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Research article

Germinated Brown Rice Preparation for Value Added Material for Healthy Brewed Vinegar

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alcohol fermentation;
acetification

Abstract

Soaking grains in water during the preparation of germinated brown rice (GBR) can result in the multiplication of bacterial contaminants. To address this problem, a system was designed to automatically change the soaking water every 6 h. A significant reduction of the contaminants was observed. GBR was saccharified by mold bran *Amylomyces rouxii*. The 5 day saccharified GBR that contained the highest α-amylase and glucoamylase activities was further used for alcohol fermentation by yeast *Saccharomyces cerevisiae*. Then, acetic acid was produced by *Acetobacter aceti* through a semi-continuous process. In the evaluation of the nutritional quality during processing, significant increments in the levels of γ-aminobutyric acid (GABA), antioxidant activities (DPPH), and total phenolic contents were found during soaking, but these levels progressively decreased in wine and were lowest in vinegar. Moreover, the soaking period from 36 h to 48 h had no significant effect on these substances. GC-MS analysis indicated that four important volatile organic compounds in the produced wine and vinegar were reported for their antimicrobial activity. Furthermore, no change in GABA was found in the vinegar over 6 months of storage. Thus, using this method could successfully produce brewed vinegar with health benefits from GBR.

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1. Introduction

Among the world's dominant crops, rice is a particularly important basic plant-based food in most Asian countries. Brown rice, also called hulled rice, can be produced by simply polishing the grains, a step which makes them more nutritious than milled rice. Interestingly, health benefits of brown rice have been shown to increas after germination [1]. During the germination of rice seeds, biochemical changes provide crucial components and energy to support seedling growth. Simultaneously, the hydrolysis of large molecules including protein, starch, and non-starch polysaccharides, occurs after the activation of hydrolytic enzymes, which results in an increment in amino acids, peptides, and simple sugars [2]. Brown rice is normally germinated in a humid and warm environment, and such condition can also encourage the multiplication of microorganisms [3]. Soaking brown rice in water for a certain duration can support the growth of native microbial biota, which can affect the quality of germinated brown rice (GBR) [4]. Hence, the soaking preparation of GBR using simple technology is crucial for economic purposes and is one of the aims of this study.

GABA (γ-Aminobutyric acid) is the dominant healthy compound among the compounds isolated from germinated grains such as rice [5], barley [6], and soybean [7]. Naturally, GABA is found in plants, animals, and some microorganisms, and has great potential health benefits because of its diverse physiological functions. GBR contains various important bioactive compounds, including γ-oryzanol, tocotrienols, ferulic acid, potassium, magnesium, zinc, and prolyl endopeptidase inhibitor, and dietary fiber [8].

The production of vinegar requires two microbiological processes. However, some vinegars, such as rice vinegar, require a third fermentation for the degradation of starch. When rice is used as the raw material, a mold starter (a fungal amylase preparation produced by growing mold on moist wheat bran) called Koji, or mold bran, is commonly used. Meanwhile, the acetification processes, used worldwide for vinegar production, is a submerged semi-continuous process [9]. At the end of each production cycle, when the ethanol in the fermentation medium is at 5 g/l or below, a given volume of fermentation medium (such as 40% v/v) is discharged as the finished product. Subsequently, the process is then recharged with the same volume of fresh wine and nutrients.

The aims of the present study were to: (1) evaluate the soaking process in order to reduce bacterial contaminants during the germination of brown rice grains, and to provide the highest yield of GABA; (2) develop a healthy brewed vinegar from GBR by a semi-continuous process; (3) determine the changes in GABA, antioxidant activities (in terms of DPPH), and total phenolic content of GBR during yeast fermentation by *Saccharomyces cerevisiae* M30, and acetic acid fermentation by *Acetobacter aceti* WK; and (4) determine the generated volatile organic compounds that contribute to the applicable antimicrobial activity of GBR vinegar.

2. Materials and Methods

2.1 Samples

Brown jasmine rice (BR) was provided by a co-operative farmer in Surin province, Thailand. BR was dried again at 50°C in the laboratory, until the moisture content was reduced to 13%. To protect against mold and grain beetles, BR was vacuum-packed in plastic bags and kept at 30±2°C before use.

2.2 Microorganisms

The DK strain of the fungus *Amylomyces rouxii* was isolated from Thai sweetened fermented rice (Khao-Mak) and cultured on Potato Dextrose Agar (PDA; Himedia, India) slants for 3 days. It was kept at 5±2°C until use. Mold bran was prepared by using 11 g fine wheat bran, 1 g rice husk, 5 ml basal medium, and 7 ml of distilled water. All ingredients were placed in a 250 ml Erlenmeyer flask and sterilized for 1 h at 121°C. A sterilized basal medium (g/l) comprising distilled water, 0.05 KH_2PO_4, 0.05 $MgSO_4$, 0.01 $FeSO_4$, 1.55 KNO_3 [10] was prepared. One PDA slant of *A. rouxii* DK was added together with sterile distilled water (5 ml). The mixture was aseptically transferred to a flask of the sterile mold bran medium and mixed well before incubating for 7 days at 30±2°C. Both α-amylase and glucoamylase were determined in the mold bran, which was cultured for 7 days and sampled every 24 h starting from day 0.

