

Antioxidant Activity of Alcoholic Beverages Made From Various Cereal Grains Using *Koji* Made with Thai *Amylomyces Rouxii* YTH3 as Saccharifying Agent

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Abstract

Microbial starter called *koji* in Japanese was made with fungal strain *Amylomyces rouxii* YTH3 isolated from Thai microbial starter, *loog pang*. *Koji* made with *A. rouxii* YTH3 was applicable for saccharifying agent for ethanol fermentation. Novel alcoholic beverage was made from various cereal grains and this *koji*, and analyzed. Ethanol concentration, pH and acidity were 8.2 to 10.8% (v/v), 3.5 to 4.4 and 3.0 to 5.2 ml. An alcoholic beverage made from black rice (*Oryza sativa* var. *Japonica* cv. *Shiun*) having anthocyanin showed brilliant red color. An alcoholic beverage made from black rice showed rather a higher antioxidant activity comparing other alcoholic beverage. The DPPH radical scavenging activity of the alcoholic beverage made from black rice was around 1800 μ M Trolox equivalent, while other alcoholic beverages were 300 to 600 μ M Trolox equivalent.

Keywords: *Amylomyces rouxii*, *loog pang*, black rice, alcoholic beverage, *koji*

1 Introduction

In Japan and other East Asian countries, a microbial starter called *koji* is prepared to brew alcoholic beverages and other fermented foods [1]. In Thailand, microbial starter called *loog pang* was also applied for production of traditional fermented beverage *sato*, *lao khao* and *khao mag* [2]. *Sato* is a kind of rice wine, *lao khao* is a distilled rice spirits and *khao mag* is a non-alcoholic sweet fermented rice. We have been trying to isolate and utilize microbial resources from the indigenous alcoholic beverages [3-6]. In this study, we tried to use *Amylomyces rouxii* YTH3 as the saccharifying agent for ethanol fermentation. We applied various cereal grain such as black rice, red rice, green rice, wild rice and polished white rice for the material for ethanol fermentation. Black rice and red rice had been cultivated in East Asian countries. In the ancient time, noble people in China and Japan ate black rice, thought to be having physical advantages. We tried to analyze and determine the characteristics of resulting alcoholic beverages made from various cereal grains to develop a novel alcoholic beverage using Thai microbial resources.

2 Experimental

2.1 Microorganisms

Amylomyces rouxii YTH3 isolated from Thai microbial starter, *loog pang* and maintained on the plate of PDA (Potato Dextrose Agar "Nissui", Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

This fungus was used for the preparation of *koji*. Industrial Japanese sake brewing yeast, *Saccharomyces cerevisiae* K7, purchased from the Brewing Society of Japan (Tokyo, Japan), was used as the fermentation yeast.

2.2 Cereal grains

Black rice (*Oryza sativa* var. *Japonica* cv. *Shiun*), Red rice (*Oryza sativa* var. *Japonica* cv. *Engimai*) and green rice (*Oryza sativa* var. *Japonica* cv. *Midorinoka*) were purchased from Kajiware Beikoku Co. Ltd, Kyoto, Japan (Figure 1). Wild rice (*Zizania aquatica*) was purchased from Suzusho Co. Ltd, Tokyo, Japan. The rice is a Native American's food, which is taxonomically different from other cereals at the genus level. Unpolished whole grain of those cereals was used for ethanol fermentation. A commercially polished non-glutinous rice grain (*O. sativa* var. *Japonica* cv. *Hinohikari*) was used for comparative study and was designated as polished white rice for convenience. White rice is eaten as a staple food in Japan, it is rice shaved about 10% outside the brown rice and contains only the starch portion. The varieties called *Hinohikari* used in this study is made in Miyazaki Prefecture and are widely eaten in Japan.

2.3 Chemicals

The compound DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Nacalai Tesque (Kyoto, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was

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purchased from Sigma-Aldrich Inc. (St Louis, Mo, USA) and BHT (2,6-di-tert-butyl-p-cresol) was purchased from Tokyo Kasei Co. Ltd (Tokyo, Japan).



