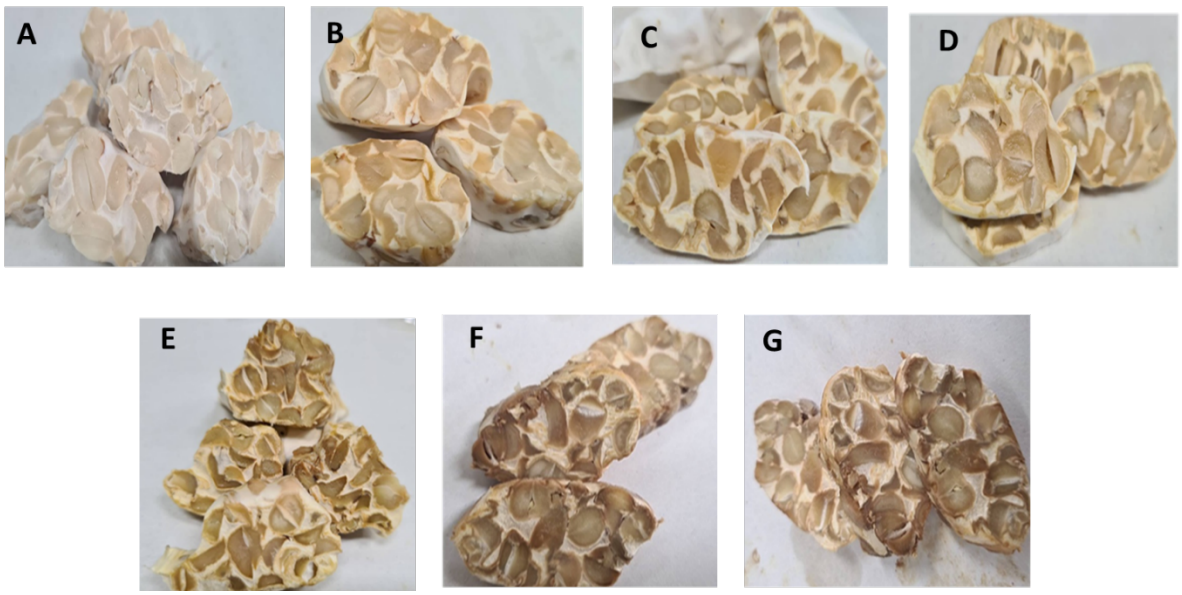

Figure 4. The appearances of fermented TSP after cultivation with 10^7 spores of *R. microsporus* /50 g, at 35◦C for 1 Day (A), 2 Days (B), 3 Days (C) 3, 4 Days (D), 5 Days (E), 6 Days (F), and 7 Days (G).

Some nutritional values of fermented TSP

The proximate analysis of fermented TSP produced under the optimal process compared to unfermented TSP was compiled in Table 1. The results demonstrated that crude protein content was 30.5% (by DW), which was enhanced by approximately 17% after fermentation. However, crude protein content increased due to the loss of carbon components during fungal metabolism, especially carbon dioxide molecules. Thus, the overall percentage of crude protein increased. According to the Indonesian National Standard (Nabilah et al., 2021), this quality met the protein standard of Tempe products, which requires a minimum protein content of 15%. The obtained protein content of our fermented TSP was also higher than the report of Matsuo (2006), who produced a peanut Tempe (Hana 17, China) by *R. oligosporus* fungus. Tempe contained 25.3% (by DW) of protein and was not different from the content of unfermented peanuts. On the other hand, fermentation slightly decreased the fat, carbohydrate, and crude fiber contents of TSP by about 3.5, 5.9 and 4.2%, respectively. At the same time, their calculated energy values were almost similar, between 538 – 539 kcal/100 g DM.