A flocculate yeast, *Saccharomyces cerevisiae* M30, was obtained from Laboratory of Yeast Biotechnology, Department of Microbiology, Kasetsart University in Thailand. After keeping in a Yeast Malt (YM; Himedia, India) agar slant at 5±2°C, yeast cells were cultured in sterilized YM medium composed of distilled water (g/l), 10 malt extracts, and 10 yeast extracts, and then incubated for 1 day at 30±2°C in a 100 rpm rotary shaker. Yeast cells (approximately 7 log CFU/ml) were achieved as a starter for GBR wine processing.

High acid-tolerant strain *Acetobacter aceti* WK, which had been screened naturally from over-ripened pineapple and adapted under high acetic acid condition for many years, was used for vinegar production. A starter of *A. aceti* WK was prepared under aeration at 4.5 l/min by culturing in a sterilized complex medium comprising the following (g/l): distilled water, 5 yeast extract, 50 glucose, 0.5 $(NH_4)_2HPO_4$, and 0.2 $MgSO_4.7H_2O$ for 7 days at 30±2°C [11].

2.3 Determination of bacterial contaminants during soaking for preparation of germinated brown rice

A modified gaseous germination condition described by Komatsuzaki *et al*. [5] was applied for BR grains. BR grains were cleaned and soaked in water at 1:5 (w/v) ratio in a stainless steel bath (1 m × 1 m × 0.1 m; Figure 1a) with a stainless steel screen (0.3 mm pore size) for 48 h at 30±2°C. During the soaking of BR grains, the water was aerated at 4.5 l/min. The results obtained using two soaking processes, namely, automatically changing the water every 6 h and not changing the water, were compared for 48 h.

Bacterial contamination during soaking of BR for 48 h with and without changing the soaking water was investigated. A sample of soaking water was collected every 6 h. When the soaking water used was changed, a sample was collected after 6 h. The samples were packed aseptically in sterile test tubes and kept at 5±2°C for a time duration not exceeding 1 h. Serial dilutions of each sample suspension with sterile 1% peptone water (Himedia, India) were prepared for the analysis of total aerobic and lactic acid bacteria (LAB) using Total Plate Count (TPC; Himedia, India) agar and de Man Rogasa Sharp (MRS; Himedia, India) agar, respectively, through a spreading technique. Plates were incubated at 30±2°C, whereas MRS plates were kept under micro-aerophilic conditions.

During the germination process in the automatically changed soaking water, grains were taken randomly at 0, 6, 12, 24, 36, and 48 h. Each sample was dried for 8 h at 50°C and kept at 30±2°C before the analysis of GABA content.

2.4 Wine preparation using germinated brown rice

GBR (12 kg) was used in each set of 100 l fermentation vessels (1 kg/tray; 12 trays per 100 l). GBR was soaked in water at a ratio of 2:1 GBR grains to water for 3 h, drained, cooked at 85±1°C for 30

min, and then cooled to 30±2°C. Sterile water was added at 1.2 l, and the mixture was mixed well before inoculation using a flask of *A. rouxii* DK mold bran. Each tray was covered with clean cheesecloth and incubated at 30±2°C for the period that resulted in the highest saccharification [12]. The mixture of saccharified rice and sweetened liquid from 12 trays was collected and used for further alcohol fermentation. The mixture was mixed with sterile supplemented medium consisting of (g/l) 150 sugar, 0.5 di-ammonium hydrogen phosphate (DAP), and 0.2 $MgSO_4.7H_2O$. The pH was adjusted to 5.5 by 10 M NaOH and 10% citric acid solution [13], and the volume was brought to 100 l with sterile water. After mixing, alcohol fermentation was started by inoculating with the yeast starter, *S. cerevisiae* M30. During fermentation for 7 days at 30±2°C, a sample was collected to monitor total sugar, ethanol produced, and yeast cell density and to evaluate the most suitable period for production of GBR wine, which was used subsequently in the acetification process.

For the monitoring of GABA, antioxidant activity, and total phenolic content during GBR wine fermentation, samples were collected at 0, 1, 3, 5, and 7 days and analyzed. In addition, analysis of volatile organic compounds (VOCs) in the finished GBR wine was conducted using GC-MS.

2.5 Semi-continuous acetification process

Normally, acetification process comprises two phases, a startup process and an operational phase. The total solute concentration (TC) of the fermentation medium (80 g/l) was prepared by adjusting to 35±1 g/l ethanol and 45±1 g/l acetic acid. In the startup phase, 25 l of the fermentation medium was prepared in our 100 l internal Venturi injector bioreactor, as described in Krusong *et al*. [11]. After inoculation with 5% (v/v) *A. aceti* in the startup, the cells adapted to the new conditions. This phase ended when the remaining ethanol in the fermentation medium was oxidized until only 5 g/l or a lower concentration was left [9]. Then, the operational phase was initiated by adding fresh medium supplemented with nutrients; the volume was brought to 75 l by adjusting the TC to 80 g/l. Complete production was achieved when the remaining ethanol reached 5 g/l or lower. The subsequent operational phase was performed by discharging 40% of total volume (75 l) [11]. Then, fresh medium with the same volume (40%) was used to recharge the bioreactor. Excluding the startup phase, nine cycles were run while the acid and ethanol contents in each cycle were determined. In addition, GBR vinegar samples from the last three cycles were collected for the analysis of GABA, antioxidant activity, and total phenolic content. The VOCs of the GBR vinegar were determined using GC-MS. In addition, samples of GBR vinegar were analyzed for GABA, antioxidant activity, and total phenolic content during storage at 30±2°C in the dark for 2, 4, and 6 months.

