**Food Research 6 (Suppl. 4) : 109 - 116 (2023)**
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

# Effect of soaking conditions on gamma-aminobutyric acid (GABA) and amino acids content in mung bean

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***Article history:***
Received: 2 April 2022
Received in revised form: 13 May 2022
Accepted: 3 August 2022
Available Online: 14 April 2023

***Keywords:***
Amino acids,
GABA,
Mung bean,
Soaking conditions

***DOI:***
https://doi.org/10.26656/fr.2017.6(S4).007

## Abstract

Gamma-aminobutyric acid (GABA) offers numerous beneficial effects on human health, especially as blood pressure regulator and stress control. This functional compound is usually found in grains including mung bean. Mung bean (*Vigna radiate*) is one of the famous grains in Southeast Asia and is usually used in cooking as porridge or dessert by local people. It is highly nutritious with protein and antioxidants. In this study, the optimum soaking conditions were determined to enhance GABA content in mung beans. An RSM with a five-level, three-factorial, Central composite Rotatable Design (CCRD) was used to determine the soaking parameters to obtain maximum GABA content, essential amino acid (EAA) and total amino acids (TAA) in mung bean. The factors that were included in the RSM analysis were temperature, time and weight of beans to water ratio. GABA and amino acids were determined by using UPLC. The optimum condition for significant GABA production ($p$<0.05) was determined as 40°C, 4 hrs and 1:5 of temperature, time and soaking water level, respectively with the highest GABA content quantified as 130.12 mg/100 g dry weight (DW). Model optimization and validation were done according to the optimum conditions from the RSM analysis resulting in 124.17 mg/100 g DW of GABA, closer to the predicted value (130.07 mg/100 g DW). The optimum conditions determined by RSM could be beneficial to the industry, especially functional food production to cater for the demand for health-promoting products in the market.

## 1. Introduction

Legumes are widely known as cheap and highly nutritious food with a good source of valuable proteins, micro-nutrients and vitamins (Tiansawang *et al.*, 2016; Chaudhary *et al*., 2020). Mung beans (*Vigna radiata*), the small and green legumes contain 24% protein, 1% fat, 63% carbohydrate, and 16% dietary fibre (US Department of Agriculture, 2001). Mung beans are also rich in vitamins, and minerals and are usually considered an inexpensive source of protein, carbohydrates, fibre and phytochemical substances, including GABA (Saleh *et al.*, 2016). Mung bean is a good candidate for GABA production due to its high protein content. These characteristics of mung bean attract consumers of consuming mung bean in their daily diet with high nutritional values with health-promoting effects.

Gamma-aminobutyric acid (GABA) is a four-carbon free amino acid found in microorganisms, plants and vertebrates with metabolic function in the Krebs cycle of plants and it works as a powerful brain signal transmitter in vertebrates (Diana *et al.*, 2014). GABA is synthesized by glutamic acid decarboxylase (GAD) through the GABA shunt and polyamine degradation pathway in plants (Yang *et al.*, 2016). GABA provides many health-promoting effects such as anti-stress and anti-oxidant (Yeap *et al.*, 2014), antiageing (Leventhal *et al*., 2003), relieving anxiety, depression and decreasing high blood pressure (Chuang *et al*., 2015; Yamatsu *et al.*, 2015; Ma *et al*., 2015).

Consequently, the need of soaking seeds in water to rehydrate them before germination (Idowu *et al.*, 2020). Soaking activates the germination processes, including the glutamate decarboxylase enzyme, which converts glutamic acid to GABA (Figure 1) (Komatsuzaki *et al.*, 2007). Soaking conditions had an important role in accumulating GABA by enhancing the activity of

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eISSN: 2550-2166 
Published by Rynnye Lyan Resources

glutamic acid decarboxylase (Reggiani *et al.*, 1989) which is responsible for GABA production in mung bean seeds. Factors like seed weight and water level ratio, time and temperature are important considerations before soaking the seeds because excessive soaking can lead to microbial enrichment and undesirable fermentation (Ray *et al.*, 2016) while inadequate soaking does not promote the changes in phytochemical content (Chaiyasut *et al.*, 2017). Several factors of soaking that affect the GABA content in mung beans have been reported for instance soaking pH (Truong *et al.*, 2017), soaking time (Hoan *et al.*, 2020) and soaking solutions (Ji *et al.*, 2020). Truong *et al.* (2017) found that the combination of soaking at pH 6.3 and 12 hrs germination at 35°C was found to yield the highest GABA content (1519.07±19.58 ppm) with a value of 25.32 times higher GABA content in raw mung bean.

