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SCREENING OF BROWN RICE VARIETIES AND OPTIMIZATION OF GERMINATION CONDITIONS FOR HIGH γ-AMINOBUTYRIC ACID YIELD

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Abstract

One of the important active components in germinated brown rice (GBR) is γ-amino-butyric acid (GABA), which may lower blood pressure and has anti-depression effects. This study aimed to improve the GABA yield of GBR, and to investigate GABA-rich germinated brown rice in terms of rice variety screening and optimization of germination conditions. After the screening process, the six varieties with the highest GABA yields in GBR were identified as 1D67, 1D117, 1D31, N84, 1D71 and 1D70 with GABA contents (mg/ml) of 29.12, 26.49, 26.09, 24.82, 23.80, and 22.84, respectively. The GABA yield of GBR under optimal germination conditions determined by both a single factor optimization and an orthogonal test design was 28.94 mg/100 g, 16.6% higher than before germination condition optimization. Results of a range analysis showed that the order of significance for five factors affecting GABA yield of N84 germinated brown rice were as follows: germination temperature > soaking liquid pH > soaking time > soaking temperature > germination time. The optimal germination conditions for producing GABA-rich germinated brown rice were soaking solution pH 4.7, soaking 9 hrs at 20°C, then germination 34 hrs at 39°C.

Introduction

Germinated brown rice (GBR) is a brown rice product obtained by germinating brown rice under a specific temperature and humidity. During the germination process, the chemical components of the brown rice changes significantly, where various hydrolases are activated, and macromolecules, such as starch, non-starch polysaccharides, and proteins are decomposed into smaller molecular compounds to provide nutrients for seedling growth and development. These changes lead to the production of more monosaccharides, peptides, amino acids, such as γ-aminobutyric acid (GABA) and essential amino acids, vitamins, and dietary fiber in brown rice seedlings after germination (Moongngarm and Saetung 2010, Patil and Khan 2011).

GABA is a non-protein amino acid and is known as a major inhibitory neurotransmitter of the mammalian cortex (Cho and Lim 2016). GABA plays a physiological role *in vivo* mainly through a receptor site on the postsynaptic membrane, which specifically recognizes and binds GABA. When GABA is bound, the ion permeability of the cell membrane is altered (Shelp *et al*. 1999). GABA receptors can be categorized into three subtypes: $GABA_A$ (Shelp *et al*. 1999), $GABA_B$ (Kaupmann *et al*. 1998) and $GABA_C$ (Prada *et al*. 2005). The physiological functions of GABA include lowering blood pressure (Kawakami *et al*. 2018), anti-depression activity (Jacobson *et al*. 2018), delaying of nerve cell aging (Leventhal *et al*. 2003), and improvement of liver and kidney functions (Hata *et al*. 2019). Atack *et al*. (2006) established that GABA is able to bind and activate an anxiolytic brain receptor, and then work synergistically with other biomolecules to prevent anxiety-related information from reaching the information processing centers in the prefrontal cortex. Therefore, GABA may have a calming effect, which leads to reduced anxiety.

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In plants, GABA is produced by the catalyzation of decarboxylation of L-glutamic acid by glutamate decarboxylase, which impacts plant seed vigor, cell development, and stress metabolism (Narayan and Nair 1990). The GABA content of GBR is related to the germination conditions of the brown rice (Patil and Khan 2011, Cho and Lim 2016). For example, even simple water soaking before germination was capable of increasing GABA yield in seedlings (Saikusa *et al*. 1994a). Saikusa *et al.* (1994a, 1994b) found that the content of free GABA in brown rice was significantly increased after water immersion. During immersion, the temperature and pH of the water are also significant factors that affect GABA accumulation in the rice germ. As previously mentioned, glutamate decarboxylase (GAD) is a key enzyme in the GABA synthesis pathway. When temperatures are below 30°C, GAD cannot be fully activated; at exceedingly high temperatures (≥ 60°C) GAD is inactivated. When the pH exceeds 6.5, GAD activity is also inhibited, however, the activity of the GABA lytic enzyme is increased, which results in a decrease in GABA content. Previous studies have shown that when brown rice is immersed for 4 hrs at a pH of 5.0 - 6.0 at 40°C, the maximum GABA yield is obtained. Additionally, some abiotic stressors, such as mechanical stimulation, mechanical damage, barometric pressure treatment, cold shock, heat shock, and hypoxia, during germination can also significantly increase the content of GABA in brown rice (Shelp *et al*. 1999, Komatsuzaki *et al*. 2007, Thuwapanichayanan *et al*. 2015).