Figure 1 Picture of polished white rice (upper left), black rice (upper middle), wild rice (upper right), green rice (bottom left) and red rice (bottom right).

2.4 Procedure for koji preparation

The procedure for preparing *A. rouxii* YTH3 koji is shown in Figure 2. One hundred grams of polished white rice grains and 100 ml of deionized water were dispensed into 1000 ml Erlenmeyer flask and autoclaved at 121°C for 15 min to prepare cooked rice. Cooked rice was cooled to ambient temperature. Then, 4 g of the *A. rouxii* YTH3 colony on the PDA plate was cut off using a sterile surgical knife, inoculated onto the cooked rice, and incubated at 30°C for 3 days to prepare the koji.

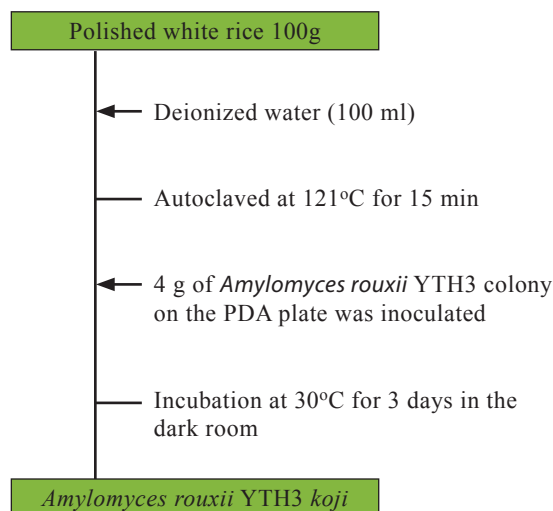


Figure 2 Procedure for preparation of *A. rouxii* YTH3 koji.

2.5 Determination of glucoamylase activity

Glucoamylase activity was assayed using the official analytical method of the National Tax Administration Agency of Japan [7]. One unit of glucoamylase activity was defined as the amount of enzyme that produced 1 mg of glucose in the rice koji in 60 min.

2.6 Ethanol fermentation procedure

Ethanol fermentation with cooking was performed according to the following procedure (Figure 3). Rice grains were ground to particles of 2 to 3 mm in diameter with an electric grinder. Twenty-seven grams, 21 g and 15 g of ground grains and 27 ml, 21 ml and 15 ml of deionized water were dispensed into a 300 ml Erlenmeyer flask and autoclaved at 121°C for 15min. After cooling to ambient temperature, the cooked rice was mixed with 3 g (10%), 9 g (30%) and 15 g (50%) of koji made with *A. rouxii* YTH3 as the saccharifying agent. Total amount of rice and koji in the initial mash was 30 g. Further 60 ml of deionized water, and 10 ml of *S. cerevisiae* K7 yeast suspension, which had previously brought the population of yeast in the initial mash to 3.0×10^7 cells/ml was added. Ethanol fermentation was conducted in the dark at 25°C. The decrease in the weight of the Erlenmeyer flask and its contents because of the evolution of the CO₂ gas was measured every 24 h.