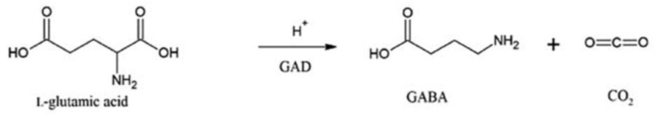


Figure 1. Decarboxylase of L-glutamic acid into gamma-aminobutyric acid (GABA) by glutamic acid decarboxylase (GAD) (adapted from Rojanarata *et al.*, 2017).

Response surface methodology (RSM) is a mathematical and statistical strategy for organising experiments, developing models, establishing the ideal circumstances for acceptable answers, and assessing the relative relevance of numerous independent variables. In this study, a five-level, three factorial Central Composite Rotatable Design (CCRD) was used to evaluate the interactive effects of the three soaking factors namely time, temperature and water level. Furthermore, the optimization verification model was used to determine the conditions for the maximum GABA production in mung beans. Therefore, this research aimed to study the optimised soaking condition of mung bean for the production of high GABA content by the response surface methodology (RSM) method.

## 2. Materials and methods

### *2.1 Preparation of germinated mung bean*

Mung beans (*Vigna radiata*) seeds were purchased from a local store in Serdang, Selangor (2.9848°N, 101.6702°E). A total of 100 g of mung beans was washed and rinsed thrice before soaking in distilled water at different ratios of bean's weight to water ratio, soaking temperature and time. The mung bean seeds were soaked in a black germination box, sealed and placed in an incubator at respective temperatures and times.

### *2.2 Experimental design and optimisation*

The germination parameters and the ranges used to maximise the GABA content were as follows: temperature of incubation (30-50°C); time (2-6 hrs) and mung bean to water ratio (1:3 to 1:7) as shown in Table 1. A five-level, three factorial CCRD was employed in this study. The fractional factorial design consisted of 8 factorial points, 6 axial points and 6 centre points, for a total of 20 experiments. The data obtained experimentally were analysed by using Stat-Ease Design Expert 13.0.1.0 version and were analysed by using three main steps as reported by Amin *et al.* (2018) which were analysis of variance (ANOVA) followed by regression analysis performance and response surface plotting with the level of significance for all tests was set at 95% confidence level.

Table 1. Ranges of the three independent variables used for the optimization of germination conditions on the accumulation of g-aminobutyric acid of germinated mung bean

| Ranges | Symbol | Code level | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| Temperature (°C) | A | 30 | 40 | 50 |
| Time (hr) | B | 2 | 4 | 6 |
| Weight of MB to water ratio | C | 3 | 5 | 7 |

### *2.3 Analysis of amino acid and GABA content*

#### *2.3.1 Preparation of mung bean extract*

The dried germinated mung bean powder was finely ground (1.0 mm) and extracted following the method by Ali (2013). Dried germinated mung bean powder (1 g) was mixed with 20 mL of hot deionized water. The mixture was vigorously shaken at 300 rpm for 30 mins and followed by centrifugation at 5000 rpm for 5 mins and the supernatant was filtered by using 0.2 µm and stored at -20°C prior to GABA analysis.

