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Effects of Anaerobic Germination and Enzymatic Saccharification on Chemical Compositions of Functional Drink from Riceberry Rice

Pitchaya Pothinuch[1*], Kankanit Jongrattanavit[2], Kingkamol Leelajaruwan[1], and Nattapong Prichapan[1]

[1]Faculty of Food Technology, Rangsit University, Pathum Thani, 12000 Thailand
[2]School of Culinary Art, Suan Dusit University, Bangkok, 10300 Thailand

*Corresponding author: E-mail: pitchaya.p@rsu.ac.th

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Abstract

Anaerobic germination and enzymatic saccharification significantly affected the chemical compositions of riceberry rice-based functional drink. Anaerobic germination significantly affected the moisture and carbohydrate contents in germinated riceberry rice (GRR), whereas the contents of crude protein, crude fat, ash and crude fiber were not changed significantly. During anaerobic germination, production of ATP was limited; therefore, the enzyme activity in the seed could be delayed in order to conserve the nutrients. With increasing germination time, gamma-aminobutyric acid (GABA) increased, reaching a maximum percentage of 58.6% in 96-hour GRR but total phenolics and total anthocyanins significantly decreased with a loss percentage of 26.5% and 44.4%, respectively. Enzymatic saccharification using α-amylase and α-glucoamylase significantly increased sugar (total sugar, reducing sugar and glucose) contents in GRR extract, depending on incubation duration of both enzymes. The 0.5 hour-incubation with α-amylase in combination with the 12 hours-incubation with α-glucoamylase was the condition under which the extract contained the highest amount of reducing sugar (22.5 g/L), glucose (11.1 g/L) and total phenolics (22.5 g gallic acid equivalent/L). As the result, anaerobic germination in combination with enzymatic saccharification might be applied as a green process for the production of a functional drink from riceberry rice.

Keywords**:** *rice; anaerobic germination; biochemical processes; chemical compositions; green process; rice beverage.*

1. Introduction

Rice is an important agricultural product of Asian countries including Thailand since it acts as staple food and a main exported product. In Thailand, many varieties of rice have been developed and improved to make them resistant to climate changes, pest and diseases. In addition, an aim of developing rice varieties was to produce high nutritious rice. One of the popular nutritious Thai rice is riceberry rice, a pigmented variety of rice. Riceberry rice is produced by crossbreeding between fragrant black rice (Hom nil) and Jasmine rice (Hom mali rice 105). The characteristics of riceberry are non-glutinous and long-grain rice that possess dark purple to black. Riceberry rice has been cultivated in every planting area throughout the year. The yield per square meter of riceberry rice in Thailand is approximately 0.19-0.35 kg. The riceberry rice is a good source of energy, nutrients and antioxidants, especially phenolic compounds and is also recommended to be used as functional food ingredient. Generally, riceberry rice is popularly consumed as whole kernel. Currently, the global rice market is highly competitive. It is necessary to add value to Thai rice by creating higher-value rice products including food and non-food products. Thai rice is diverse in nutrition; therefore, it is possible to develop healthy rice food products such as cereal bars, bakery

products and athletic beverages using food technology and innovation.

In this study, riceberry rice was used as a main functional ingredient to produce natural functional drink. The rice-based functional drink is becoming popular as an alternative because of its hypoallergenic properties. To enhance nutritional quality and health benefits of rice-based drink, germination was applied in the production process. Rice is the only cereal that can germinate under both aerobic and anaerobic conditions, unlike other cereals such as wheat, barley and oat (Wunthunyarat et al., 2020). Rice seed has adaptive mechanisms in order to germinate anaerobically (Shen et al., 2015). An α-amylase in rice could hydrolyze endosperm starch into fermentable sugars to generate ATP in an anaerobic condition; however, the amount of ATP is much lower when compared to that in aerobic condition (Lasanthi-Kudahettige et al., 2007; Pucciariello, 2020). Metabolism of rice starch in anoxia is relate to the α-amylase gene RAMY3D (Lasanthi-Kudahettige et al., 2007). Anaerobic germination is a stress condition leading to bioactive compound accumulation. For example, Shen et al. (2015) found a significant increase in polyphenols and GABA. Maisont, & Nakrungsa (2010) reported that germination significantly enhanced GABA, dietary fiber, total phenolics and antioxidant capacity, while fat, starch, amylose contents slightly decreased. In addition, enzymatic saccharification has also been used in production of food and beverage in order to enhance the level of reducing sugars from starch hydrolysis (Guo et al., 2018). Wongkhalaung, & Boonyaratanakornkit (2000) developed a yogurt-type product from saccharified jasmine rice using 0.2% α-amylase and, after 20-21 hours, the maximum amount of 23.8% glucose was achieved. To retrieve reducing sugar efficiently, a two-step enzymatic hydrolysis was also applied in production of rice beverage. For example, Beaulieu et al. (2020) produced enzyme-treated brown rice beverage using both α-amylase (300 μg/100 g starch) and glucoamylase (300 μg/100 g starch), resulting in 15 °Brix after incubation of 24 hours. Banyavongsa et al. (2019) found that total sugar reached a peak of 13 °Brix with α-amylase at 0.15% and an incubation time of 55 min and the Brix value increased slowly with a glucoamylase concentration of 0.15% at 40 min. To our knowledge, there is limited information on the application of anaerobic germination in food processes. Therefore, to develop natural functional drink from rice, anaerobic germination was applied. A two-step enzymatic saccharification was also applied in the process in order to increase simple sugar in the rice-based drink without adding sugar.