2.6 Analytical methods

The amylolytic activity in the mold bran was determined by extraction with 50 ml of cooled 0.1 M sodium citrate buffer (pH 5.0), rotary shaking at 200 rpm for 60 min at 25±2°C, and filtration through a cheesecloth. After centrifuging at 7,500 × *g* for 15 min at 4°C, the clear filtrate as crude enzyme of each sample was evaluated for α-amylase and glucoamylase activities. α-Amylase activity was measured using a mixture of 0.1 ml 0.1 M phosphate buffer (pH 7), 1 ml 1% soluble starch, and 1 ml crude enzyme. The enzyme reaction was achieved after incubating at 30°C for 30 min to allow the release of reducing sugar. Then, 0.1 M HCl (0.25 ml) was added to stop the reaction, and the amount of released reducing sugar was determined by the dinitrosalicylic acid method at 540 nm. The definition of amylase activity was that "one unit is the amount of enzyme liberating glucose 1 µmol/min from soluble starch under standard assay conditions" [14]. In addition, glucoamylase analysis was conducted using 0.5 ml crude enzyme, which was added into a solution consisting of 1.5 ml 0.1 M sodium acetate buffer (pH 5.0), and 0.5% soluble starch. Reaction conditions were controlled at 55°C for 15 min. Then, 1 ml of 3,5-dinitrosalicylic acid was added to stop the reaction. The solution was heated further at 80°C for 3-5 min, cooled to room temperature,

and measured at an absorbance of 540 nm. One unit of glucoamylase activity is the amount of enzyme liberating glucose (1 µmol/min) from soluble starch under standard assay conditions [15].

Total sugar was measured by the phenol-sulfuric acid colorimetric procedure with glucose as a standard. Concentrated sulfuric acid can breakdown the total soluble sugars in the sample to monosaccharides, which can be quantified by measurement of absorbance at 480 nm using a spectrophotometer (UV-1601 Shimadzu, Japan).

Ethanol was detected by gas chromatography (SHIMADSU 8D, Japan), using a Carbowax 20M capillary column with 0.2 µm Chromosorb. An FID detector was used [16]. Acidity (% v/v) by acid-base titration with 0.1 M NaOH with phenolphthalein was conducted [9].

Modified solid phase micro-extraction (SPME) was used for volatile compound determination using the method of Vas and Vékey [17]. The sample (5 ml) was put in a glass bottle (25 ml) with a 20 ml volume of headspace. Then, 3 g NaCl was added to the bottle before sealing with a Stableflex PDMS/DVB septum cap (60 µm fiber size) (Supelco Inc., Bellefonte, PA, USA). The extract was left to stand at 37°C for 60 min prior to analysis by GC-MS (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) using a Stabilwax column (ID, 0.25 mm; length, 30 m; film, 0.25 µm). The inlet temperature was set at 250°C and splitless injection at 75 ml/min was controlled. Meanwhile, helium gas as carrier at 1.2 ml/min was used. After introducing samples and maintaining them for 5 min at 40°C, the temperature was increased to 230°C at 5°C/min and maintained at this level for 5 min. An electron ionization mode with 230°C ion source temperature was used for MS analysis with 35-300 amu scan mass range and 0 min solvent delay time. MS transfer line was at 240°C. Identification of volatile organic compounds depended on mass spectra fragmentation patterns, the retention times, and correlation with the Wiley, 275.L data library [18].

The Folin-Ciocalteau method was used to determine the total phenolic content [19]. Folin-Ciocalteau reagent (5 ml) was added to 1 ml gallic acid as the standard phenolic compound or 1 ml of a diluted solution sample of BR extract, GBR extract, GBR wine, or vinegar, and mixed well for 30 s. Each sample was diluted with de-ionized water at ratios of 1:2, 1:5, and 1:10. Then, 1 M Na_2CO_3 (15 ml) was added before the volume was brought up to 100 ml with distilled water. After allowing the mixture to stand for 1 h, phenols were measured with colorimetry at 765 nm using a visible spectrophotometer (Thermo Fisher, Thermo Scientific™ model, GENESYS 20, USA). Total phenol content as gallic acid equivalent (mg GAE/ml) was measured by comparing with a standard curve of gallic acid solution at 0, 50, 100, 150, 200, and 250 mg/ml.