Although there have been many previous reports on improving the GABA content of germinated brown rice, most involve only single factor optimization studies or are associated with high cost treatments (Roohinejad *et al*. 2011, Saikusa *et al*. 1994b, Eamarjharn *et al*. 2016). Additionally, studies have shown that GABA yield may vary greatly depending on the rice variety (Karladee and Suriyong 2012). Therefore, based on the screening of high GABA producing brown rice varieties, the current study sought to elucidate the effects of five factors, including soaking temperature, soaking time, soaking liquid pH, germination temperature, and germination time, on the GABA yield in brown rice in combination with orthogonal test. It was anticipated that this study would demonstrate the optimal germination conditions which result in GABA-rich germinated brown rice to be used for practical application in production.

Materials and Methods

A total of 60 varieties, including 14 white rice, 12 red rice, and 34 black rice varieties, were obtained from the Key Laboratory of Crop Biotechnology at Fujian Universities (Fujian Agriculture and Forestry University).

Sixty rice varieties of the previously mentioned brown rice samples, were planted in the rice field of Fujian Agriculture and Forestry University in Minhou, Fuzhou. Field management was carried out according to the general rice planting method. Each variety was randomly sampled in three portions when rice seeds were mature. The rice samples were then separately polished and prepared into brown rice for further use.

Ten grams of brown rice were placed in a conical flask, washed with purified water, drained, immersed in a 1% hypochlorous acid solution for 5 min for disinfection, and were then subsequently washed with sterile water and drained. Next, the rice was soaked in sterile water (pH 5.0), and the conical flask was placed in water bath at 30°C for 6 hrs. Afterwards, the brown rice was removed, spread in a sterile Petri dish, covered with sterile gauze, and placed in a 30 °C incubator for 24 hrs. The gauze, which was used to cover the brown rice was pre-soaked with a 0.5 mmol/l $CaCl_2$ solution. The gauze was sprayed with 0.5 mmol/l $CaCl_2$ solution every 4 hrs during the 24 hrs period to maintain proper moisture.

After the incubation period, the germinated brown rice was then placed in an 800 W microwave oven for 30 sec, dried in an oven at 50°C for 12 hrs, and then pulverized with a

high-speed pulverizer to obtain the final germinated brown rice flour product. After screening through a 100-mesh sieve, the flour was stored in a refrigerator at 4°C for future use.

Five g germinated brown rice flour was used as starting material, 30 ml 60% ethanol was added, then water bath at 55°C for 6 hrs, during which time it was shaken for 30 sec at a 30 min interval. Then the mixture was centrifuged at 3000 g for 15 min. The supernatant was transferred and concentrated to 5 ml, then filtered with 0.45 μm membrane to obtain the sample to be tested.

Derivatization of the sample was then accomplished using a acetonitrile solution, which contained 1% 2,4-dinitrofluorobenzene (FDNB) as a derivatizing agent. One milliliter of the sample concentrate, 1 ml of a 0.5 mol/l $NaHCO_3$ solution (pH 9.0), and the derivatizing agent were added to a 10 ml volumetric flask, which was then placed in a water bath at 60°C for 1 hr in the dark. After cooling, a phosphate buffer (pH 7.0) was added to volume. The flask was mixed well and then the contents were filtered through a 0.45 μm filter to obtain the injection sample (Xiong *et al*. 2019).