2. Objectives

Many consumers are concerned about saturated fat, lactose and gluten tolerance in food and beverages as well as the environmental impact in food production. Therefore, the objectives of this study were to develop functional beverages from riceberry rice using green processes, namely anaerobic germination and enzymatic saccharification. The effect of duration of anaerobic germination on chemical compositions in GRR was investigated. In addition, the effect of incubation duration of α-amylase and α-glucoamylase on sugar contents was also determined. Finally, the product from this study would be naturally functional drinks with characteristics of lactose free, gluten-free, cholesterol-free and no sugar added which are suitable for health-conscious consumers.

3. Materials and methods

3.1 Materials

Riceberry rice was purchased from Farming Community Enterprise Nong Salai, Kanchanaburi, located in Kanchanaburi Province, in February 2020.

3.2 Chemicals

The enzymes, α-amylase (EC 3.2.1.1) obtained from *Bacillus licheniformis* and α-glucoamylase (EC 3.2.1.3) obtained from submerged fermentation of *Aspergillus niger*, were purchased from Reach Biotechnology Co., Ltd., Thailand. The 1.15 unit of α-amylase and 4.5 unit of α-glucoamylase were prepared for the experiment. One unit of α-amylase is the amount of enzyme need to hydrolyze 1 mg of starch in 1 min at pH 6 and 70 ˚C. One unit of α-glucoamylase is the amount of the enzyme which hydrolyze soluble starch to 1 mg glucose at 40 ˚C and pH 4.6 in one hour. Ethanol (AR grade), methanol (HPLC grade) and acetronitrile (HPLC grade) were purchased from RCI Labscan Ltd., Thailand. Petroleum ether, sodium hydroxide (NaOH), hydrochloric acid (HCl), copper sulfate (Cu_2SO_4), sodium acetate (CH_3COONa), potassium chloride (KCl), sodium carbonate (Na_2CO_3), sodium potassium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and sulfuric acid were purchased from Qrëc, New Zealand or Daejung, Korea or Merck, USA. Folin-Ciocalteu's reagent and phenol were purchased from Loba

Chemi, India. Boric acid and glucose were purchased from KEMAUS, Australia. Gallic acid was purchased from Acros organics, Belgium. The standards of glucose and maltose were purchased from Sigma-Aldrich, USA. The 3,5-dinitrosalicylic acid (DNS) was also purchased from Sigma-Aldrich, USA.

3.3 Determination of germination percentage

Whole hulled riceberry rice was cleaned with distilled water and the floated or defective grains were eliminated. The hulled rice was soaked in distilled water for 4 hours at ambient temperature. After that, the soaking water was drained from the hulled rice. A hundred grains of soaked hulled riceberry rice were put into 24x19x8.5 cm plastic box containing wet tissue paper without overlapping and then covered with a lid. After 3 days, the hulled riceberry rice with small root was counted to determine the germination percentage (as shown in equation 1). To indicate the integral grain, germination percentage should be higher than 80%. This process was done in triplication:

$$\text{Germination percentage (\%)} = \frac{N_{GRR}}{N_T} \text{ x } 100 \quad (1)$$

where N_{GRR} is the numbers of hulled riceberry rice with small roots and N_T is the total numbers of hulled riceberry rice.

3.4 Preparation of germinated riceberry rice

The anaerobic germination or water soaking method was used in this study. Hulled riceberry rice was soaked in distilled water for 4 hours at room temperature. The hulled rice was cleaned with distilled water before germination. The hulled riceberry rice was immersed in distilled water at a ratio of 1: 3 (hulled rice: distilled water) in a 24x19x8.5 cm plastic box for 48-96 hours at room temperature. The distilled water was changed at 8 hours intervals. After that, the hulled germinated riceberry rice (hulled GRR) was dried at 60 °C until the moisture content was lower than 14%. The dried and hulled GRR was dehulled in a rubber roll in dehusking machine for 2-3 times. Dehulled GRR was ground and sieved using stainless steel grinder and 80 mesh sieves. The powder of dehulled GRR was kept at -18 °C before extraction.