Using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay according to the method of Gorinstein *et al*. [20], the antioxidant activities of BR, GBR, and GBR wine or vinegar extract were determined. The sample (100 µl) was placed in 2.9 ml of 0.1 mM ethanolic solution of DPPH. After vigorous mixing in a vortex mixer, the reaction solution was incubated at room temperature for 30 min. The absorbance of the sample solution was measured at 515 nm in a visible spectrophotometer, and ascorbic acid was used as the positive control. Radical scavenging activity (% inhibition) was calculated using the following formula:

$$\% \text{ inhibition} = (Ac - As)/Ac \times 100$$

where As = absorbance of tested samples, and Ac = absorbance of the control

GABA content was measured by using a modified method from Srisang *et al*. [21]. Finely ground BR, GBR grain samples, GBR wine, and GBR vinegar were extracted by 3% sulfosalicylic acid at a ratio of 0.5 g per 200 ml and incubated for 1.5 h at room temperature. Then, each clear filtrate was removed by centrifuging at 5,000 × *g* at 4°C for 10 min. The 50 µl filtrate was mixed well with 50 µl $NaHCO_3$ and 200 µl dimethylaminoazobenzene before incubation for 10 min at 70°C. The 250 µl of ethanol and 250 µl of KH_2PO_4 were added into the solution, which was filtered through 0.45 µm pore-size nylon filters and then analyzed by HPLC (Agilent 1100 Series, Agilent Technologies, California, USA) equipped with Supelcosil-LC-DABS column, where column

temperature at 35°C and the detector at 465 nm were set. Acetonitrile as a mobile phase was supplied at 1 ml/min.

2.7 Statistical analysis

The data from three replicates were analyzed using one-way analysis of variance (ANOVA). A mean comparison test was performed using the Tukey's Test. SPSS Version 10.0 for Windows pocket program was used for these analyses.

3. Results and Discussion

Although soaking BR grains in water is the normal process for preparing GBR, it may lead to multiplication of bacterial contaminants, especially when the BR grains are soaked at 30°C-35°C for more than 20 h [5]. The results (Table 1) in our present study further confirmed this. High total aerobic count (6.8±0.1 log CFU/ml) and low amount of LAB (2.6±0.4 log CFU/ml) were found under the condition in which soaking water was not changed. An unacceptable smell of BR was observed at 24 h of soaking. We did not determine the number of bacterial contaminants after 24 h, because spoilage has already occurred.

Treatment processes for reduction of microorganism multiplication during GBR production, including ultraviolet and electrochemical disinfection, have been recommended [22]. However, cost problems related to the multiplication of bacterial contaminants still remain. Therefore, to address these problems, we designed a system to remove the soaked water automatically every 6 h. The results, shown in Table 1, revealed that the loads of total aerobic bacteria and LAB were significantly reduced to 3.1±0.6 and 1.5±0.3 log CFU/ml, respectively, after 24 h of soaking when this automatic system was in operation. In addition, little change in these bacterial contaminants was found in subsequent soaking time up to 48 h.

BR germination is necessary for the analysis of GABA content. Komatsuzaki *et al.* [5] recommended that GABA should be allowed to accumulate during germination. When BR was soaked with aeration at 4 l/min and with automatic changing of soaking water every 6 h, the BR contained 2.81±0.7 mg/kg GABA, which increased significantly ($p \leq 0.05$) after prolonged soaking. The highest amount of GABA (125.6±7 mg/kg) was reached after 48 h of soaking, but no significant difference ($p \leq 0.05$) from the level after 36 h soaking (124±6 mg/kg) (Figure 1b) was found. Therefore, we decided to use the GBR obtained after 36 h soaking in wine fermentation. The levels of GABA produced using the aeration processes were high but were lower than those obtained by Komatsuzaki *et al.* [5] (249 mg/kg). However, their method increased the amount of surface microorganisms. This lower yield of GABA using the aeration process could have been partially due to the difficulties in the germination of brown rice, which included low germination rates and spoilage. After the removal of the BR husk, some broken kernels were possibly found; these may have made it easy for enzymes or microorganisms to contaminate the sample, leading to spoilage during soaking in water [23]. The automatic removal of the soaking water every 6 h could reduce the spoilage of GBR by bacterial contaminants.

Saccharification of GBR was carried out by the action of the enzymes α-amylase and glucoamylase in mold bran of *A. rouxii* DK. The highest activities of α-amylase (2,345 unit/g) and glucoamylase (1,450 unit/g) were found after 5 days of saccharification; the α-amylase activity was higher than the glucoamylase activity over the whole period of saccharification (Figure 2a). Therefore, the method of saccharification of GBR by mold bran for 5 days at 30±2°C was used for further GBR wine fermentation.