HPLC chromatographic conditions were as follows: the chromatographic column was Hypersil ODS2 C18 (250 nm × 4.6 nm, 5 μm), acetonitrile : water : phosphate buffer (pH 7.2) = 25 : 20 : 55 (v/v) was used as mobile phase, the flow rate was 1.0 ml/min, the wavelength was 360 nm and column temperature was 28°C, the sample injected was 10 μl (Xiong *et al*. 2019).

The effects of soaking temperature, soaking time, soaking liquid pH, germination temperature, and germination time on the GABA yield of brown rice during germination were evaluated. Then, an orthogonal design method was used to determine the optimal germination conditions to produce GABA-rich brown rice. Results were statistically analyzed using SPSS 18.0 software.

Results and Discussion

After germinating 60 rice varieties of brown rice, the GABA content for each was determined by HPLC. The results indicated that the six varieties with the highest GABA contents were 1D67, 1D117, 1D31, N84, 1D71 and 1D70, with GABA contents of 29.12, 26.49, 26.09, 24.82, 23.80 and 22.84 mg/ml, respectively (Table 1). Considering the types of rice varieties and the quantity of seed stocks, brown rice of the N84 black rice variety was selected as the raw material for subsequent experiments.

Five temperature levels, 15, 20, 25, 30 and 35°C were set for soaking of N84 brown rice. Soaking temperature of the brown rice affected significantly on the GABA yield of the germinated brown rice, as shown in Fig. 1. When the immersion temperature was 20°C, the GABA yield of the germinated brown rice was highest, reaching to 16.95 mg/100 g. However, when the soaking temperature was greater than 20°C, the GABA yield decreased significantly ($p < 0.05$).

N84 brown rice was soaked for four different time intervals, which included 3, 6, 9 and 12 hrs. Soaking duration of brown rice had a significant influence on the GABA yield of germinated brown rice, as shown in Fig. 2. When the brown rice was soaked for 9 hrs and then germinated, the GABA yield was highest, which was 22.51 mg/100 g. When the soaking time was prolonged or shortened, the GABA yield decreased significantly ($p < 0.05$).

Four levels of pH, 3.0, 4.0, 5.0, and 6.0, were set for soaking liquid of N84 brown rice. The pH of the soaking liquid of the brown rice exerted a significant influence on the GABA yield of germinated brown rice, as shown in Fig. 3. When the pH of the soaking liquid was 5.0, the GABA yield reached highest, which was 18.42 mg/100 g. When the pH of the soaking liquid was increased or decreased, the GABA yield decreased significantly ($p < 0.05$).

N84 brown rice was germinated at five different time intervals, which included 20, 24, 28, 32, and 36 hrs. The germination time of the brown rice acted significantly on the GABA yield of

germinated brown rice, as shown in Fig. 4. When the germination time was less than 28 hrs, the GABA yield of the germinated brown rice increased with the progression of germination. When the germination time was 32 hrs, the GABA yield reached its highest point at 28.30 mg/100 g, however, there was no significant difference in GABA yield after 28 hrs of germination. When the germination time reached 34 hrs, GABA yield decreased significantly ($p < 0.05$).

Table 1. GABA content of germinated brown rice from different rice varieties (mg/100 g).

