Enrichment of Mung Bean with L-DOPA, GABA, Essential Amino Acids *via* Controlled Biofermentation Strategy

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Abstract: L-DOPA (L-3,4-dihydroxyphenylalanine) the precursor of neurotransmitter dopamine is used in the management of Parkinson disease and effective in controlling diabetic state. Gamma-aminobutyric acid (GABA), a non-protein amino acid is known to have many pharmacological functions and plays a major role in inhibiting neurotransmitter in brain. Whereas essential amino acids can't synthesize in human body and it must be taken from foods to maintain good immune function. This study aims to evaluate the enrichment of mung bean with L-DOPA, GABA and essential amino acids *via* controlled solid state fermentation using *Rhizopus* strain 5351. Fermentation was carried out for a duration up to 48 h at 30 °C and the samples were analyzed at certain time intervals. The concentration of glucosamine and β-glucosidase, which indicated the growth of fungal was noted low at the early growth stage (0 to 10 h), but it was observing increased linearly within 18 to 48 h growth periods. The L-DOPA was produced after 10 h fermentation time (0.008 g/100 g dry weight, DW) and the highest yield of L-DOPA content (0.07 g/100 g DW) was attained at the fermentation time of 28 h. However, the concentration of L-DOPA was noted decreased after that. The protease activity, free and essential amino acids content also showed a drastic increment within the fermentation period of 10 to 38 h. The highest content of free and essential amino acids (FAAs and EAAs) and the protease activity of fermented mung bean were exhibited at 38 h incubation time, which were 3.74 g/100 g DW, 1.43 g/100 g DW and 18.4 U/g dry weight, respectively. The GABA content of fermented mung bean was found low (0.019 - 0.021 g/100 g DW) at early incubation time (0-10 h), however, it showed a drastic increment in the fermented mung bean after 18 h (0.132 g/100 g DW) and continuously increased until 38 h (0.198 g/100 g DW). This study showed the potential of solid state fermentation as a good strategy to enrich the fermented mung bean with L-DOPA, GABA and other beneficial bioactive compounds which play an important role to maintain good health as it helps to enhance our immune system and regulating neurotransmitter function.

Keywords: L-DOPA, GABA, Mung Bean, Protease, Solid State Fermentation.

INTRODUCTION

L-DOPA (L-3-,4-dihydroxyphenylalanine) is the precursor of neurotransmitter dopamine and its synthesis involved the use of tyrosinase to converts tyrosine to L-DOPA, which is a useful drug in the treatment of Parkinson disease. On the other hand, GABA is a metabolic product of L-glutamine that was produced *via* glutamate decarboxylase enzyme. GABA was reported to have various physiological activities which function as anti-hypertension [1], an-diabetic [2], anti-inflammatory [3], anti-depression [4], and as anti-inflammatory agent [5]. Both L-DOPA and GABA can be produced by prokaryotic and eukaryotic organisms. Numerous studies have shown that these two compounds exists naturally in some plant sources such as legumes and seeds [6-10] and can be produced by certain bacteria [11-13], yeast [14] and fungal [15-16] under certain circumstances.

Plant sources especially legumes and seeds are great source of phytonutrients such as proteins, fats, carbohydrates, minerals, vitamins, fiber and phenolic compounds. Regular consumption of these sources may have beneficial effects to our health and protect from serious diseases such as cancer, diabetes and hypertension [17] Mung bean is one of the legumes containing many important phytochemicals with health promoting properties. It has been widely consumed and used in the treatment of certain diseases in ASEAN region for decades. Its extract had been reported to have pharmacological properties like anti-glycation [18], anti-inflammatory [19], anti-oxidant [20], anti-fungal and anti-bacteria [21]. All these beneficial effects were associated with the presence of GABA [22], phenolic compounds, vitexin and isovitexin [19] and also bioactive peptides [21]. However, these bioactive compounds such as GABA are found too low in the native plant sources and hence various physical and chemical approaches have been conducted to increase the yield of these bioactive compounds. Heat treatment [9], cold shock [11], soaking and gaseous treatment [23], germination [24] and fermentation process [4] are some of the approaches applied by researchers to enhance the yield of these beneficial compounds in various plant sources.