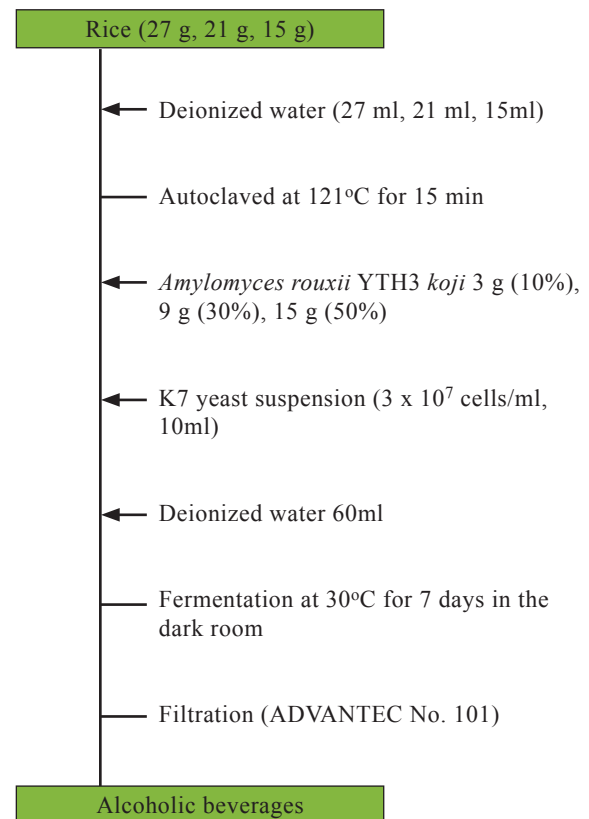


Figure 3 Procedure for ethanol fermentation made with *A. rouxii* YTH3 koji and K7 yeast.

2.7 General analytical methods

Fermented mash made from various cereal grains was centrifuged at 3000 rpm for 15 min and filtered through no. 101 filter paper (Advantec Toyo Co. Ltd, Tokyo, Japan), and the resulting alcoholic beverage was analyzed. Acidity was measured by titrating 10 mL of alcoholic beverage with 0.1M NaOH. The reducing sugar content, as glucose, was determined according to the methods of Nelson [8] and Somogyi [9]. The amount of total phenolic compounds, as gallic acid equivalent, was determined according to the Folin–Ciocalteu method [10–11]. The ethanol concentration of the alcoholic beverage was determined with a gas chromatograph (model GC-14A; Shimadzu Co., Kyoto, Japan) equipped with a 3.1m PEG-HT column (Gasukuro Kogyo, Inc., Tokyo, Japan).

2.8 Determination of antioxidative activity

The DPPH radical scavenging activity, as the Trolox equivalent, was measured on the basis of the method of Yamaguchi et al. [12]. The lipid peroxidation inhibitory activity, as the BHT equivalent, was determined using β -carotene [11].

3 Results and Discussion

Total amount of cooked rice grain and *koji* in the initial mash was 30 g. Fermentation curves of the mash containing 3 g (10%), 9 g (30%) and 15 g (50%) of *A. rouxii* YTH3 *koji* were

shown in Figures 4, 5 and 6, respectively. *Koji* made with *A. rouxii* YTH3 was applicable for saccharifying agent for ethanol fermentation. In making rice *koji*, we added 100 ml of deionized water to 100 g of white rice and made steamed rice. As the result of rice *koji* making using this steamed rice, it was possible to produce rice *koji* having glucoamylase activity of 250 u / g. It was also confirmed that the glucoamylase activity of this rice *koji* shows an activity equivalent to that of rice *koji* (*Aspergillus oryzae*) used for Japanese *sake* [7]. From the results that glucoamylase activity was decreased when steaming rice is made with low moisture content, the importance of moisture content in this rice *koji* making was also confirmed. The moisture content of steamed rice used for ordinary rice *koji* is 30 to 40%, and it is known that when the moisture content is high, the enzyme activity weakens [13].

The *A. rouxii* YTH3 used in this study suggested the possibility of exhibiting high enzyme activity in a relatively humid state. The mash composed of 30% or 50% *koji* and polished white rice, black rice, red rice and green rice contained 9.7 to 10.8% ethanol (Table 1). Ethanol concentration of the mash composed of wild rice and *koji* was lower. On the other hand, the mash composed of 10% *koji* and polished white rice, black rice, red rice and green rice contained 8.2 to 9.6 ethanol. This seems to be caused by the fact that the proportion of rice *koji* is small and it cannot be saccharified smoothly. It was also inferred that the amount of residual sugar in the mash was larger than that of other proportions (30 or 50% *koji*) and that the amount of carbon dioxide gas generation was small.