No.	Variety	GABA yield	No.	Variety	GABA yield	No.	Variety	GABA yield
1	1D97	13.90 ± 0.13	21	1A825	18.53 ± 0.22	41	1A817	17.95 ± 0.16
2	DN201	15.68 ± 0.15	22	1D54	11.79 ± 0.12	42	1D41	20.35 ± 0.18
3	1A1461	20.08 ± 0. 20	23	1A819	21.70 ± 0.20	43	1D38	16.75 ± 0.17
4	1A1476	6.20 ± 0.09	24	1D32	13.22 ± 0.13	44	1D23	17.61 ± 0.17
5	1D108	13.49 ± 0.10	25	1D09	15.05 ± 0.15	45	1D71	23.80 ± 0.18
6	1D93	13.98 ± 0.12	26	1D02	11.20 ± 0.10	46	1D20	10.93 ± 0.09
7	1D103	6.84 ± 0.09	27	1A820	10.91 ± 0.12	47	1D36	17.89 ± 0.14
8	1D96	11.88 ± 0.13	28	1D33	12.38 ± 0.12	48	1D35	19.47 ± 0.20
9	1D105	15.51 ± 0.12	29	1A814	10.20 ± 0.09	49	1D113	20.55 ± 0.20
10	1A1463	19.87 ± 0.22	30	1A816	15.93 ± 0.10	50	1D29	22.67 ± 0.20
11	1D104	11.68 ± 0.12	31	1D21	16.09 ± 0.14	51	1D69	14.08 ± 0.15
12	1A1473	9.29 ± 0.09	32	1D26	18.02 ± 0.12	52	1D05	9.44 ± 0.09
13	1D100	16.69 ± 0.13	33	1D122	18.38 ± 0.16	53	1D34	22.57 ± 0.17
14	1D94	11.20 ± 0.12	34	1D117	26.49 ± 0.23	54	1D63	18.43 ± 0.18
15	1D01	14.54 ± 0.12	35	OB607	20.75 ± 0.20	55	1D67	29.12 ± 0.20
16	JZHM	14.22 ± 0.13	36	1C45	12.16 ± 0.14	56	N84	24.82 ± 0.19
17	1D53	12.15 ± 0.13	37	1D73	21.14 ± 0.18	57	1D40	14.82 ± 0.11
18	1D31	26.09 ± 0.21	38	1D72	17.29 ± 0.17	58	1D68	20.66 ± 0.17
19	1D03	12.58 ± 0.13	39	1D70	22.84 ± 0.17	59	1C196	15.94 ± 0.14
20	1D04	12.59 ± 0.13	40	1D27	15.70 ± 0.12	60	1D66	21.02 ± 0.15

Of the brown rice samples shown, those numbering 1 - 14 were from white rice, those numbering 15 - 26 were from red rice, and those numbering 27 - 60 were from black rice.

N84 brown rice was germinated at four different temperatures, which included 25, 30, 35 and 40°C. Germination temperature of brown rice influenced the GABA yield of germinated brown rice significantly, as shown in Fig. 5. When the germination temperature was 35°C, the GABA yield of the germinated brown rice was the highest, at 23.04 mg/100 g. When the germination temperature increased or decreased, the GABA yield decreased significantly ($p < 0.05$).

According to the previously described single factor optimization results, a five-factor, four-level orthogonal test design of the GABA-rich N84 brown rice germination conditions was carried out, and a total of 16 groups, with three replicates each, were required (Tables 2 and 3). As shown in Table 3, the 11th group had the highest GABA yield for the N84 germinated brown rice. The corresponding germination conditions were as follows: soaking liquid at a pH of 4.4, soaking completed at 20°C for 9 hrs and germination completed at 39°C for 30 hrs. Under these

germination conditions, the GABA yield of the N84 germinated brown rice was 28.94 mg/100 g, which was 16.6% higher than results obtained from the previous non-optimized tests.

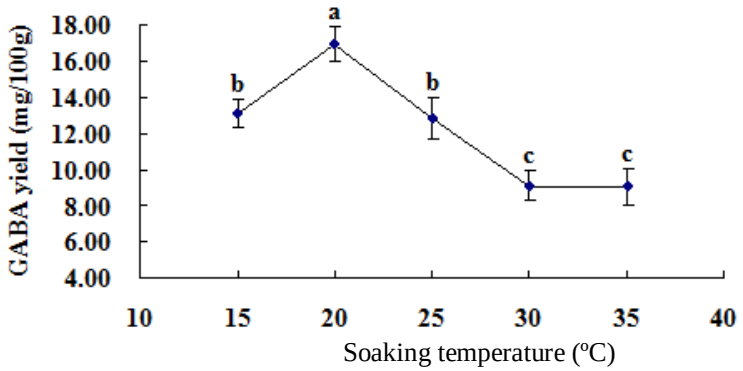

Fig. 1. The effects of the soaking temperature of brown rice on the production of GABA. Different letters in different treatments denote statistically significant differences ($p < 0.05$), the same below.