Fermentation process is well known to increase the nutritional composition and metabolic regulatory functions of various foods. This involves the hydrolysis

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of food constituents such as protein, fat and carbohydrate by protease, lipase and amylase enzymes, which are secreted by microbes during its growth period. This technique has long been used for the production of tempeh (fermented soy bean) *via* solid state fermentation and widely applied to various kinds of legumes lately to increase its nutritional value. It was reported that both L-DOPA and GABA synthesis could be enhanced through microbial solid state fermentation [25-26]. Past research findings have indicated that the nutraceutical properties of mung bean improved dramatically after subjected to solid state fermentation [7]. Furthermore, solid state fermentation can be considered as a simple and cost effective process as it requires small volume of water and the risk of contamination is low. Thus, this study aims to investigate the capability of *Rhizopus* strain 5351 in enriching bioactive compounds in mung bean *via* solid-state bioconversion.

MATERIALS AND METHODS

Preparation of Inoculum

Four days old of *Rhizopus* strain 5351 that grown on potato dextrose agar was inoculated with sterile rice flour, mixed well and incubated at 30 °C for 4 days. After 4 days incubation, it was subjected to drying process at 40 °C for a duration of 6 days before ground to powder and kept at 4 °C for future use.

Preparation of Fermented Mung Bean

The dehulled mung bean was washed and soaked for 16 h. Then, beans were rinsed and boiled for 10 min before drained and air dried to achieve the final moisture content of 50-60% (w/w). The inoculum was added at a ratio of 5 g/kg beans and mixed thoroughly before packed in perforated polyethylene bags (thickness: 15 – 20 mm, perforation distance: 1 cm apart, 100 g cooked beans per pack). The fermentation was carried out at 30 (± 2) °C for 48 h. At certain time intervals, the fermented beans was took out and oven dried to achieve the moisture content of 3-5% (w/w) before ground into fine powder using ultra centrifugal mill (Retsch, model: ZM200).

Extraction of Bioactive Compounds from Fermented Mung Bean

Chilled distilled water was added to ground mung bean (1:10, w/v) and subjected to shaking at a agitation speed of 300 rpm, 30 ^{o}C for 30 min. It was followed by centrifugation step which set at 10,000 rpm for 10 min with the temperature fix at 4 ^{o}C. The collected supernatant was then filtered through a Whatman No. 4 filter paper.

Measurement of Glucosamine Content

Glucosamine content was measured according to Desgranges *et al.*'s method [27] with slight modification. Dried fermented mung bean (0.5 g) was hydrolyzed in 10 N HCl (6 mL) at 100 °C for 16 h. The hydrolyzed mung bean was then cooled to room temperature, filtered through Whatman No. 1 filter paper and the pH was adjusted to 7.0 before the final volume was topped up to 50 mL. A mixture of hydrolyzed mung bean (1 mL) and 2% (v/v) acetylacetone (1 mL) was heated at 100 °C for 20 min. After cool down dropping to room temperature, 6 mL of absolute ethanol was added, followed by 1 mL Ehrlich reagent. The solution was remixed and then heated at 65 °C for 10 min before the absorbance was read at the wavelength of 530 nm. N-acetyl-D-glucosamine was used as a standard.

β-Glucosidase Activity Assay

The activity of β-glucosidase was measured using p-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate. A mixture comprised of 100 µL of pNPG, 800 µL of 200 mM sodium acetate buffer (pH 4.6) and 100 µL of fermented mung bean water extract were mixed thoroughly before subjected to agitation rate of 150 rpm for 15 min at 50 ^{o}C. The reaction was stopped by adding 1000 µL of 0.1 M sodium carbonate and the amount of p-nitrophenol (pNP) released was measured at the wavelength of 400 nm. One unit of enzyme activity was defined as the amount of β-glucosidase that released 1 µmol of pNP per min under the above mentioned assay conditions. pNP was used as a standard to calculate the β glucosidase activity.