Table 1. Bacterial contaminants in water during soaking of brown rice for 48 h at 30±2°C

Soaking period (h)	No Soaking Water Change*			Soaking Water Change*		
	pH	TPC (log CFU/ml)	LAB (log CFU/ml)	pH§	TPC (log CFU/ml)	LAB (log CFU/ml)
0†	6.5	2.5±0.1^{A}	1.1±0.2^{a}	6.5	2.5±0.3^{A}	1.1±0.5^{a}
6†	5.1	2.8±0.3^{A}	1.5±0.1^{a}	5.0	2.7±0.5^{A}	1.1±0.4^{b}
12†	3.5	4.1±0.2^{A}	2.1±0.3^{a}	5.0	2.9±0.4^{B}	1.3±0.5^{b}
18†	3.1	5.6±0.2^{A}	2.2±0.3^{a}	4.8	2.9±0.1^{B}	1.5±0.4^{b}
24†	2.8	6.8±0.1^{A}	2.6±0.4^{a}	4.5	3.1±0.6^{B}	1.5±0.3^{b}
30	ND	ND	ND	4.5	3.2±0.4	1.8±0.1
36	ND	ND	ND	4.2	3.2±0.2	1.8±0.5
42	ND	ND	ND	4.2	3.2±0.5	2.0±0.4
48	ND	ND	ND	4.0	3.3±0.7	2.1±0.5

* Mean ± one standard deviation; mean comparison is analyzed by Tukey's test. † Means with different upper-case letters within the same row showed significant difference ($p \leq 0.05$) in the total aerobic count on the TPC plate. † Means with different lower-case letters within the same row showed significant difference ($p \leq 0.05$) in lactic acid bacteria on MRS plates. § pH of soaking water was determined at the end of the soaking period before changing the soaking water. Abbreviations: TPC, Total Plate Count; LAB, Lactic Acid Bacteria; ND, Not determined.

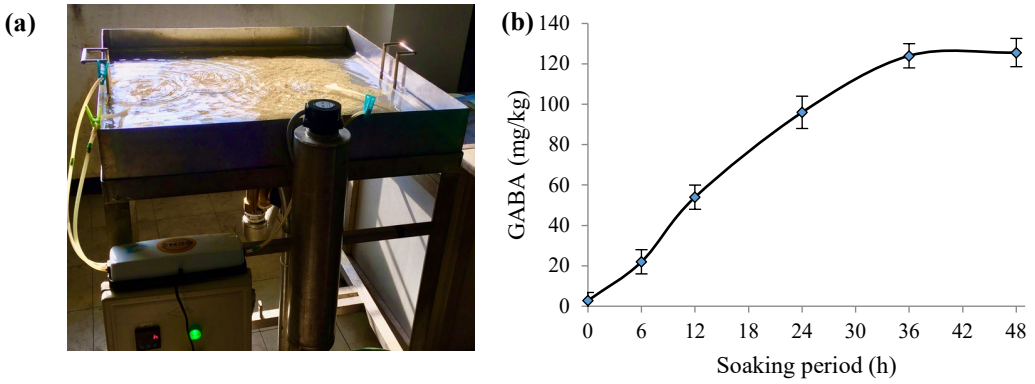

Figure 1. Soaking under aeration at 4 l/min for germination of brown rice: (a) soaking equipment used, removal automatically of soaking water every 6 h; (b) γ-aminobutyric acid (GABA) accumulation during soaking period in the designed soaking equipment

The breakdown of starch in plant cells is normally due to extracellular enzymes, such as α-amylase and glucoamylase, which can achieve almost complete starch saccharification. Molds, which are significant sources of both α-amylase and glucoamylase, can be screened and isolated, because their enzyme activities can be used to degrade gelatinized starch. Consequently, amylolytic activity during saccharification of GBR by the mold bran of *A. rouxii* DK was determined. The mold bran of *A. rouxii* DK was used as a source of both enzymes. After 5 days of saccharification resulting in the breakdown of starch to sugar, the saccharified GBR was fermented simultaneously with the yeast *S. cerevisiae* M30. The results in Figure 2b showed that *S. cerevisiae* M30 was effective in the fermentation process at 30±2°C and produced 92±3.5 g/l ethanol in 5 days with high cell density (12 log CFU/ml). Subsequently, this GBR wine was used for acetic acid production using *A. aceti* WK.