Fermented TSP contained a total phenolic content (TPC) content higher than that of TPS about 2.6 folds after fungal fermentation. These results corresponded to their ABTS scavenging antioxidant capacity. It was found that fermentation could improve the ABTS inhibition capacity (eq. mg Trolox/g DW) and the EC_{50} (mg DW/mL) of TPS approximately 1.9 folds, as the results are shown in Table 2 and Figure 5. It has long been reported and confirmed by many reports that fermentation is an effective technique for the antioxidant improvement of natural raw materials, especially cereals and leguminous seeds. The study of fermented soybean (Lo et al., 2022; Wongputtisin et al., 2007), and fermented peanut press cake (Sadh et al., 2018) are examples. Various indigenous phenolic compounds of TSP are the main compounds responsible for antioxidation activities. Radhakrishnan et al. (2014) reported the existence of many phenolic compounds, such as daidzin, genistin, quercetin, isorhamnetin, and rutin, in peanut seeds. These active compounds could be increasingly released from peanut cells because of the activity of such cell structure-degrading enzymes (cellulases, hemicellulases, and proteases). Moreover, the proteolytic activity of fungus produces short-chain peptides and free amino acids, especially arginine, tyrosine, methionine, histidine, lysine, and tryptophan that are generally accepted to be antioxidants (Wang and Mejia, 2005; Taheri et al., 2023). The reducing sugars liberated from polysaccharide hydrolysis are also accepted as potent antioxidants. The enzymatic transformation of glycoside isoflavones to aglycone isoflavones by the activity of *Rhizopus* β-glucosidase might be another mechanism to explain the increasing of antioxidant capacity. Aglycone isoflavones are the forms with higher bioactivities and bioavailability than those of glycoside forms (Baú and Ida, 2015). The antitumor, antimenopausal (female) osteoporosis and anti-aging properties, improvement of learning and memory skills of menopausal women, prevention and treatment of heart disease and diabetes are the functionalities of isoflavones (Wang et al. , 2013; Lante et al., 2018). However, this invented fermented TSP needs to investigate its specific active compounds and functionalities further before being introduced to the market with high competitiveness

Table 1. Proximate composition of tiger stripe peanut and fermented tiger stripe peanut product (%w/w)

Component	Unit	Tiger stripe peanut	Fermented tiger stripe peanut
Moisture	%	3.84	2.59
Fat	%	32.74	31.59
Protein	%	26.01	30.53
Carbohydrate	%	35.09	32.99
Ash content	%	2.29	2.27
Crude fiber	%	4.24	4.06
Energy	kcal/100 g	539	538

Table 2. The total phenolic content (TPC) and ABTS scavenging antioxidant capacity of tiger stripe peanut and its fermented product

Samples	Tiger stipe peanut	Fermented TSP
Total phenolic content (mg eq. gallic acid/g DW)	2.50 ± 0.03	6.59 ± 0.09
ABTS scavenging antioxidant capacity		
- ABTS inhibition (eq. mg Trolox/g DW)	8.29 ± 0.99	16.00 ± 1.57
- EC_{50} (mg DW/ml)	39.28	20.47

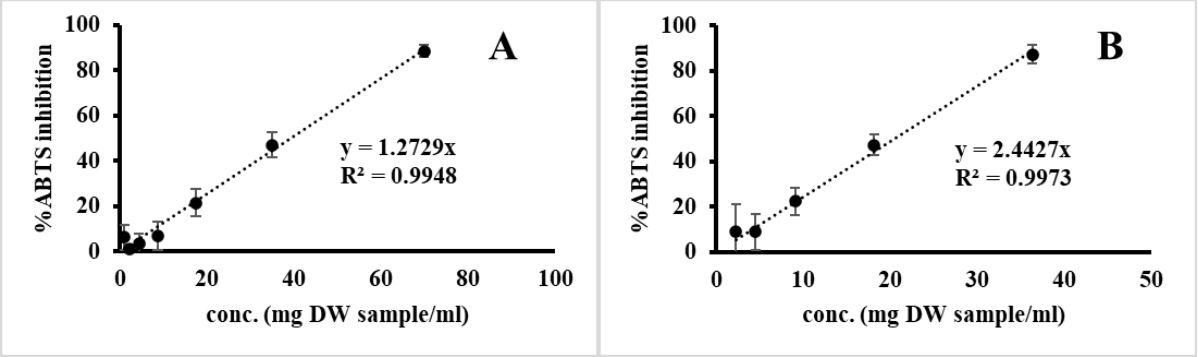

Figure 5. The ABTS inhibition capacity of tiger strip peanut extract (A) and fermented tiger peanut extract (B) at various concentrations.

CONCLUSIONS

Based on the above results, it could be concluded that the optimal fermentation conditions for fermented TSP production included an inoculation size of 10^7 spores/g, incubation at 35 ºC, and two days of fermentation period using *Rhizopus microsporus* BG5. The fermented TSP produced under these optimal conditions exhibited satisfactory nutritional values. Protein and polyphenolic contents and antioxidant capacity (ABTS scavenging activity) were significantly improved after the fermentation. This fermented TSP will be a new nutritious food product serving the healthy food market. However, further study on its active compounds, functionality, and safety are required before commercial application.

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