Measurement of L-DOPA, GABA and Amino Acids Profile using Ultra Performance Liquid Chromatography (UPLC)

The L-DOPA, GABA and amino acids profile of fermented mung bean were analyzed using AccQ-Tag^{TM} Ultra column (2.1 mm x 100 mm, 1.7 µm) with the flow rate of 0.7 mL/min. The column temperature was controlled at 55 °C and the UV spectra is quantified at the wavelength of 260 nm. A total of 10 µL water extract from fermented mung bean was derivatized with 70 µL AccQ-Tag^{TM} Ultra borate buffer and mix vigorously. Then, 20 µL AccQ^{TM} Fluor reagent was added and vortex for a while, followed by heating

at 55 °C for 10 min before injecting 1 µL solution into the UPLC system as described in the UPLC amino acid analysis application solution. The gradient elution consisting of AccQ-Tag™ Ultra Eluent A and AccQ-Tag™ Ultra Eluent B. Gradient elution was conducted as follows: from 0 to 0.54 min, maintained at 99.9% A; from 0.54 to 5.74 min, linear gradient from 99.9 to 90.9% A; from 5.74 to 7.74 min, linear gradient from 90.9 to 78.8% A; from 7.74 to 8.50 min, linear gradient from 78.8 to 40.4% A and then hold for 0.3 min at 40.4% A; from 8.80 to 8.90 min, linear gradients from 40.4 to 99.9% A and then maintained at 99.9% for another 2.1 min. Quantification was made using calibration curves obtained by injecting known amounts of amino acids standards, L-DOPA and GABA as external standards with known retention times. The total essential amino acids were calculated based on the sum of histidine, phenylalanine, threonine, methionine, leucine, isoleucine, tryptophan, lysine and valine. All analyses were performed in triplicate.

Protease Activity Assay

Protease activity was measured according to Brock *et al.*'s method [28] with minor modification. An amount of 1 mL 0.8% (w/v) azocasein in 50 mM acetate buffer pH 5.5 was preincubated at 40 °C for 5 min. The enzyme reaction was initiated by adding 1 mL fermented mung bean extract and the reaction was allowed to continue for 15 min at 40 °C with the agitation rate of 200 rpm. Then, the reaction was stopped by adding an equal volume of 10% (w/v) trichloroacetic acid (TCA). After 15 min, the mixture was subjected to centrifugation to obtain the supernatant and then mixed with an equal volume of 1 N NaOH. The absorbance of the mixture was read at 450 nm. Control was assayed in the same manner except the mung bean extract was added after TCA.

Statistical Analysis

Data was analyzed using Statistical Package for Social Science (SPSS) for Windows version 11.0. One way analysis of variance (ANOVA) at 95% significance level was used to determine significant different ($P \leq 0.05$) between the means.

RESULTS AND DISCUSSION

Glucosamine Content and β-Glucosidase Activity

N-acetyl-glucosamine is a hydrolytic product of chitin which is present in the cell wall of fungal [29]. During the solid state fermentation, the growth of *Rhizopus* strain 5351 was monitored by measuring the concentration of glucosamine. Fermentation of mung bean was carried out for the duration of 48 h at 30 °C and the samples were analyzed at the certain time intervals. The concentration of glucosamine, which indicated the growth of fungal was found to be low at the early growth stage (0 to 10 h). However, the glucosamine content in fermented mung bean was increased linearly at 10 h onward (Figure **1**). The content of glucosamine was noted increased from 17.2