#### *2.3.2 Determination of GABA and amino acids content*

The filtered extract was derivatized with 70 µL of AccQ-Tag™ Ultra borate buffer followed by 20 µL of AccQ™ Flour reagent. All analyses were performed on Ultra Performance Liquid Chromatography, UPLC (Waters, USA) system that consists of a binary solvent manager a sample manager fitted with 2 µL sample loop and UV-PDA detector set at 260 nm. The analysis data were analyzed by using Waters Empower™ 2 Software. Amino acid and GABA were profiled separately using Acquity UPLC Taq Ultra Column (2.1 mm internal diameter × 100 mm × 1.7 mm particle size). The gradient elution comprised of AccQ Taq Ultra Eluent A and AccQ Tag Ultra Eluent B with a gradient condition of 0 - 0.54 min, 0 - 0.1% B; 0.54 - 5.74 min, 0.1 – 9.1% B;

https://doi.org/10.26656/fr.2017.6(S4).007 

5.74 – 7.74 min, 9.1 - 21.2% B; 7.74 – 8.8 min, 21.2 – 59.6% B; 8.8 – 11 min, 59.6 – 0.1% B; and 0.1% B isocraticly; 2.1 mins. The flow rate, injection volumes and temperature were set at 0.7 mL/min, 1.0 µL and 55° C accordingly. Quantification was made by using the calibration curves obtained by injecting amino acids standard and GABA as external standards with known retention times. The total essential amino acids were calculated based on the sum of phenylalanine, threonine, methionine, leucine, isoleusine, lysine, and valine (Mubarak, 2005).

*2.4 Statistical analysis*

Each experiment was done in triplicate (n = 3) and analysed statistically by the software Stat-Ease Design Expert 13.0.1.0 used for the optimization of GABA and amino acid content.

## 3. Results and discussion

In general, soaking time, temperature and water ratio at 1:5 was found to have an impact on GABA production. GABA content increased slightly when the soaking temperature was increased from 50 to 57°C and got the highest value of 170.8 mg/100 g DW at 4 hrs soaking. The GABA content decreased with an increase in soaking time. Similarly, the GABA content increased when germination time increased from 6 to 7.36 hrs and reached the highest value of 161.4 mg/100 g DW at 40°C incubation temperature.

The GABA content was generally increased after soaking at different conditions. The GABA content in mung bean was increased up to 170 mg/100 g DW from the initial content of 7.7 mg/100 g DW in raw mung bean. This might be due to the conversion of glutamic acid to GABA as the glutamate decarboxylase enzyme was activated in the process of decomposition of stored protein in grains and supplied to the growing part of the seedlings. Consequently, the GABA content was increased as the germination process took place. This result is in line with a report by Tiansawang *et al.* (2016) that germinated mung bean produced higher GABA content than non-germinated mung bean which increased from 13.25 mg/100 g dry matter to 80.68 mg/100 g dry matter after 6 hrs incubation.

*3.1 Effect of soaking conditions on GABA content*

The effect of soaking conditions (temperature, time and water ratio) on GABA content in germinated mung bean are shown in Table 2. The variables were fitted to the second model equation and examined for the goodness of fit. A test of lack of fit was used were in a low F-value indicating that the model equation is an adequate approximation for the data (Amin *et al*., 2018).

The results of ANOVA of the GABA content are shown in Table 3. To determine the significance of the polynomial model, an ANOVA was conducted. The coefficient of determination, $R^2$ explains the overall predictive capability of a model. According to Table 3, the $R^2$ value of the GABA content in germinated mung bean was 0.92. This value was close to 1.00 indicating the close performance of the model equation of the system and can be used for interpolation in the experimental domain. Whereas, the small value of *p*