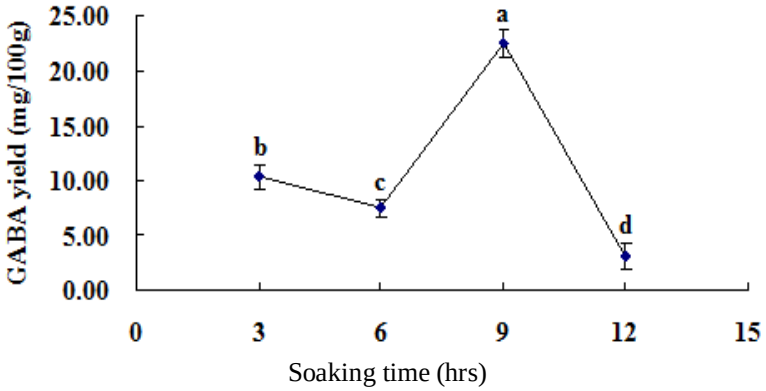

Fig. 2. The effects of the soaking time of brown rice on the production of GABA.

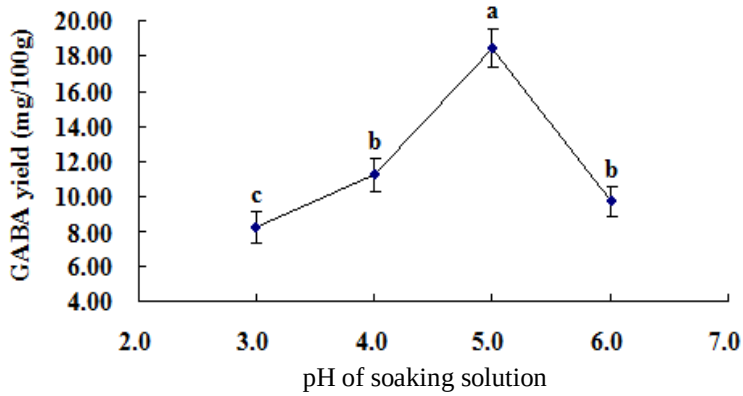

Fig. 3. The effects of pH of the soaking liquid on the production of GABA.

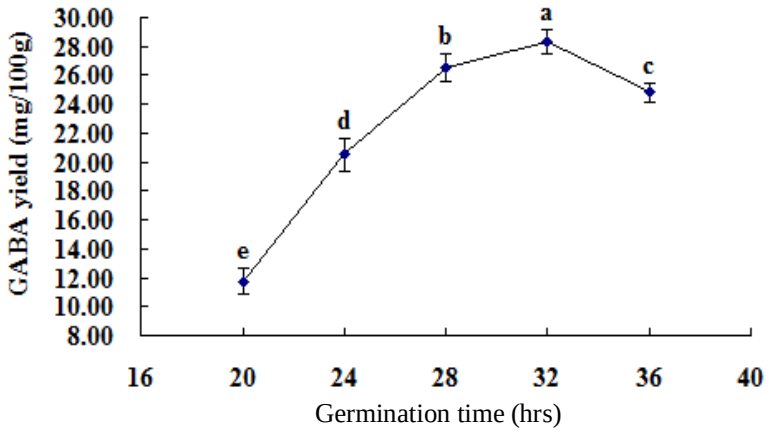

Fig. 4. The effects of germination time of brown rice on the production of GABA.