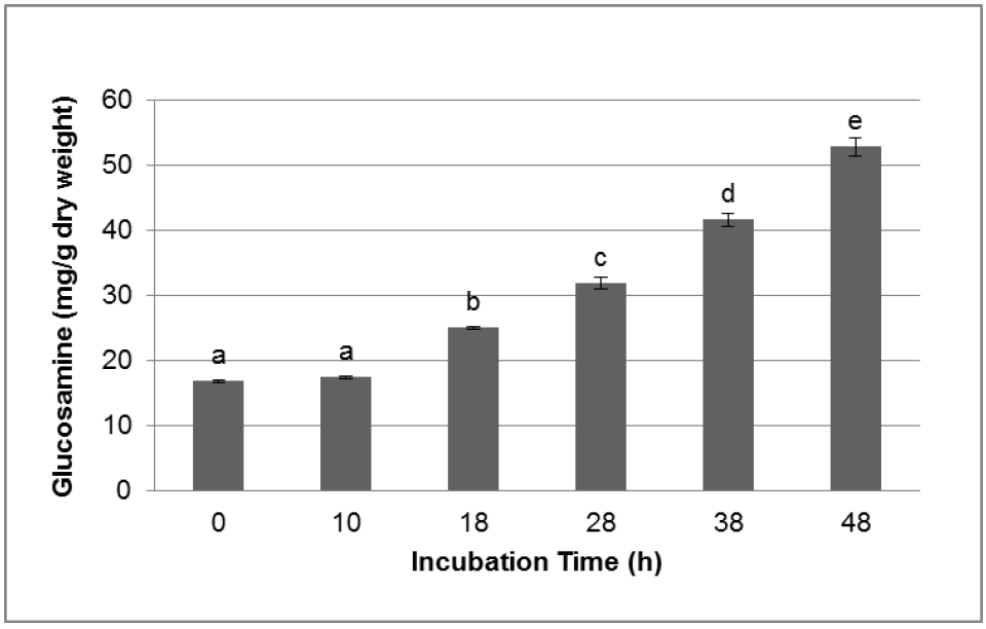

Figure 1: Changes of glucosamine content in mung bean during solid state fermentation by *Rhizopus* strain 5351. Bars represent the standard error of the means (n=3), different letters are significantly difference at $P \leq 0.05$.

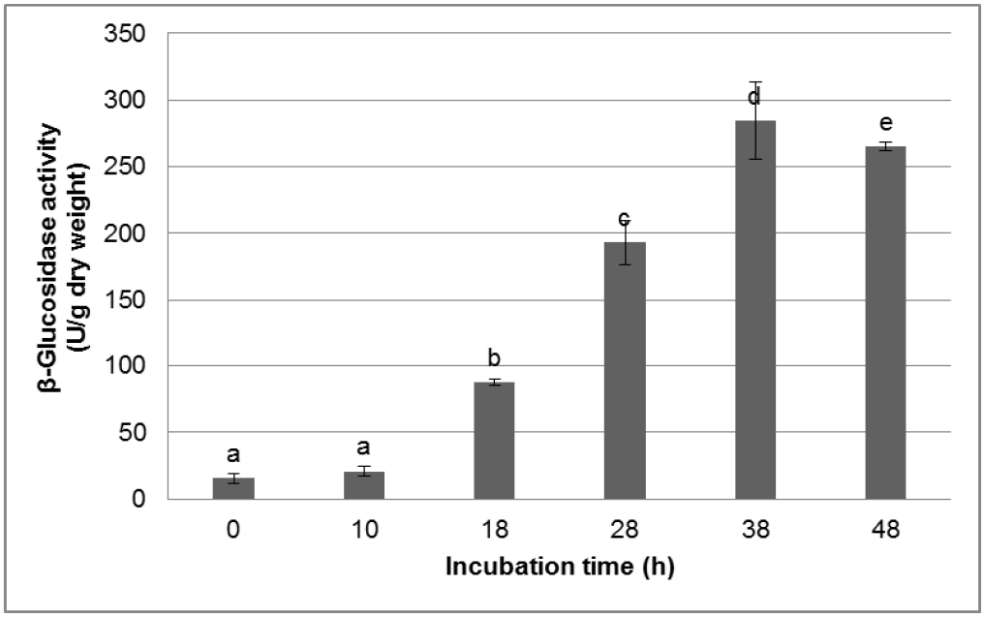

Figure 2: Changes of β-glucosidase activity in mung bean during solid state fermentation by *Rhizopus* strain 5351. Bars represent the standard error of the means (n=3), different letters are significantly difference at $P \leq 0.05$.