Table 2. Experiment data for GABA content from germinated mung bean

| No. | Experimental design | A | B | C | GABA (mg/100 g DW) |
|---|---|---|---|---|---|
| 1 | Factorial | 30 | 2 | 3 | 54.2 |
| 2 | Factorial | 50 | 2 | 3 | 103.8 |
| 3 | Factorial | 30 | 6 | 3 | 90 |
| 4 | Factorial | 50 | 6 | 3 | 122.2 |
| 5 | Factorial | 30 | 2 | 7 | 53.6 |
| 6 | Factorial | 50 | 2 | 7 | 94.4 |
| 7 | Factorial | 30 | 6 | 7 | 129.2 |
| 8 | Factorial | 50 | 6 | 7 | 129.6 |
| 9 | Axial | 23.1821 | 4 | 5 | 73.6 |
| 10 | Axial | 56.8179 | 4 | 5 | 170.8 |
| 11 | Axial | 40 | 0.636414 | 5 | 63.8 |
| 12 | Axial | 40 | 7.36359 | 5 | 161.4 |
| 13 | Axial | 40 | 4 | 1.63641 | 79.4 |
| 14 | Axial | 40 | 4 | 8.36359 | 118.4 |
| 15 | Center | 40 | 4 | 5 | 135.8 |
| 16 | Center | 40 | 4 | 5 | 132.4 |
| 17 | Center | 40 | 4 | 5 | 124.8 |
| 18 | Center | 40 | 4 | 5 | 135.4 |
| 19 | Center | 40 | 4 | 5 | 132.8 |
| 20 | Center | 40 | 4 | 5 | 120.8 |

A: soaking temperature, B: soaking time, C: mung bean weight to water ratio

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($p$<0.05) obtained from the ANOVA analysis showed that the reduced cubic polynomial model was highly significant and can be used to represent the actual relationship between the response of GABA content and the significant variables.

According to the RSM results, response surface modelling was significant, as shown in Table 3 under polynomial regression function and predicting the response of GABA content in the samples was according to the following equation:

$$\text{GABA content} = -305.51709 + 9.298A + 35.66149B + 41.4315C - 0.36125AB - 0.25375AC + 1.76875BC - 0.056083A^2 - 2.25060B^2 - 3.46152C^2$$

The soaking temperature, time and water level were important factors that affected the GABA content in germinated mung beans. The three-dimensional (3D) surface plots were developed using a fitted reduced cubic model (modified model) by holding one independent variable at a constant and changing the levels of the other two variables to evaluate the interaction relationship between the variable factors. The surface plots showed the interaction of each-paired element and the optimization values for producing maximum GABA content (Figures 2, 3 and 4).

Figure 2 shows the response plots of the GABA content in germinated mung beans based on the effect of the temperature, time and their mutual effect with the water ratio set at 100 g MB: 500 ml water. It was observed that GABA production increased as the temperature increased from 30 to 50°C. The temperature and time of soaking exhibited an important inter-relationship in determining the GABA content. Higher GABA content was obtained as a result of soaking at a higher temperature and increased soaking time from 2 to 6 hrs.

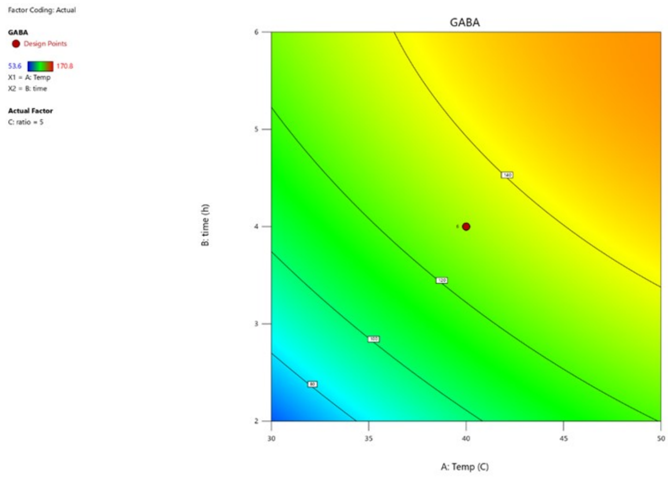


Figure 2. Response surface plots of the GABA content in germinated mung bean based on the effect of the temperature, time and their mutual effect with the water ratio set at 100 g MB: 500 mL water

Figure 3 shows the response surface plots of the GABA content in germinated mung beans based on the effect of the temperature, water ratio and their mutual effect, with time set at 4 hrs. A dome shape was observed from the plot. According to the response plot, a higher water ratio was more suitable to maximise the GABA content only when the temperature is increased from 45 to 50°C. In this case, lower production of GABA content was obtained at a lower water level, whereas higher GABA was produced at a higher water level when the time was set at 4 hrs and the soaking temperature was increased.