3.5 Effect of germination duration on the chemical composition

In this study, GRR was germinated by anaerobic germination (soaking in distilled water). The germination was carried out according to the method of Singh et. al. (2017) and Thitinunsomboon et.al. (2013) with some modifications. The effect of soaking time (48-96 hours) on proximate compositions (crude protein, crude fat, crude fiber, moisture, ash and carbohydrate), sugar contents (total sugars, reducing sugar, maltose and glucose), total phenolics, total anthocyanins and GABA was investigated.

Dehulled GRR was extracted using the method of Chow, & Landhäusser (2004) with some modification. Briefly, two grams of GRR powder was mixed with 13 mL of 80% ethanol and extracted using ultrasonic bath (4 °C) for 15 min. The extraction temperature was controlled at 80 °C. Then, the mixture was centrifuged at 5,000 rpm for 20 min. The supernatant was kept and the residue was re-extracted with 12 mL of 80% ethanol. After centrifugation, the second extract was mixed with the first extract and then filtrated using No.1 Whatman® filter paper. The filtrate was stored at -18 °C until analysis. The experiment was done in triplication.

3.6 Effect of enzyme incubation on chemical compositions

The enzymes, α-amylase and α-gluco-amylase were used in this study. The effect of incubation time of the two enzymes on sugar contents and total phenolics was investigated. The incubation time for α-amylase and α-glucoamylase were in the range of 0.5-2 hours and 12-48 hours, respectively. For the preparation of GRR-based drink, a hundred grams of GRR powder were added into 1,000 mL of distilled water with a pH of 5.5. The mixture was gelatinized by heating at 90 °C for 15 min. Then, 1.15 unit of α-amylase was mixed homogeneously and incubated at 90 °C for 0.5-2 hours. Consequently, the mixture was centrifuged at 7,000 rpm for 10 min at 25 °C. The supernatant was collected. After that, the 4.5 unit of α-glucoamylase was added into the supernatant and incubated at 60 °C for 12-48 hrs. The enzyme was inactivated by heating at 80 °C for 10 min and immediately cooling. The sample was centrifuged at 7,000 rpm for 10 min at 25 °C. The supernatant was collected and stored at -18 °C until analysis. The experiment was done in triplication.

3.7 Analysis of chemical compositions

3.7.1 Proximate analysis

Proximate compositions, including moisture, crude fat, crude protein, crude fiber, and ash contents, were determined according to the Association of Official Analytical Chemists methods (AOAC, 2000). Carbohydrates were calculated by difference as shown in equation 2. The analysis was done in triplication and the results were expressed as percentages (%).

$$\text{Carbohydrate (\%)} = 100 - (\text{\% moisture} + \text{\% crude fat} + \text{\% crude protein} + \text{\% ash}) \quad (2)$$

3.7.2 Determination of sugar

Total sugar was determined using a phenol-sulfuric acid assay which was modified from Chow, & Landhäusser (2004). The extract 2 mL was mixed with 2 mL of 5% phenol solution, followed by 5 mL of concentrated H_2SO_4, on a Vortex test tube mixer. The mixture was held in the fume hood for 10 min. The absorbance was measured at wavelength of 490 nm. The concentration of total sugar was calculated from standard curve of glucose solutions (0-60 mg/L). The analysis was done in triplication.

Reducing sugar was determined using a DNS assay according to the method of Garriga et al. (2017). The extract 1 mL was mixed with 3 mL DNS reagent. The preparation of DNS reagent was done following: 1 g DNS was added in 20 mL of 2 M NaOH and mixed well (solution A). Then, 30 g sodium potassium tartrate was dissolved in distilled water (solution B). Solution A and B were mixed, heated and then the volume was adjusted to 100 mL by adding distilled water. The mixture was boiled at 90 °C for 10 min and, then cooled down to ambient temperature. The absorbance was measured at wavelength of 540 nm. The concentration of reducing sugar was calculated by using standard curve of glucose (0-300 mg/L). The analysis was done in triplication.

Determination of glucose and maltose was executed using Agilent Technologies 1260 Infinity Serie HPLC system consisting of a gradient pump, a degassing system, an autosampler, a thermostat column and equipped with the Agilent 1260 Infinity evaporative light scattering detector (Agilent Technologies, Palo Alto, CA, USA). The column used was a 5 mm ZORBAX carbohydrate column (4.6x150 mm) (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was isocratic of water (solvent A: 10%) and acetonitrile (solvent B: 90%) with 1 mL/min at 30 °C. The injection volume was 20 µL. For ELSD parameters: nebulizer temperature, evaporation temperatures, ELSD gain and ELSD filter were set at 45 °C, 80 °C, 5 s, and 10 s, respectively. The glucose and maltose content in the extract was calculated by comparing the sample peak area with the standard curve. The glucose and maltose contents of each sample was determined in duplication. The analysis was done in replication.