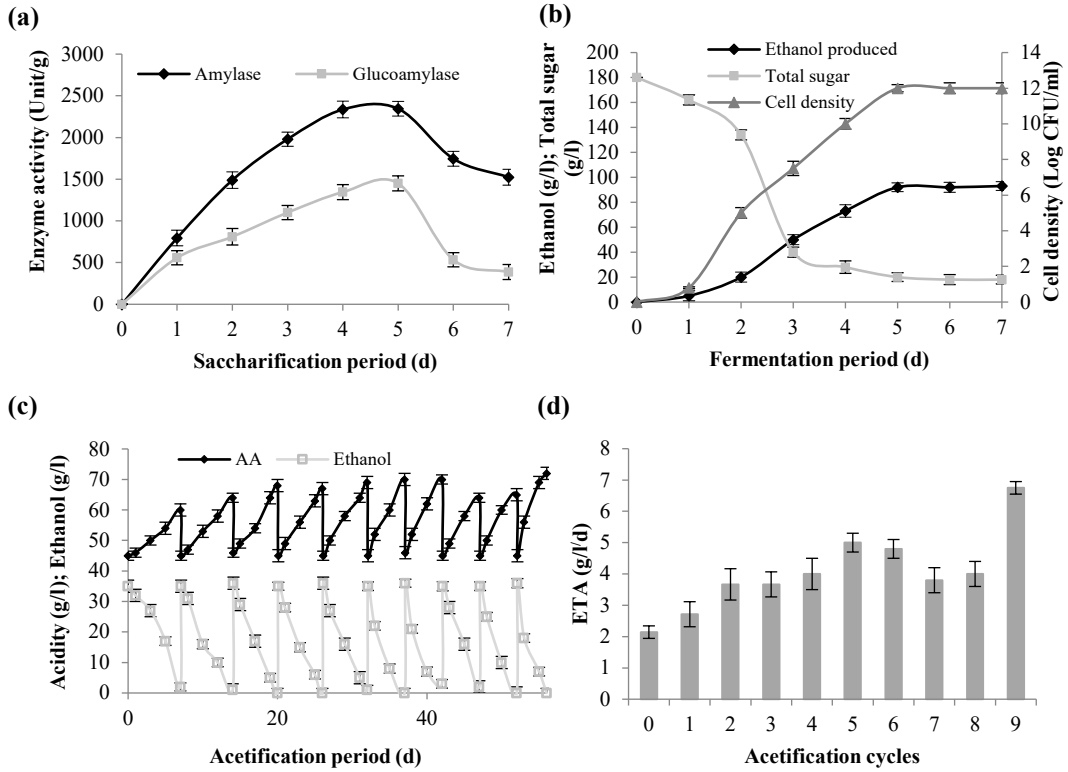

Figure 2. Germinated brown rice as substrate for vinegar production: (a) α-amylase and glucoamylase activities during saccharification of GBR by mold bran of *Amylomyces rouxii* DK at 30±2°C for 7 days; (b) GBR wine, simultaneous fermentation of 5 day saccharified GBR by yeast *Saccharomyces cerevisiae* M30 at 30±2°C for 7 days: (c) acid-produced and ethanol utilized in nine semi-continuous cycles of acetification of GBR wine by *Acetobacter aceti* WK in a 100 l internal Venturi injector bioreactor at 30±1°C; (d) acetification rate of each cycle. Cycle No. 0 means the startup phase, whereas cycles No. 1-9 were operational (or production) cycles. Abbreviation: AA, acetic acid

Semi- continuous processes are commonly used in commercialized vinegar production. A key to the success of this operation is aeration in the bioreactor. Using the internal Venturi injector 100 l bioreactor, as described by Krusong *et al.* [11], a high amount of fine air bubbles was generated and supplied for the acetification of GBR wine by *A. aceti*.

Normally, cells of acetic acid bacteria (AAB) require 7-14 days after inoculation to adapt during the startup acetification process because of the effect of dilution under new fermentation conditions [16]. In our study, the adaptation period of *A. aceti* WK under a high initial acetic acid concentration (Figure 2c) was only 7 days, which implied that GBR wine medium was a good source of nutrition to support cell growth under abundant fine air bubbles. The results obtained from the nine semi-continuous cycles (Figure 2c) revealed that AAB cells had 4-7 days for each cycle with 64±1.5 g/l to 72±2 g/l acetic acid content. However, in the 9th cycle, the acetification period was shortened, which improved the acetification rate of GBR vinegar (Figure 2d).

The results in Figure 2c imply that *A. aceti* WK developed the ability to tolerate the negative effect of high acetic acid content, which was an additional key for successful acetification. A

previous work reported that inhibition occurs at above 20 g/l acetic acid level, reduction of growth rate occurs at above 40 g/l acetic acid, and strong inhibition (approximately 70%) occurs at 60 g/l [24]. However, some strains of *Acetobacter* can gradually adapt to and resist increasing acetic acid concentrations, thereby making them suitable for the commercialized acidification process [25].

The reduction of ETA observed in the 7th and 8th cycles was likely due to the oscillation of AAB during long fermentation period (Figure 2d). Krusong *et al*. [26] reported that AAB cells decreased during the 5th and 6th cycles which resulted in the reduction of ETA. Besides, the high acetic acid concentration of the end product in the medium harmed the AAB cells and affected the ETA as well.

As mentioned in our previous study [11], acetification performance by *A. aceti* WK during the semi-continuous process decreased cell biomass in some cycles. This is due to the toxic effect of acetic acid on cells, as reported by Maestre *et al.* [27]. However, AAB cells could gradually adapt during these circumstances, which shortened the cycle period of acetification. This finding implied the effectiveness of *A. aceti* WK adaptation in the bioreactor under a high amount of fine air bubbles using GBR wine; thus, its suitability as a substrate was proven.

Monitoring of volatile organic compounds by GC- MS was performed, and the total phenolic content, antioxidant activity, and GABA content of the GBR vinegar process were investigated. As shown in Figures 3a and 3b, 38 VOCs were present in wine, whereas 34 were present in vinegar. Besides acetic acid, only three other compounds were found in both GBR wine and vinegar (Table 2), namely, (1) acetic acid ethyl ester ($C_4H_8O_2$), (2) hexanoic acid ($C_6H_{12}O_2$), and (3) menthone ($C_{10}H_{18}O$).