A prolonged duration of soaking was also required to enhance the GABA content production when germinating mung beans at higher water levels and the

Table 3. ANOVA analysis and equation formula for the response surface reduced cubic model of the GABA production in germinated mung bean

| Response: GABA content in germinated mung bean | | | | | | | |
|---|---|---|---|---|---|---|---|
| Source | | Sum of squares | DF | Mean Square | F value | Prob>F | |
| Model | | 19524.48 | 9 | 2169.39 | 12.11 | 0.0003 | Significant |
| | A | 6009.09 | 1 | 6009.09 | 33.54 | 0.0002 | |
| | B | 7932.65 | 1 | 7932.65 | 44.27 | < 0.0001 | |
| | C | 764.65 | 1 | 764.65 | 4.27 | 0.0657 | |
| | $A^2$ | 453.27 | 1 | 453.27 | 2.33 | 0.1578 | |
| | $B^2$ | 1167.93 | 1 | 1167.93 | 1.15 | 0.3087 | |
| | $C^2$ | 2762.84 | 1 | 2762.84 | 2.23 | 0.1658 | |
| | AB | 417.60 | 1 | 417.60 | 2.53 | 0.1428 | |
| | AC | 206.04 | 1 | 206.04 | 6.52 | 0.0287 | |
| | BC | 400.44 | 1 | 400.44 | 15.42 | 0.0028 | |
| Residual | | 1791.71 | 10 | 179.17 | | | |
| | Lack of Fit | 1604.30 | 5 | 320.86 | 8.56 | 0.0171 | Significant |
| | Pure Error | 187.41 | 5 | 37.48 | | | |
| Cor Total | | 21316.19 | 19 | | | | |

Significant at "Prob>F" less than 0.05, $R^2$ = 0.92
A: Temperature, B: Time, C: Water ratio

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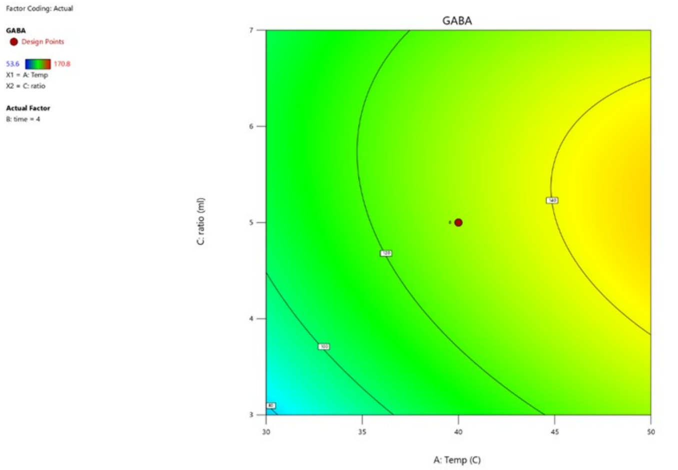


Figure 3. Response surface plots of the GABA content in germinated mung bean based on the effect of the temperature, water ratio and their mutual effect, with time set at 4 hrs

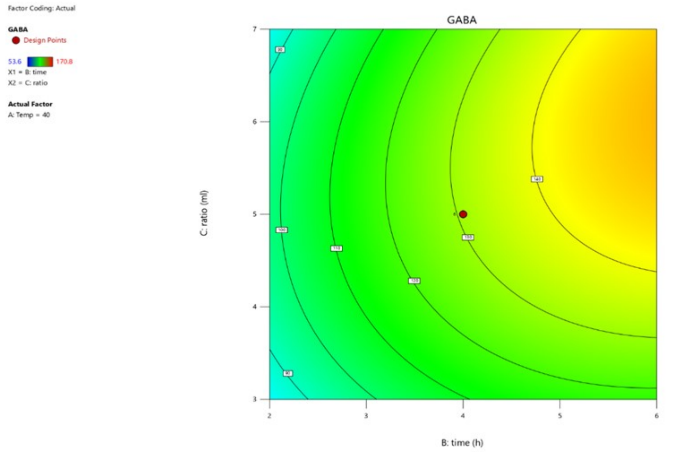


Figure 4. Response surface plots of the GABA content in germinated mung bean based on the effect of the time, water ratio and their mutual effect, with temperature set to 40°C.