3.7.3 Determination of total phenolics

Total phenolic content was determine using Folin-Ciocalteu's reagent as described in Kim et al. (2003) with some modification. Briefly, 0.1 mL of extract was mixed with 0.9 mL of water and 0.1 mL of Folin-Ciocalteu's reagent. After 6 min, 1 mL of 7% Na_2CO_3 was added and then incubated for 90 min at room temperature. The absorbance was measured at wavelength of 760 nm. TPC was expressed as mg gallic acid equivalent/g dry weight (mg GAE/g DW) and determined in triplicate.

3.7.4 Determination of total anthocyanins

Total anthocyanin content was determined using a pH differential assay according to Giusti, & Wrolstad (2005) with some modifications. Each extract of 0.5 mL was separately mixed with 4.5 mL of two buffer solutions which were 0.4 M hydrochloric acid–sodium acetate buffer (pH = 4.5) and 0.025 M hydrochloric acid–potassium chloride buffer (pH = 1). After 15 min, the absorbance at wavelength of 510 and 700 nm was measured. Total anthocyanins were calculated using equation 3:

$$\text{Total anthocyanin (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1) \quad (3)$$

where A is $(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$;

MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol); DF is the dilution factor; l is the cell length (1 cm.); ε is the molar extinction coefficient (26,900 $L \cdot cm^{-1} \cdot mol^{-1}$). The total anthocyanin contents were expressed as cyanidin-3-glucoside equivalents. The analysis was done in triplicate.

3.7.5 Determination of GABA

Determination of GABA using HPLC technique was performed. The HPLC system consisting of a reverse phase 5 mm ZORBAX Eclipse AAA (4.6x150 mm) column (Agilent Technologies, Palo Alto, CA, USA) connected with the Shimadzu LC-10 and equipped with a RF10Axl fluorescence detector was applied.

The mobile phase used was 40 mM NaH_2PO_4 pH 7.8 (solvent A) and the mixture of acetonitrile (45%): methanol (45%): water (10%) (solvent B). The solvent gradient was performed as followed: a linear step from 0% to 57% of solvent B from 1.9 to 21.1 min; 57% to 100% of solvent B from 21.1 to 21.6 min; isocratic conditions with 100% solvent B from 21.6 to 25.0 min; followed by a rapid to 0% solvent B at 25.1 min; and then isocratic conditions with 0% solvent B until complete (total 30 min). The mobile phase was delivered at flow rate of 1.5 mL/min. The derivatization of GABA in the extract/standard was done by mixing derivative reagent (10 mL methanolic OPA, 10 mL 4 N borate buffer pH 10.2 and 640 mL of solvent A) with 20 mL of extract/standard. The derivatization reaction was carried out for 10 min at 40 °C. The injection volume of the derivatized extract was 20 μL. The derivatized extract was detected using excitation and emission wavelength at 220 and 385 nm. The GABA content in the extract was calculated by comparing the sample peak area (% fluorescence) with the standard curve of derivatized GABA. The GABA content of each sample was determined in duplication.

3.8 Statistic analysis

The effect of germination time on chemical characteristics of GRR was studied using Completely Random Design (CRD) as experimental design. A factorial in CRD was applied to determine the effect of enzyme incubation on the chemical compositions of the GRR extract. Data were expressed as mean ± S.D. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the least significant different (LSD) test or independent t-test. Significant differences were statistically considered at $p<0.05$.

4. Results and Discussion

4.1 Effect of germination time on chemical compositions

In this study, hulled riceberry rice was germinated by anaerobic germination (soaking in distilled water). The effect of germination time (48-96 hours) on proximate compositions (crude protein, crude fat, crude fiber, moisture, ash and carbohydrate), sugars contents (total sugars, reducing sugar, maltose and glucose), total phenolics, total anthocyanins and GABA was investigated in GRR (Table 1 and 2). The quality of rice seed was determined by using germination percentage. In this study, riceberry rice had 82.33% of germination percentage. High quality seeds possess a viability of at least 80% (Feng et al., 2019). The characteristics of GRR during germination (48-96 hours) are shown in Figure 1.