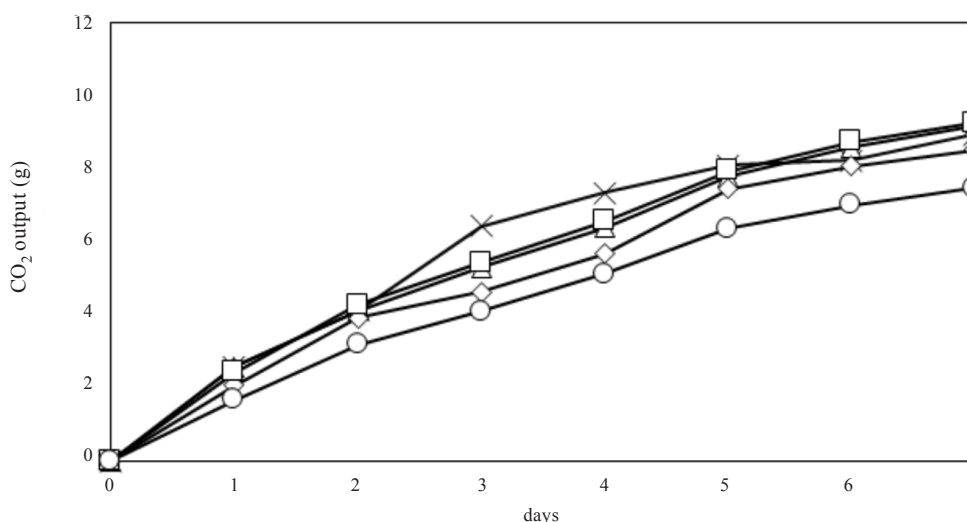


Figure 4 Time courses of fermentation of the mashes made from various grains using 3 g of *A. rouxii* YTH3 *koji* (10%) and K7 yeast. Symbols: x, polished white rice; Δ , black rice; $\diamond$, red rice; $\square$, green rice; $\circ$, wild rice. Values are the mean of triplicates.

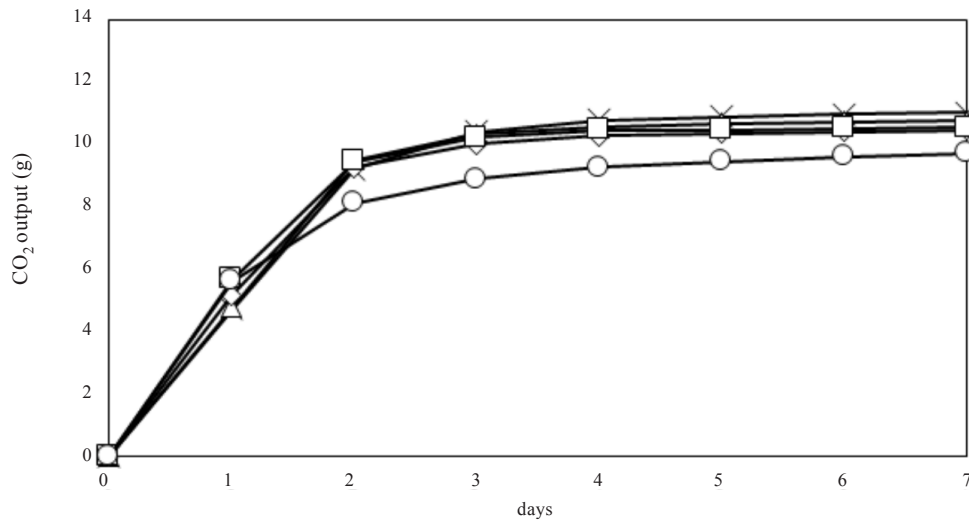


Figure 5 Time courses of fermentation of the mashes made from various grains using 9 g of *A. rouxii* YTH3 koji (30%) and K7 yeast. Symbols: x, polished white rice; Δ, black rice; ◇, red rice; □, green rice; ○, wild rice. Values are the mean of triplicates.