temperature was set at 40°C as shown in Figure 4. At a lower water level, less than 400 mL for 100 g mung beans, the GABA production decreased when the time of soaking was increased to 3 hrs. This result is in line with that reported by Hoan *et al.* (2020) that the maximum increase in GABA content was found when the soaking time was increased up to 7.8667 hrs to 4.138 mg GABA/g dry weight. This might be due to the suitable conditions provided that enhanced the GAD enzyme activity and thus increase the GABA content. Similar findings were reported by Tiansawang *et al.* (2016) that is soaking was an efficient process that helped increase the content of GABA in legumes and sesame. The authors reported the increase of GABA content was found out higher in seeds that were soaked as compared to those that were not soaked. The rapid buildup of GABA during the soaking and germination stages of soybean and black bean has been postulated as a possible cause of water stress in young tissues (Matsuyama *et al.*, 2009). The restoration of the physiological functions and the dormancy of beans and seeds might be released as a result of immersion (Jiang *et al*., 2021). The increment of GABA content in soaked beans can be explained by the induction of metabolism as a result of water imbibition that led to the activation of endogenous enzymes and GAD in the reaction and hence produces secondary metabolites, GABA and other nutrients (Liao *et al.,* 2017; Luo *et al.,* 2017).

The optimal combination of experiments on selected parameters for maximum GABA production was investigated to evaluate the accuracy of the above model. The selected conditions are shown in Table 4. The experimental value of GABA in germinated mung bean under optimum conditions was 126.13±6.11 mg/100 g DW, which was almost similar to the predicted value of 130.751±2.19 mg/100 g DW in the model. This result verified the model of GABA production in germinated mung bean prediction.

### *3.2 Effect of soaking conditions on the amino acid content*

The soaking conditions (temperature, time and seed weight/water ratio) had significant effects on the content of essential amino acids and total amino acids. Table 5 shows the variation of essential amino acids (EAA) and total amino acids (TAA) for each soaking condition in the range of 103.7 to 197.2 mg/100 g DW and 303.4 to 677.5 mg/100 g DW, respectively. The varying degree of breakdown of seed components during soaking contributed to the variation of amino acid content in soaked mung beans at different conditions. Another soaking factor that contributed to the higher amount of TAA is soaking temperature. Higher temperatures resulted in higher TAA analysed from the soaked mung bean. According to Table 5, the soaking condition that resulted in the highest level of EAA and TAA was soaking at the highest temperature (57°C). Truong *et al.*

Table 4. Optimization and model verification of GABA produced in germinated mung bean

| Optimization model | GABA content (mg/100 g DW) | |
|---|---|---|
| | Experimental Value | Predicted Value |
| Temperature (°C): 40<br>Time (hrs): 4<br>Bean: water: 100 g MB: 500 mL water<br>GABA content: Maximum<br>Optimum solution:<br>Temperature (40°C), Time (4 hrs), Water ratio (1:5) | 126.13±6.11 | 130.751 |