mg/g DW at the beginning growth period and reached 52 mg/g DW at 48 h growth phase. It was reported that the growth of fungal was related to the β-glucosidase activity [7]. The changes of β-glucosidase activity (Figure **2**) were similar to the changes in glucosamine concentration during 48 h growth periods. The β-glucosidase activity was minimal at the first 10 h (21 U/g DW), but it showed a significant increment after 18 h onward and then slowly decreased when reached at the fermentation time of 48 h. The optimal β-glucosidase activity (285 U/g DW) was obtained at 38 h fermentation period, which was 14 fold increment compared to non-fermented mung bean. β-glucosidase is the responsible enzyme that hydrolyzes the glycosidic bond of terminal non-reducing glucosyl residue from oligo or polysaccharide, thus releases reducing sugar. Therefore, the increment of the β-glucosidase may indicate the need of fungal for sugar as a carbon source to grow.

Protease Activity, Free and Essential Amino Acids Content

The effect of solid state fermentation on the protease activity of fermented mung bean is shown in Figure **3**. Initially, there is no much changes in protease activity of fermented mung bean for the first 10 h. However, a significant increment was detected within 10 to 38 h, but then showed a slightly decreased when reached at 48 h of fermentation periods. This pattern was in agreement with Ruiz-Teran and Owens (1996)'s findings [30], the same changes of protease activity trend observed in fermented soybean. The highest activity of protease (18.4 U/g DW) was obtained at 38 h fermentation which was 99% higher than non-fermented mung bean (0.1 U/g DW). Even though our findings showed that the protease activity of fermented mung bean had been slowed down after 38 h, however, higher protease activity might be obtained by prolonging the incubation time up to 120 h [30-31]. The cell wall bound protease was associated with the significant hydrolysis of protein [32], whereby the hydrolytic protein was utilized as a carbon and nitrogen source for the fungal growth or being accumulated as peptide and free amino acids [30].

Figure **4** shows the changes of free (FAAs) and essential amino acids (EAAs) content of fermented mung bean after inoculated with *Rhizopus* strain 5351. Total essential amino acids were calculated based on the sum of histidine, phenylalanine, threonine, methionine, leucine, isoleucine, tryptophan, lysine and valine. This finding showed a close relationship between the protease activity, free (FAAs) and essential amino acids (EAAs) in fermented mung bean. Overall, the FAAs and EAAs concentration were observed increased linearly up to 38 h and then maintained high concentration after that. Initially, the concentration of FAAs was 0.18 g/100 g DW and the higher concentration of FAAs was obtained at 38 h (3.74 g/100 g DW) and remained until the end of incubation period. In present study, the increment of FAAs concentration in fermented mung bean was 21 folds after 38 h fermentation, which was higher than