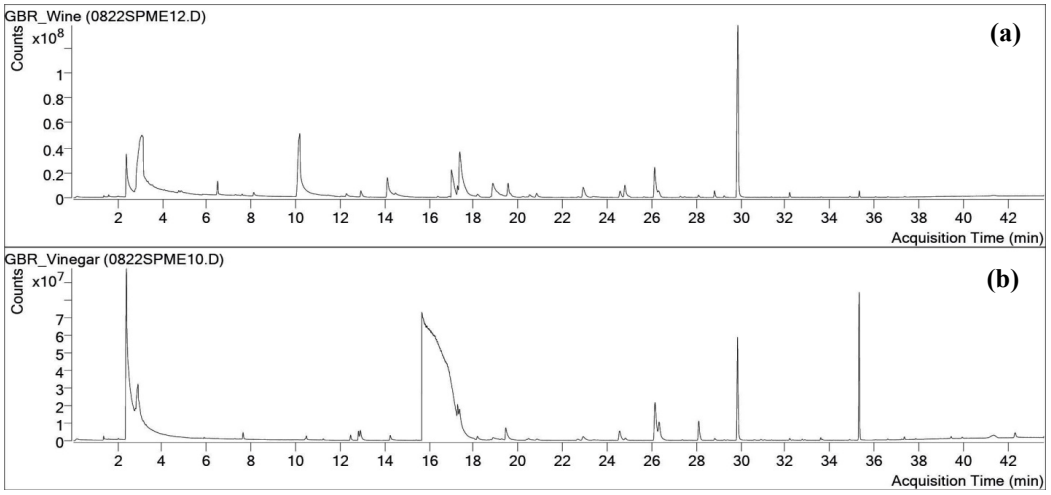

Figure 3. GC-MS chromatogram of major volatile organic compounds detected in: (a) germinated brown rice wine, and (b) germinated brown rice vinegar

Interestingly, the menthone detected in both GBR wine and vinegar is an essential oil found in an edible medicinal plant *Ziziphora clinopodioides*. This oil has antibacterial, antifungal, and antioxidant activities [28]. Therefore, menthone may result in the increment of antibacterial and antioxidant activities of GBR vinegar.

Moreover, all three major VOCs, namely, (1) acetic acid ethyl ester, (2) acetic acid, and (3) hexanoic acid, are also found in GBR vinegar. These VOCs have antimicrobial activity. Ethyl acetate (or acetic acid ethyl ester [$C_4H_8O_2$]) is the most abundant ester commonly produced in wine. It is also the most common volatile acid that occurs with acetic acid, and it is generated during

Table 2. The major volatile organic compounds of germinated brown rice in wine[†] and vinegar[†] that were detected using GC-MS.

Retention time[ψ] (min)	Chemical name	Formula	Relative concentration (%)	
			wine	vinegar
2.68	**Acetic acid ethyl ester**	**$C_4H_8O_2$**	**5.9**	**36.12**
2.92	Methane, oxybis-	C_2H_6O	-	7.74
3.06	Dimethylamine	C_2H_7N	19.5	-
10.18	1-Butanol, 3-methyl-	$C_5H_{12}O$	12.83	-
14.12	Propanoic acid, 2-, hydroxy-, ethyl ester	$C_5H_{10}O_3$	2.88	-
15.68	**Acetic acid**	**$C_2H_4O_2$**	-	**8.29**
17.01	Butanoic acid, 2-hydroxy-3-Methyl-, ethyl ester	$C_7H_{14}O_3$	4.91	-
17.36	endo-4-Deuterio-exo-4,5-epoxy-endo-tricyclo[5.2.1.0(2,6)]dec-8-en-3-one	$C_{10}H_9DO_2$	11.57	-
18.86	1-Phenyl-1,2-propandione	$C_9H_8O_2$	3.32	-
19.45	Propanoic acid	$C_3H_6O_2$	-	1.75
19.55	2,3-Butanediol	$C_4H_{10}O_2$	1.65	-
22.92	Butanoic acid	$C_4H_8O_2$	1.68	-
24.57	Pentanoic acid	$C_5H_{10}O_2$	-	1.42
24.79	Butanedioic acid, diethyl ester	$C_8H_{14}O_4$	1.86	-
26.11	**Menthone**	**$C_{10}H_{18}O$**	**4.01**	**5.61**
29.46	**Hexanoic acid**	**$C_6H_{12}O_2$**	**17.21**	**8.23**

[†] Total volatile compounds detected in wine: 38; in vinegar: 34.

[ψ] Data of each retention time are correlated to fragmentation chromatogram, as shown in Figure 3.

acetification. Chebouat *et al.* [29] reported that ethyl acetate is active against either Gram-negative (*Escherichia coli*) or Gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) bacteria at a concentration of 1000 µg/l. However, Roy *et al.* [30] reported no antifungal ability against *Aspergillus niger*, *A. ochraceus*, and *Candida albicans*.

Acetic acid is a short-chained organic acid that is considered a weak acid; it disassociates incompletely in water. The antimicrobial activity of acetic acid occurs at the level of undissociated molecules. The undissociated molecules destroy microbial cells after passing readily through membranes, resulting in changes in membrane composition and enzyme inactivation [24]. Hexanoic acid has strong inhibitory effects on *Listeria monocytogenes* (10 strains) and *L. innocua* (2 strains) [31].