4.1.1 Proximate compositions

Table 1 showed that germination time significantly affected moisture and carbohydrate contents ($p<0.05$), whereas the contents of crude protein, crude fat, ash and crude fiber were not significantly different among germination durations ($p\geq0.05$). An average content of moisture of GRR (5.9%) was approximately two times lower than that in ungerminated riceberry rice (11.2%) since the GRR was dried after germination (4 hours). The carbohydrate content was determined as the difference between 100 and the sum of moisture, crude protein, crude lipid and ash contents. Therefore, their moisture contents affected the carbohydrate content in the ungerminated riceberry rice GRR. Changes of crude protein, crude fat, carbohydrate, ash and crude fiber were not significantly different in riceberry rice during anaerobic germination. Average amounts of crude protein, crude fat, ash and crude fiber of GRR were 7.0, 5.2, 1.3 and 0.1%, respectively. Under anaerobic germination, only 2 ATP/mol of glucose is produced via fermentation, whereas aerobic germination produces 36 ATP/mol glucose of glucose (Ma et. al., 2020). Insufficient ATP could delay synthesis of hydrolase especially α-amylase in rice (Lee et al., 2014). As the results, anaerobic germination could conserve nutrients in GRR. Singh et al. (2017) reported that moisture and crude protein contents in germinated brown rice were insignificantly influenced by soaking time and temperature. In contrast, Wunthunyarat et al. (2020) found that protein content in germinated brown rice increased with increasing germination duration (48-96 hours) but lipid and ash decreased in both aerobic and anaerobic conditions. Biochemical and chemical changes during germination might depend on rice cultivar, rice type and germination conditions.

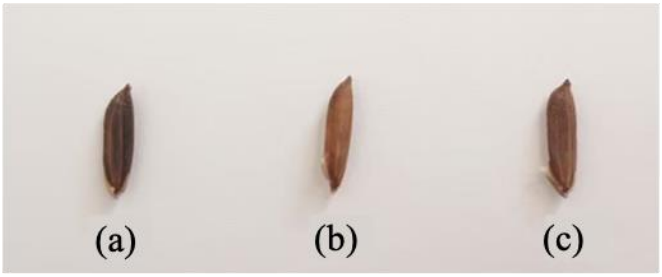

Figure 1 Appearance of germinated riceberry rice with germination time of 48 hours (a), 72 hours (b) and 96 hours (c).

Table 1 Nutritional compositions of germinated riceberry rice at different germination durations

Compositions	Germination duration (hour)			
	0	**48**	**72**	**96**
Moisture (% by weight)	11.2±0.3[a]	5.9±0.0[b]	5.4±0.1[b]	6.4±0.1[b]
Crude protein (% by weight)	7.0±0.0[a]	7.0±0.3[a]	7.0±0.5[a]	7.1±0.4[a]
Crude fat (% by weight)	5.3±0.8[a]	5.8±0.3[a]	5.1±1.8[a]	4.8±1.0[a]
Ash (% by weight)	1.4±0.0[a]	1.3±0.0[a]	1.2±0.1[a]	1.3±0.0[a]
Carbohydrate (% by weight)	75.1±1.1[b]	80.0±0.0[a]	81.2±1.3[a]	80.5±1.5[a]
Crude fiber (% by weight)	0.4±0.2[a]	0.2±0.0[a]	0.1±0.0[a]	0.1±0.0[a]
Total sugar (mg/100 g)	936.3±28.1[a]	690.8±9.4[b]	776.0±48.5[ab]	898.7±74.2[a]
Reducing sugar (mg/100 g)	462.8±36.5[c]	449.2±52.3[c]	555.3±24.9[b]	636.8±26.2[a]
Glucose (mg/100 g)	383.7±5.0[c]	409.8±4.7[b]	422.3±0.3[b]	444.6±10.7[a]
Maltose (mg/100 g)	468.9±1.4[a]	nd	nd	nd

[a-c] Different small letter indicates significant difference among germination durations at p£0.05. nd: not detectable.

4.1.2 Sugar contents

Changes in sugar in riceberry rice under anaerobic germination were observed. Total sugar, reducing sugar, glucose and maltose contents in GRR were shown in Table 1. The duration of anaerobic germination significantly affected total sugar, reducing sugar, glucose and maltose contents. Total sugar contents increased as germination processed. The higher total sugar contents were found in 72-hour and 96-hour GRR. Total sugar in ungerminated riceberry rice was insignificantly different from 72- and 96-hour GRR. It might be due to the effect of other components such as phenolic compounds, which could interfere in phenol-sulfuric acid analysis for total sugar determination. Regarding reducing sugar and glucose contents, the 96-hour GRR contained the highest contents, followed by the 72-hour GRR. The ungerminated riceberry rice had the lowest amount of glucose. Maltose was not observed in GRR but found only in the ungerminated riceberry rice. It is possible that maltose of GRR was degraded to glucose. An increase in glucose with increasing germination duration occurred when amylose was degraded to provide energy for biochemical and physiochemical changes during germination (Chinma et al., 2015).