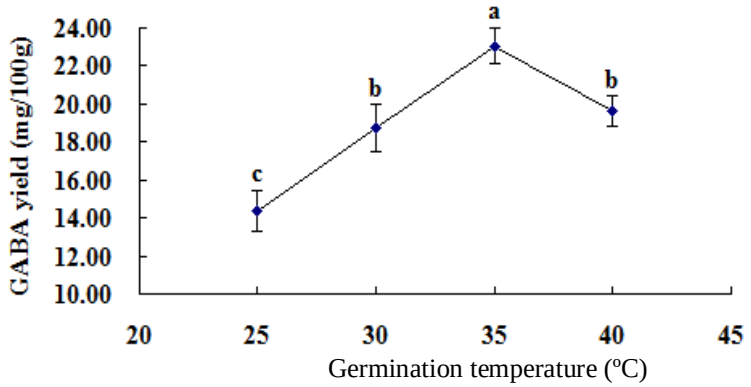

Fig. 5. The effects of the germination temperature of brown rice on the production of GABA.

Table 2. Factors and levels of the L_{16} (4^5) orthogonal test.

Number	Factors	Levels			
		1	2	3	4
A	Soaking temperature /°C	16	18	20	22
B	Soaking time/hr	8.0	8.5	9.0	9.5
C	pH of soaking solution	4.4	4.7	5.0	5.3
D	Germination time/hr	28	30	32	34
E	Germination temperature/°C	33	35	37	39

The primary and secondary relationships between the five factors affecting the GABA yield in N84 brown rice were analyzed by a range analysis method (Table 4). The results showed that the order of significance of the factors affecting the GABA yield were as follows: germination temperature > soaking liquid pH > soaking time > soaking temperature > germination time. The range values for these factors were 8.47, 7.05, 5.50, 2.84 and 2.53, respectively. Considering the

single factor optimization and the orthogonal design results, the optimal germination conditions for producing GABA-rich N84 brown rice were as follows: soaking liquid pH of 4.7, soaking for 9 hrs at 20°C, and then germination for 34 hrs at 39°C.

Table 3. Arrangement and results of the L_{16} (4^5) orthogonal test.

Group	Factors					GABA yield (mg/100 g)
	A	B	C	D	E	
1	1	1	1	1	1	18.83
2	1	2	2	2	2	19.00
3	1	3	3	3	3	13.38
4	1	4	4	4	4	24.88
5	2	1	2	3	4	24.46
6	2	2	1	4	3	17.30
7	2	3	4	1	2	20.60
8	2	4	3	2	1	6.20
9	3	1	3	4	2	15.34
10	3	2	4	3	1	16.95
11	3	3	1	2	4	28.94
12	3	4	2	1	3	17.22
13	4	1	4	2	3	19.94
14	4	2	3	1	4	20.82
15	4	3	2	4	1	23.27
16	4	4	1	3	2	15.89

Table 4. Range analysis of the germination conditions of the germinated brown rice with high GABA yield.

Factors	A	B	C	D	E
K1	19.02	19.64	20.24	19.37	16.31
K2	17.14	18.52	20.99	18.52	17.71
K3	19.61	21.55	13.94	17.67	16.96
K4	19.98	16.05	20.59	20.2	24.78
Range	2.84	5.5	7.05	2.53	8.47
Results of range analysis	22°C	9 h	pH 4.7	34 h	39°C
Results of orthogonal test	20°C	9 h	pH 4.4	30 h	39°C
Results of comprehensive consideration	20°C	9 h	pH 4.7	34 h	39°C

Brown rice undergoes a series of physiological changes during the germination process. Hydrolases, such as amylase, protease and phytase are activated which involve corresponding enzymolysis processes. Therefore, brown rice contains more nutrients after germination, and