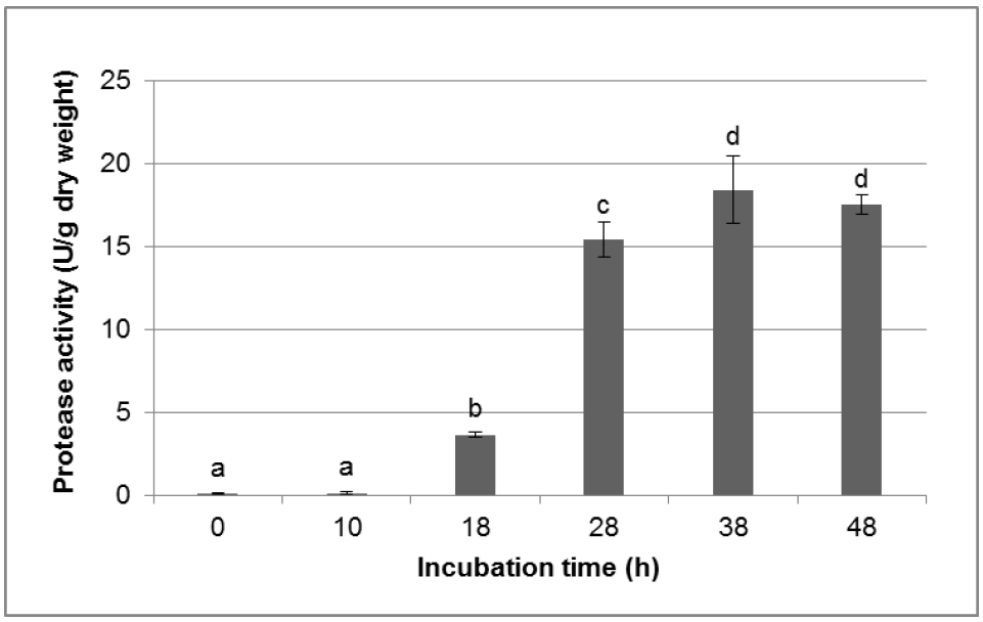

Figure 3: Changes of protease activity in mung bean during solid state fermentation by *Rhizopus s*train 5351. Bars represent the standard error of the means (n=3), different letters are significantly difference at $P \leq 0.05$.

fermented soya bean [31] and fermented faba bean [33]. The latter fermented products only showed an increment of five-fold (final concentration, 19.6 mg/g DW) and three-fold (final concentration, 17.66 g/kg DW) respectively after fermentation. Analysis on the amino acids production by Bauman and Bisping (1995) [31] showed that it was strain dependent with the highest amino acids producer was *Rhizopus oligosporus,* followed by *R. oryzae* and *R. stolonifer*.

During mung bean fermentation, the concentration of EAAs was increased drastically from 3 to 27-fold with respect to non-fermented mung bean. The concentration of EAAs for non-fermented mung bean was 0.055 g/100 g DW and showed a significant increment at the beginning of 10 h to 28 h fermentation time with the value of 1.31 g/100 g DW, followed by slight increased to 1.51 g/100 g DW when reached 48 h fermentation time. The improvement of EAAs in fermented mung bean was in agreements with other fermented legumes [33-35] even though it was fermented with different the starter culture, temperature and incubation time.

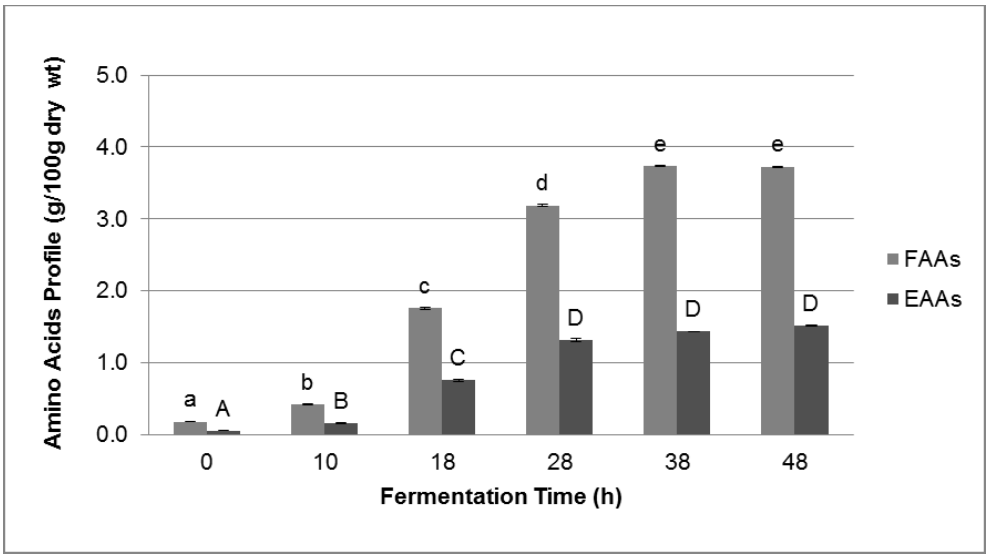

Figure 4: Changes of Free (FAAs) and Essential Amino Acid (EAAs) content in mung bean during solid state fermentation by *Rhizopus s*train 5351. Bars represent the standard error of the means (n=3), different letters are significantly difference at $P \leq 0.05$.