4.1.3 Total phenolics, total anthocyanins and GABA

Total phenolics and total anthocyanins in GRR were revealed in Table 2. The results showed that both contents were higher in ungerminated riceberry rice than GRR. The reduction of total phenolics and total anthocyanins might be due to their water-soluble property. During anaerobic germination, anthocyanins, a water-soluble phenolic pigment mainly present in riceberry rice, could dissolve in the soaking water. At 96 hour-germination, the loss percentages of total phenolics and total anthocyanins in GRR were 26.5% and 44.4%, respectively. GABA was also determined in GRR. GABA is a naturally bioactive compound that had several health benefits associated with lowering blood pressure, positive mood, less anxiety, depression and stress (Varanyanond et al., 2005; Watanabe et al., 2016). The result is shown in Table 2 and GABA chromatograms of GRR are illustrated in Figure 2. The content of GABA escalated with the increase in germination time. The 96-hour GRR had the highest GABA contents that was 58.6% higher

than that of the ungerminated riceberry rice. The results were similar to Singh et. al. (2017) who studied the effects of soaking temperature and time on GABA content of brown rice (Phitsanulok 2 rice) and they found that an increase of soaking time (5 hours) at 33ºC provided the highest GABA content (18.7 mg/100 g). Thitinunsomboon et al. (2013) and Trung et al. (2017) reported that hypoxia-anaerobic germination could boost GABA accumulation in germinated brown rice and legume seeds, respectively. The level of GABA rapidly increases in plant response to various forms of stress, including anaerobic conditions, that accelerates the accumulation ability of plants.

Table 2 Bioactive compounds of germinated riceberry rice at different germination durations

Compositions	Germination duration (hour)			
	0	**48**	**72**	**96**
Total phenolic content (mg gallic acid equivalent/100 g)	192.9±19.4^{a}	174.1±14.5^{ab}	155.8±18.3^{bc}	141.8±5.8^{c}
Total anthocyanin (mg cyanidin-3-glucoside equivalent/100 g)	126.1±11.1^{a}	53.2±14.3^{c}	87.1±3.4^{b}	70.1±7.3^{bc}
GABA (mg/100 g)	8.6±0.1^{d}	18.8±0.2^{b}	14.0±0.0^{c}	20.8±0.3^{a}

$^{a-d}$ *Different small letters indicate significant difference among germination durations at p<0.05.*

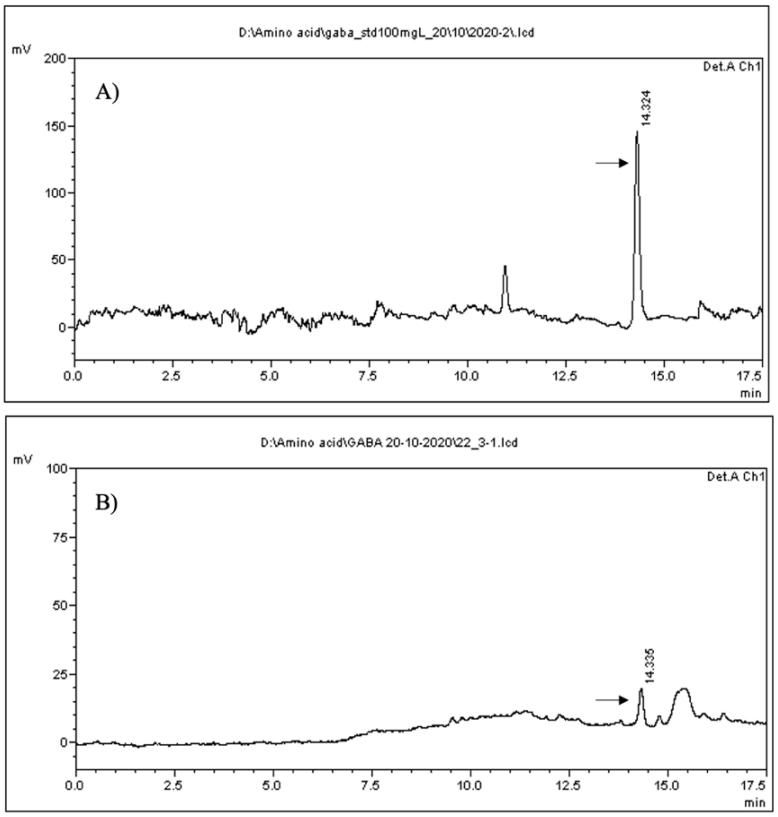

Figure 2 Representative chromatogram of GABA standard (100 mg/L) (a) and GRR (b). The GABA peak is marked with an arrow at a retention time of 14.3 min.

Figure 3 Characteristics of germinated riceberry rice extract incubated with a-amylase and a-glucoamylase

4.2 Effect of enzyme incubation on chemical compositions

The 96-hour GRR was selected to produce GRR extract incorporated with utilization of a-amylase and a-glucoamylase since the sample contained higher contents of GABA and glucose. The appearance of the obtained extract was an orange-pink color (Figure 3).