GABA is one of the important active ingredients in GBR (Moongngarm and Saetung 2010, Cho and Lim 2016). Studies have shown that the GABA yields of different rice varieties vary greatly after germination (Karladee and Suriyong 2012). Varanyanond *et al.* (2005) investigated the effects of water soaking on the GABA yield of six rice varieties in Thailand (Khao Dawk Mali 105, Pathum Thani 1, Chai Nat 1, Suphan Buri 1, Leuang Pratew 123 and Plai Ngahm). Results indicated that the different rice varieties had significantly different GABA accumulation rates during immersion. For example, after 1 hr of soaking the GABA content of Chai Nat 1 was gradually stabilized, while the GABA content of Khao Dawk Mali 105 increased at a high rate within 4 hrs of soaking, and the GABA content of Leuang Pratew 123 did not significantly change during soaking. Roohinejad *et al.* (2011) categorized 18 brown rice varieties into high medium and low GABA groups according to the respective initial GABA content, and then they were germinated. It was found that brown rice with a high initial GABA content had a higher GABA content after germination than the GBR with a low initial GABA content. Therefore, the GABA content of brown rice after germination was also related to the initial GABA content.

Germination conditions of brown rice, such as soaking time, soaking temperature, and soaking liquid pH, have also been shown to have significant effects on the GABA yield of GBR (Patil and Khan 2011, Cho and Lim 2016). Saikusa *et al.* (1994b) found that the GABA yield of GBR was highest in the rice variety Koshihikari (*Oryza sativa* L. subsp. japónica Kato) when it was soaked for 8 hrs. Thiitunsomboon *et al*. (2012) used the rice variety PTT1 (Indica rice) as a raw material, and found that 3 hrs was the optimal soaking time. The results of these two studies were quite different. The former was consistent with the results of the present study. The results of the latter were significantly different from both the former study and this study. These differences may be attributed to the different varieties used in each study. However, additional factors leading to these differences may be elucidated through further experimentation.

Currently, there are a few studies focused on the optimization of the soaking temperature of brown rice, and the soaking temperature adopted by most scholars is between 28 - 40°C (Komatsuzaki *et al*. 2007, Thitinunsomboon *et al.* 2012, Saikusa *et al.* 1994b, Cáceres *et al*. 2017). In the present study, using both a single-factor optimization and an orthogonal experimental design, the optimal soaking temperature was found to be 20°C, and furthermore, GABA yield was shown to be greatly reduced when the soaking temperature exceeded 20°C. It was surprising to see that the results of this study were so different from the soaking temperatures that are supported by other studies.

In some studies, the pH of the soaking liquid of the brown rice was adjusted by adding various buffers. Eamarjharn *et al.* (2016) showed that, when used at the same concentration (50 mmol/l), phosphate buffer was superior to other tested buffers, such as tris, boric acid, and citric acid-based. For example, when 80 mmol/l phosphate buffer was added, the GABA yield of the GBR was increased by 2.7 times. This might be that the free phosphate ions in the buffer system promote the formation of Schiff base by acting as both a proton donor and acceptor (Huang *et al*. 2001). Schiff base serves as an intermediate of the irreversible formyl decarboxylation reaction during the synthesis of GABA by glutamic acid and facilitates GABA accumulation (Toney 2005).

As shown from the single factor optimization results of this study, the optimal conditions for the five factors affecting GABA yield in N84 germinated brown rice were soaking temperature 20°C, soaking time 9 hrs, soaking liquid pH 5.0, germination time 28 - 32 hrs, germination temperature 35°C. After the comprehensive single factor optimization, the orthogonal design, and the range analysis, the optimal germination conditions obtained were as follows: soaking solution pH 4.7, 9 hrs of soaking at 20°C, and 34 hrs of germination at 39°C. Among these experiments and results, the single factor optimization results for germination time and germination temperature were greatly related to the results of the orthogonal design. This demonstrates that it is

necessary to combine single factor optimization and orthogonal experimental design when optimizing the factors influencing brown rice germination conditions.

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