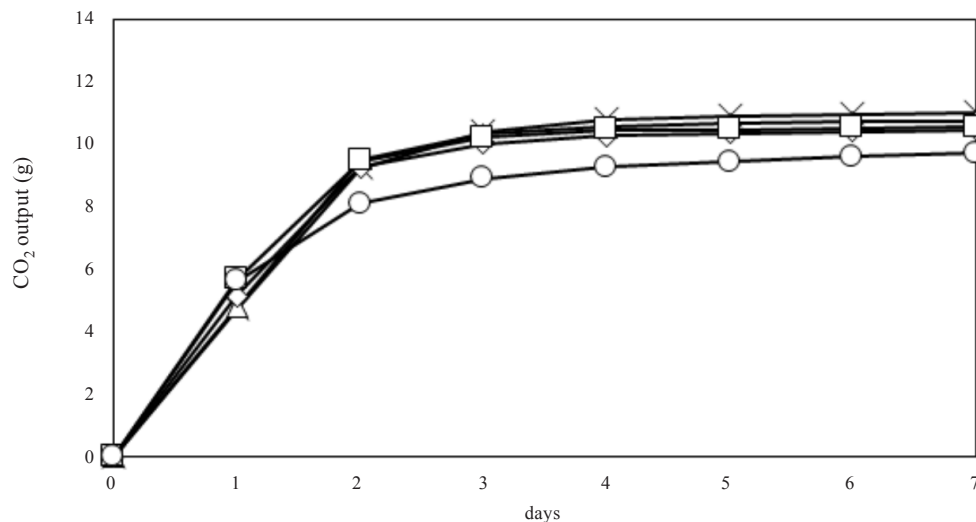


Figure 6 Time courses of fermentation of the mashes made from various grains using 15 g of *A. rouxii* YTH3 koji (50%) and K7 yeast. Symbols: x, polished white rice; Δ, black rice; ◇, red rice; □, green rice; ○, wild rice. Values are the mean of triplicates.

The alcoholic beverages made from polished white rice, red rice, green rice and wild rice showed pale color (Figs. 7, 9, 10, 11). The alcoholic beverage made from black rice grain showed red color (Figure 8) and contained 1.30 to 1.72 μg/ml anthocyanin as cyanidin-3-glucoside (Table 1). The color tones of the alcoholic beverage made from polished white rice and green rice were similar. Compared to these alcoholic beverage,

wild rice showed a somewhat dark yellow color. On the other hand, red rice became a slightly reddish from pale color as the proportion of rice *koji* increased. However, anthocyanins were not included. As the percentage of rice *koji* in black rice increased, the red color became thin, and it was confirmed that the content of anthocyanin decreased as can be seen from Table 1.

Table 1 Characteristics of alcoholic beverages made from various grains using *A. rouxii* YTH3 polished white rice *koji* and K7 yeast

	Polished white rice			Black rice			Red rice			Green rice			Wild rice		
	10%	30%	50%	10%	30%	50%	10%	30%	50%	10%	30%	50%	10%	30%	50%
Final pH	3.5	3.8	3.7	4.0	4.2	4.2	4.0	4.1	4.1	4.0	4.2	4.2	4.4	4.3	4.3
CO ₂ output (g)	6.9	10.5	10.8	7.3	10.2	9.1	6.9	9.8	10.3	7.5	10.1	10.4	5.9	8.5	9.3
Filtrate (ml)	70	75	77	68	71	75	59	68	70	68	69	70	49	60	67
Acidity (ml)	3.0	3.7	4.3	5.2	5.0	5.1	4.2	4.5	4.6	4.8	4.7	4.8	3.7	4.1	4.4
Ethanol concentration (% v/v)	9.3	10.8	10.6	8.2	9.9	10.0	9.0	9.7	10.2	9.6	10.0	10.2	8.5	9.2	8.9
Reducing sugar content (µg/ml, glucose eq.)	6591	310	334	5748	440	409	6049	344	332	6631	454	443	2364	559	525
Total phenolic compounds (µg/ml, GA equivalent)	115	234	308	485	521	502	252	321	366	237	332	386	306	382	420
Anthocyanin content (µg/ml, Cy3-glc eq.)	ND	ND	ND	172	15.0	13.0	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values are the means of three replicates