4.2.1 Sugar content

Adding a-amylase and a-glucoamylase during production of GRR extract helps to degrade rice starch into small molecules of sugar such as glucose, a monosaccharide that can be quickly absorbed into body and easily metabolized as energy. The a-amylase randomly breaks down glycosidic bonds within polymer chains of starch molecules at a-1,4-glycosidic position, resulting in smaller molecules of oligosaccharides and disaccharides (Steup, 1990). The enzyme a-glucoamylase continually digest to achieve only monosaccharides including glucose (Steup, 1990). The sweetness level of glucose is less than sucrose, a sugar commonly used in food processing. The relative sweetness of glucose and sucrose are 0.59 and 1.0, respectively. Thus, the riceberry rice-based functional drink in this study is naturally sweet which is less sweet than general drinks on the market that usually contain sucrose.

The samples' total sugar and reducing sugar contents were analyzed by phenol-sulfuric acid and DNS methods, respectively. Table 4 shows total sugar and reducing sugar contents in GRR extracts. The total sugar in GRR extract was in range of 32.4-66.1 g/L. The contents of sugars in samples were significantly affected by incubation time with both a-amylase and a-glucoamylase. The results showed that high total sugar content significantly increased when the incubation duration with a-amylase extended up to 1.5 hours and then the contents decreased after 2 hours of incubation. The sample incubated with a-amylase for 1.5 hours contained higher contents of total sugar (> 51 g/L) than the others (0.5, 1 and 2 hours) at all incubation times with a-glucoamylase (12-48 hours). The maximum total sugar content (66.1 g/L) was detected in the sample with incubation of a-amylase and a-glucoamylase for 1.5 and 12 hours, respectively. Total sugar levels of sample incubated with a-amylase for 2 hour were lower than 36.4 g/L. GRR extract had the content of 19.0-22.5 g/L for reducing sugar. Incubation with a-amylase and a-glucoamylase significantly modify reducing sugar content. For all durations of a-glucoamylase incubation, the GRR extract incubated with a-amylase for 0.5 hours was higher, except 48 hour-incubation with glucoamylase. In addition, the GRR extract with 12-hour incubation with a-glucoamylase was higher in all duration of a-amylase incubation except 2.0-hour incubation. Enzymes including a-amylases and a-glucoamylase catalyzed reaction undergo completion in an optimal time. When an enzyme is incubated with its substrates for a long time, a greater amount of product will be formed. The accumulation of reaction products generally decreases the enzyme velocity. The product might bind to the active site of the enzyme. Furthermore, enzyme might suffer denaturation and loss of catalytic activity with time. Therefore, GRR extracts incubated with a a-amylase for 0.5-1.5 hours in combination with 12 and 24 hour of incubation with a-glucoamylase were selected to determine glucose and maltose contents.

Table 4 Total sugar and reducing sugar contents (g/L) of germinated riceberry extracts at different incubation time with a-amylase and glucoamylase.

Incubation time with α-amylase (hour)	Incubation time with α-glucoamylase (hour)			
	12	**24**	**36**	**48**
Total sugar (g/L)				
0.5	$41.2\pm8.1^{B,a}$	$43.9\pm6.7^{B,a}$	$37.4\pm7.8^{B,a}$	$48.7\pm8.1^{B,a}$
1.0	$47.3\pm10.1^{B,a}$	$37.2\pm10.1^{B,ab}$	$34.1\pm7.1^{B,b}$	$47.0\pm7.1^{B,a}$
1.5	$66.1\pm4.7^{A,a}$	$55.2\pm1.5^{A,b}$	$51.5\pm2.4^{A,c}$	$58.6\pm1.9^{A,b}$
2.0	$35.9\pm3.1^{C,a}$	$32.4\pm3.8^{BC,a}$	$36.4\pm3.8^{B,a}$	$35.5\pm3.2^{C,a}$
Reducing sugar (g/L)				
0.5	$22.5\pm0.2^{A,a}$	$20.3\pm0.3^{A,b}$	$19.7\pm0.2^{A,c}$	$19.0\pm0.2^{B,c}$
1.0	$20.9\pm0.3^{B,a}$	$19.6\pm0.3^{B,b}$	$19.4\pm0.2^{B,b}$	$19.2\pm0.3^{B,c}$
1.5	$20.7\pm0.2^{B,a}$	$20.0\pm0.2^{A,b}$	$19.6\pm0.2^{A,c}$	$19.7\pm0.4^{A,bc}$
2.0	$19.6\pm0.2^{C,b}$	$20.1\pm0.3^{A,a}$	$19.8\pm0.2^{A,b}$	$19.7\pm0.2^{A,b}$

$^{A-C}$Different capital letters indicate significant difference among incubation time with a-amylase at $p<0.05$.

$^{a-c}$Different small letters indicate significant difference among incubation time with a-glucoamylase at $p<0.05$.