https://doi.org/10.26656/fr.2017.6(S4).007 

Table 5. Essential amino acids and total amino acids for each soaking condition

| Temperature | Time | Weight/Water ratio | Essential Amino Acid (mg/100 g DW) | Total Amino Acids (mg/100 g DW) |
|---|---|---|---|---|
| 30 | 2 | 3 | 105.5 | 387.2 |
| 50 | 2 | 3 | 122.3 | 407.9 |
| 30 | 6 | 3 | 158.4 | 550 |
| 50 | 6 | 3 | 166.3 | 437 |
| 30 | 2 | 7 | 103.7 | 388.8 |
| 50 | 2 | 7 | 130.5 | 442.3 |
| 30 | 6 | 7 | 106.67 | 303.4 |
| 50 | 6 | 7 | 175.7 | 443.9 |
| 23.1821 | 4 | 5 | 106.4 | 401.4 |
| 56.8179 | 4 | 5 | 197.2 | 677.5 |
| 40 | 0.636414 | 5 | 119.2 | 409.3 |
| 40 | 7.36359 | 5 | 159.9 | 511.8 |
| 40 | 4 | 1.63641 | 158.4 | 506.5 |
| 40 | 4 | 8.36359 | 133.6 | 461.9 |
| 40 | 4 | 5 | 144.7 | 401.8 |
| 40 | 4 | 5 | 117.8 | 486.9 |
| 40 | 4 | 5 | 121.7 | 458.7 |
| 40 | 4 | 5 | 140.3 | 505.8 |
| 40 | 4 | 5 | 167.7 | 570 |
| 40 | 4 | 5 | 130.8 | 401.9 |

(2017) reported that free amino acid content was affected by incubation temperature as higher temperatures at 40°C produced 2.2 times more free amino acid than incubation at 35°C during mung bean germination.

Figure 5 shows the profile of amino acids from the soaking condition with the highest level of GABA production as compared to amino acids in raw mung bean. As shown in Figure 5, the amino acid profile exhibits the increment of essential amino acids in mung bean after soaking. All of the essential amino acids increased as a result of soaking with the highest increase in tryptophan content up to 4.14 times than the content in raw mung beans. This is in line with a report by Mubarak (2005) that the germination process enhanced the content of total EAA such as leucine, isoleucine, tryptophan and lysine.

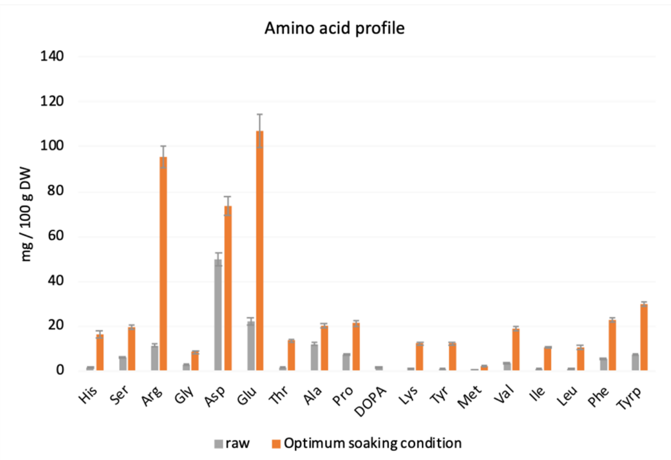


Figure 5. Amino acids profile for raw mung bean and mung bean soaked at optimum soaking conditions (Temperature: 40°C; time: 4 hrs; Ratio bean to water: 100 g MB: 500 mL water) His: histidine, Ser: serine, Arg: arginine, Gly: glycine, Asp: aspartic acid, Glu: glutamic acid, Thr: threonine, Ala: alanine, Pro: proline, DOPA: dopamine, Lys: lysine, Tyr: tyrosine, Met: methionine, Val: valine, Ile: isoleucine, Phe: phenylalanine, Tyrp: tryptophan

## 4. Conclusion

The study presented the optimization condition for producing the highest GABA content with soaking time (4 hrs), soaking temperature (40°) and bean-to-water ratio at 100 g MB to 500 ml water. The prediction (130.751±2.87 mg/100 g DW) and the actual GABA content (126.13±6.11 mg/100 g DW) were insignificantly different. The surface model was suitable and significant ($p$<0.05). The soaking factors namely temperature, time and water ratio strongly influenced the GABA production in germinated mung beans. These factors can be further applied to producing high GABA products that can be potentially used for functional food and health supplement products.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

The research work was funded by the Malaysian Agricultural Research and Development Institute (MARDI) (KMD-289) for financial support.

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