ND, Not detected

Phenolic compounds have been found in various colored rice, and it has been reported that there is a positive correlation between DPPH radical scavenging activity and total phenolic compounds content [14]. In the alcoholic beverage made in this study, as the proportion of rice *koji* became higher, the amount of phenolic compound content also increased (Figure 12), but the DPPH radical scavenging activity of the alcoholic beverage did not change, conversely, it decreased in the alcoholic beverage made from black rice (Figure 13). The alcoholic beverage made in this study contains phenolic compounds derived from rice bran or rice *koji*, and it seems that various types of phenolic compounds are present. In this study, no positive correlation was found between phenolic

compound and DPPH radical scavenging ability. This may be due to the difference in DPPH radical scavenging activity depending on the type of phenolic compound. With respect to black rice, as the proportion of rice *koji* increased, there was no change in the amount of phenolic compound content, but a decrease in DPPH radical scavenging ability was observed. This may be due to a decrease in anthocyanin content. From now on, it seems necessary to investigate the relationship between phenolic compound type of various colored rice and DPPH radical scavenging activity. On the other hand, no significant difference was observed in all the alcoholic beverage as to the lipid peroxidation inhibition activity (Figure 14).

**Figure 7** Picture of alcoholic beverages made from polished white rice using *A. rouxii* YTH3 *koji* and K7 yeast. From left to right; alcoholic beverage made with 3 g (10%), 9 g (30%) and 15 g (50%) of *koji*.

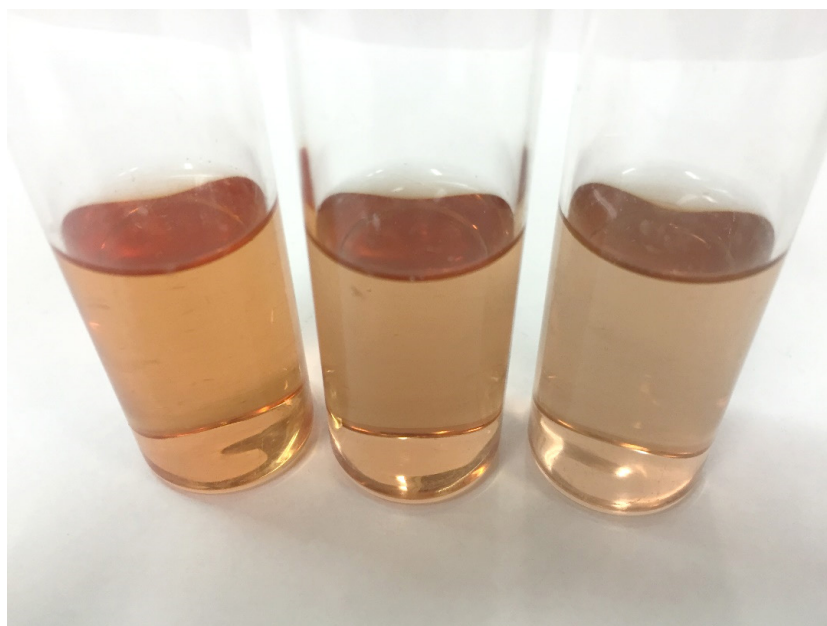


Figure 8 Picture of alcoholic beverages made from black rice using *A. rouxii* YTH3 *koji* and K7 yeast. From right left to left right; alcoholic beverage made with 3 g (10%), 9 g (30%) and 15 g (50%) of *koji*.