Table 5 Glucose and maltose contents (g/L) of germinated riceberry extracts at different incubation time with α-amylase and α-glucoamylase

Incubation time with α-amylase (hour)	Incubation time with α-glucoamylase (hour)	
	12	**24**
Glucose (g/L)		
0.5	$11.1\pm0.0^{A,a}$	$7.5\pm0.2^{B,b}$
1.0	$10.6\pm0.1^{AB,a}$	$8.0\pm0.1^{A,b}$
1.5	$9.6\pm0.3^{B,a}$	$7.7\pm0.0^{AB,b}$
Maltose (g/L)		
0.5	$3.6\pm0.1^{B,a}$	$3.8\pm0.2^{A,a}$
1.0	$4.3\pm0.1^{A,a}$	$3.8\pm0.1^{A,b}$
1.5	nd	nd

$^{a-b}$Different small letters indicate significant difference among incubation time with a-glucoamylase at $p<0.05$.

$^{A-B}$Different capital letters indicate significant difference among incubation time with a-amylase at $p<0.05$.

nd: not detectable

The contents of glucose and maltose in the GRR extract with incubation after α-amylase and a-glucoamylase are shown in Table 5. Glucose and maltose in the GRR extract were in range of 7.5-11.1 g/L and 3.6-4.3 g/L, respectively. The results showed that incubation time with α- amylase and a-glucoamylase significantly affected the content of glucose and maltose. The samples incubated with α-amylase for 0.5 and 1.0 hour, in combination with a 12 hour incubation with a-glucoamylase, were not significantly different and contained the maximum glucose content. Maltose was found in low amount in the GRR extract and was not found in longer duration of incubation of the enzymes. Therefore, the incubation conditions of 0.5 hour with α-amylase and 12 hours with a-glucoamylase were selected for rice-base functional drink.

4.2.2 Total phenolic content

Total phenolic content of the GRR extract after incubation with α-amylase and a-glucoamylase is shown in Table 6. The results showed that total phenolics depended on incubation time of both enzymes. The maximum amount of total phenolics was found in the sample that incubated with α-amylase and a-glucoamylase for 0.5 and 12 hours, respectively. According to the chemical compositions in GRR extract, the incubation condition of α-amylase and a-glucoamylase was selected at 0.5 and 12 hours, respectively for production of rice-based functional drink because this condition provides the highest total phenolics and the shortest production time.

Table 6 Total phenolic content (g gallic acid equivalent/L) of germinated riceberry extracts at different incubation time with α-amylase and α-glucoamylase

Incubation time with α-amylase (min)	Incubation time with α-glucoamylase (hour)			
	12	24	36	48
0.5	$22.5\pm0.2^{A,a}$	$20.3\pm0.3^{A,b}$	$19.7\pm0.2^{A,c}$	$19.0\pm0.2^{B,c}$
1.0	$20.9\pm0.3^{B,a}$	$19.6\pm0.3^{B,b}$	$19.4\pm0.2^{B,b}$	$19.2\pm0.2^{B,c}$
1.5	$20.7\pm0.2^{B,a}$	$20.0\pm0.2^{A,b}$	$19.6\pm0.2^{A,c}$	$19.7\pm0.4^{A,bc}$
2.0	$19.6\pm0.2^{C,b}$	$20.1\pm0.3^{A,a}$	$19.8\pm0.2^{A,b}$	$19.7\pm0.2^{A,b}$

$^{A-C}$Different small letters indicate significant difference among incubation time with a-amylase at $p<0.05$.

$^{a-c}$Different capital letters indicate significant difference among incubation time with a-glucoamylase at $p<0.05$.

5. Conclusions

This research introduced green process for production of riceberry rice-based functional drink using anaerobic germination and enzymatic saccharification. However, optimal duration of anaerobic germination and enzymatic hydrolysis should be critically considered in order to obtain higher health-related components. Using anaerobic germination could conserve nutrients including crude proteins, crude fats, ash and crude fibers in GRR. As germination time increased, GABA enhanced to a maximum percentage of 58.6% in 96-hour GRR, but total phenolics and total anthocyanins significantly decreased with loss percentage of 26.5% and 44.4%, respectively. Therefore, in this study, 96 hours of anaerobic germination should be recommended. For enzymatic saccharification, incubation time significantly affected sugar contents, including total sugar, reducing sugar, maltose and glucose. The optimal incubation with α-amylase and α-glucoamylase should be at 0.5 and 12 hours, respectively. The extract from the 0.5 hour-incubation with α-amylase combined with the 12-hour incubation with α-glucoamylase contained the highest amounts of reducing sugar (22.5 g/L), glucose (11.1 g/L) and total phenolics (22.5 g gallic acid equivalent/L). Finally, the product from this study would be naturally sweetened functional drink with high-health components. The information could be an alternative process to add-value to Thai rice in order to compete in global rice market.

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