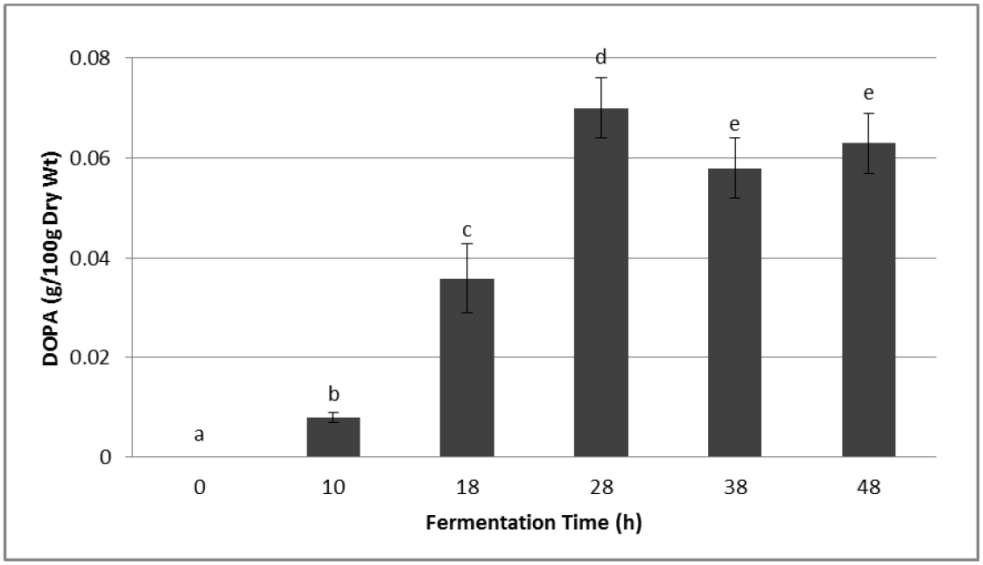

Figure 5: Changes of L-DOPA content in mung bean during solid state fermentation by *Rhizopus* strain 5351. Bars represent the standard error of the means (n=3), different letters are significantly difference at $P \leq 0.05$.

L-DOPA Concentration

It was reported that solid state fermentation could improve L-DOPA concentration in beans [25]. L-DOPA was not detected in non-fermented mung bean and the productions of L-DOPA was started after 10 h fermentation with the yield of 0.008 g/100 g DW and then reached its maximal production at 28 h fermentation time with the yield of 0.07 g/100 g DW before decreased to 0.063 g/100 g DW after 48 h (Figure **5**). The reduction of L-DOPA after 28 h fermentation time might be due to conversion of L-DOPA to other metabolites such as dopachrome [36] and quinone [14]. The highest yield of L-DOPA (0.07 g/100 g DW) at 28 h fermentation time was coincided with high protease activity presents in the fermented mung bean (Figure **3**). The high yield of L-DOPA could be related to the high protease activity that resulted more proteins being hydrolyzed to produce free amino acids (FAAs) such as tyrosine which is an important substrate for the production of L-DOPA by tyrosinase enzyme. Previous study had reported that the yield of L-DOPA was increased with an increment in the tyrosine content [37] and tyrosinase activity [13]. Furthermore, increment in the cell biomass or mycelium concentration also played an important role in the production of L-DOPA as it was related to tyrosinase activity [14]. In the present study, the rapid increased of glucosamine content in fermented mung bean was due to rapid growth of *Rhizopus* strain 5351 mycelium that might contribute to the higher production of tyrosinase enzyme, thus, indirectly increased the L-DOPA concentration.