The results of monitoring the total phenolic content, antioxidant activity (DPPH), and GABA of GBR wine and GBR vinegar, in comparison with the original brown rice and GBR (control) (Table 3), showed that GBR had a total phenolic content of 50.7±2.8 mg GAE/ml, DPPH of 47.5±3.6% inhibition, and GABA of 124±0.07 mg/kg, which were higher than those of brown rice ($p \leq 0.05$). A significant reduction of these parameters in both GBR wine and GBR vinegar observed was due to the dilution effect. In GBR wine, slight increment in both total phenolic content and DPPH was found after 3-7 days of fermentation. These findings may be due to the effect of

extraction by the high concentration of ethanol produced when fermentation time increased. Moreover, no significant changes were found in the levels of GABA in GBR wine.

GABA analysis during the processing steps of GBR vinegar production (Table 3) showed different GABA levels ($p \leq 0.05$) among brown rice, GBR, and processed GBR (wine and vinegar). Moreover, significant detrimental changes in both wine and vinegar were observed. In GBR and processed GBR, the decreases were due to the effect of dilution during the preparation step of the fermentation medium in both wine and vinegar processes.

Table 3. Total phenolic content, DPPH and GABA of GBR during vinegar processing

Source	Total Phenolic Content† (mg GAE/ml)	DPPH† (% inhibition)	GABA† (mg/kg)
Brown rice (BR)	38.4±3.4 [b]	35.8±4.7 [b]	2.81±0.02 [d]
Germinated brown rice (GBR)	50.7±2.8 [a]	47.5±3.6 [a]	124±0.07 [a]
GBR wine:			
Day 0	8.7±1.2 [c]	10.4±0.8 [c]	28.9±0.12 [b]
Day 1	8.7±1.4 [c]	10.7±0.9 [c]	28.5±0.14 [b]
Day 3	10.1±1.6 [c]	11.7±1.2 [c]	28.9±0.16 [b]
Day 5	10.5±1.2 [c]	12.2±1.6 [c]	28.6±0.09 [b]
Day 7	10.8±1.7 [c]	13.7±1.9 [c]	28.9±0.08 [b]
GBR vinegar			
Cycle 1	8.8±1.2 [c]	9.2±0.6 [d]	14.1±0.09 [c]
Cycle 2	8.9±1.0 [c]	9.3±0.5 [d]	14.5±0.08 [c]
Cycle 3	8.9±0.6 [c]	9.5±0.9 [d]	14.3±0.14 [c]
Average (without start up)	8.9±0.9 [c]	9.3±0.7 [d]	14.3±0.1 [c]
Storage at 30±2°C			
2 months	9.6±1.3 [c]	12.4±1.3 [d]	14.5±0.6 [c]
4 months	9.9±1.9 [c]	15.2±1.7 [d]	14.7±0.9 [c]
6 months	10.6±1.4 [c]	18.8±1.9 [d]	14.7±0.7 [c]

† Mean ± one standard deviation; mean comparison is analyzed by Tukey's test; means with different letters within the same column were significantly different ($p \leq 0.05$).
Abbreviations: GABA, γ-aminobutyric acid; DPPH, 1-diphenyl-2-picryl hydrazyl radical assay

The long-term quality of GBR vinegar was investigated. Samples of GBR vinegar were kept at 30±2°C for 6 months. GABA, total phenolic content, and DPPH were monitored every 2 months. As shown in Table 3, no changes in GABA (14.3±0.1 to 14.7±0.7 mg/kg) were observed. These findings confirmed those of Di Cagno *et al*. [32], who also reported that GABA possesses resistance to acidity, thereby explaining why GABA could remain in the brewed vinegar during protracted storage at 30±2°C for 6 months. However, a slight increment in total phenolic content (from 8.9±0.9 to 10.6±1.4 mg GAE/ml) and DPPH (from 9.3±0.7 to 18.8±1.9% inhibition) was observed during storage at 30±2°C for 6 months. Interestingly, these results could be seen as supportive of claims of the benefits of GBR vinegar for consumer health.

4. Conclusions

The soaking process, which reduced the growth of microbial biota in brown rice, was the key to successful improvement of the nutritional properties, including total phenolic and GABA contents and antioxidant properties, of GBR. Well-prepared GBR is recommended as a valuable raw material for vinegar production. After testing, the nutritional level was maintained to some degree during the three-stage production of vinegar from GBR, but the levels of all three-stages were progressively reduced during germination, wine production, and vinegar production after the initial increase during germination. The production of vinegar via the three-stage production process produced a product with better health benefits and overcame contamination by microorganisms. During fermentation of wine and vinegar, 38 and 34 volatile organic compounds respectively were generated. Four major volatile compounds were common to GBR vinegar: acetic acid ethyl ester (or ethyl acetate), acetic acid, menthone, and hexanoic acid. All of these organic volatiles have previously been reported to have antimicrobial activities, which means they can be applied to GBR vinegar production. Individual major volatile compounds are maintained over the long term, thereby making GBR vinegar a healthy product with distinctive flavor and aroma, and antimicrobial activity.

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