GABA Concentration

GABA is synthesized from glutamate using L-glutamic acid decarboxylase and pyridoxal phosphate as a cofactor. Figure **6** shows the changes of GABA content in mung bean during fermentation. In the first 10 h fermentation, there was no change in the concentration of GABA (0.021 g/100 g DW). However, the GABA content was increased drastically to 0.132 g/100 g DW after 10 h incubation and achieved 0.198 g GABA/100 g DW when incubated for 38 h before decreased slightly after that. The reduction of GABA content after 38 h fermentation might be due to the conversion of GABA to succinic semialdehyde and then further hydrolyzed to succinic acid by GABA transaminase and succinic semialdehyde dehydrogenase, respectively [38]. The trend of GABA content in fermented mung bean during 48 h incubation period showed the similar phenomenon as observed in the protease activity (Figure **3**) and FAAs (Figure **4**) profile as discussed earlier. The hydrolysis of protein by protease had increased the FAAs content and produce high amount of glutamate. Furthermore, accumulation of glutamate decarboxylase enzyme during fungal growth might also contribute to the increment of GABA content in fermented mung bean. The development of GABA enriched food is of interest nowadays to prevent various health problems linked to oxidative stress. To date, several fermented foods with improved GABA content have been reported, including kidney bean [39], faba bean [33], black soybean [4], lentil [40], adzuki bean [11] and rice [41]. Several scientific studies have shown that fermented mung

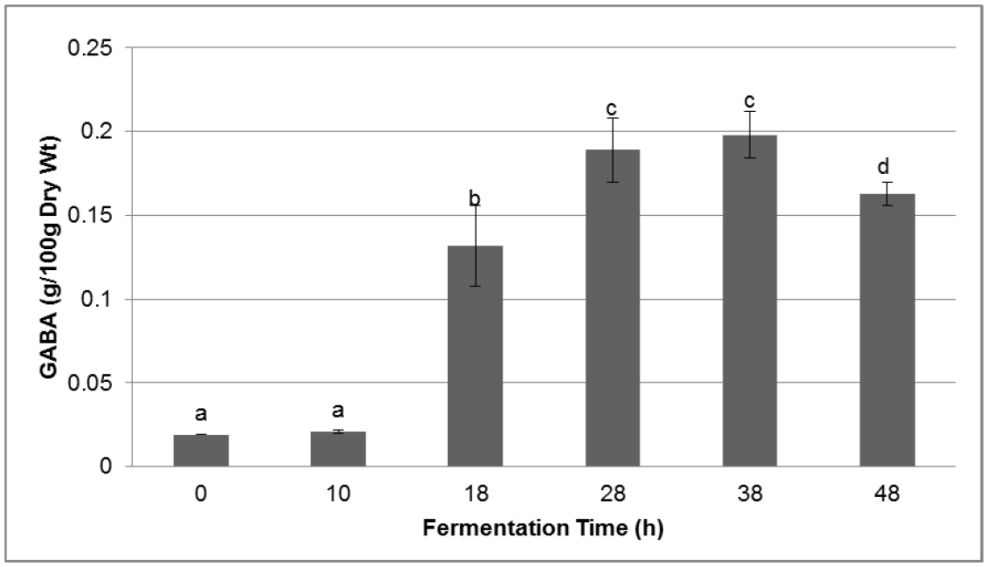

Figure 6: Changes of GABA content in mung bean during solid state fermentation by *Rhizopus* strain 5351. Bars represent the standard error of the means (n=3), different letters are significantly difference at $P \leq 0.05$.

bean possesses various health promoting properties such as hepatoprotective effect [42], chemopreventation effect [43], antihyperglycemic effect [22] and anti-stress treatment [44].

CONCLUSION

Overall, the concentration of L-DOPA, GABA, free and essential amino acids content of fermented mung bean may be improved drastically during solid state fermentation by *Rhizopus* strain 5351. This study showed the potential of solid state fermentation as a good strategy to enriched mung bean nutritive content with L-DOPA, GABA and other health beneficial compounds.

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