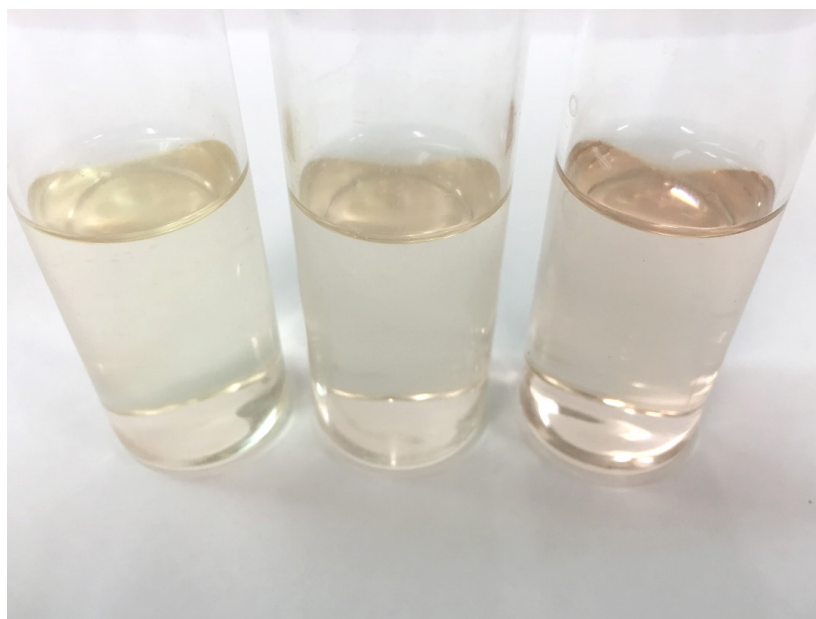


Figure 9 Picture of alcoholic beverages made from red rice using *A. rouxii* YTH3 *koji* and K7 yeast. From right left to left right; alcoholic beverage made with 3 g (10%), 9 g (30%) and 15 g (50%) of *koji*.



Figure 10 Picture of alcoholic beverages made from green rice using *A. rouxii* YTH3 *koji* and K7 yeast. From right left to left right; alcoholic beverage made with 3 g (10%), 9 g (30%) and 15 g (50%) of *koji*.



Figure 11 Picture of alcoholic beverages made from wild rice using *A. rouxii* YTH3 *koji* and K7 yeast. From right to left; alcoholic beverage made with 3 g (10%), 9 g (30%) and 15 g (50%) of *koji*.

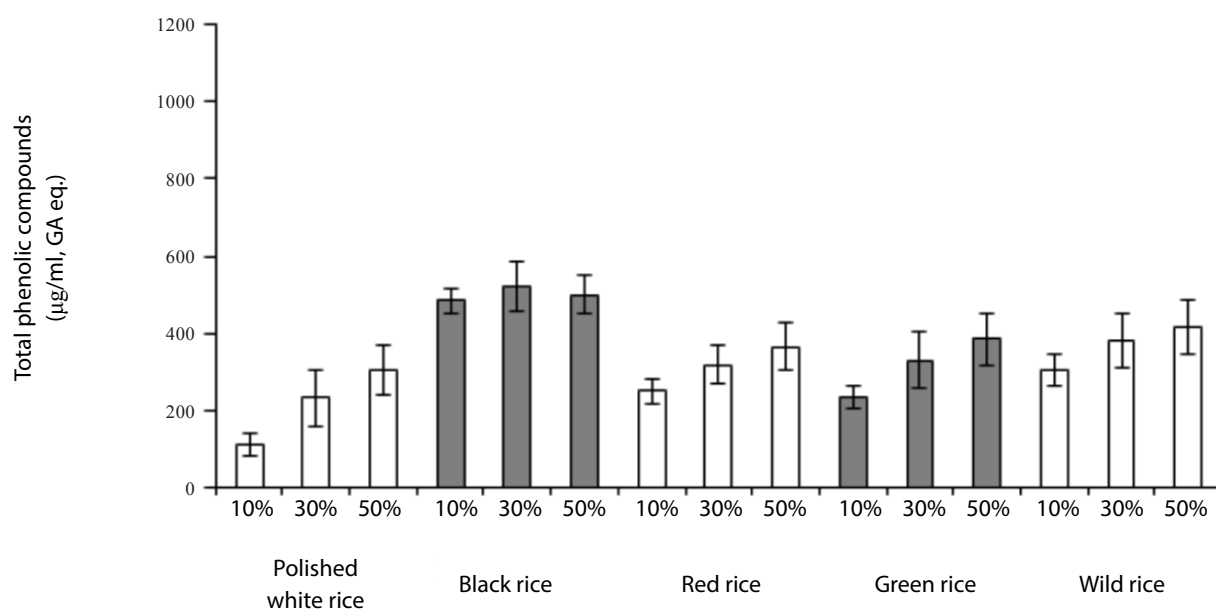


Figure 12 Total phenolic compounds of alcoholic beverages made from various grains using *A. rouxii* YTH3 koji and K7 yeast. Each value is the mean $\pm$ S.D. (n=3).

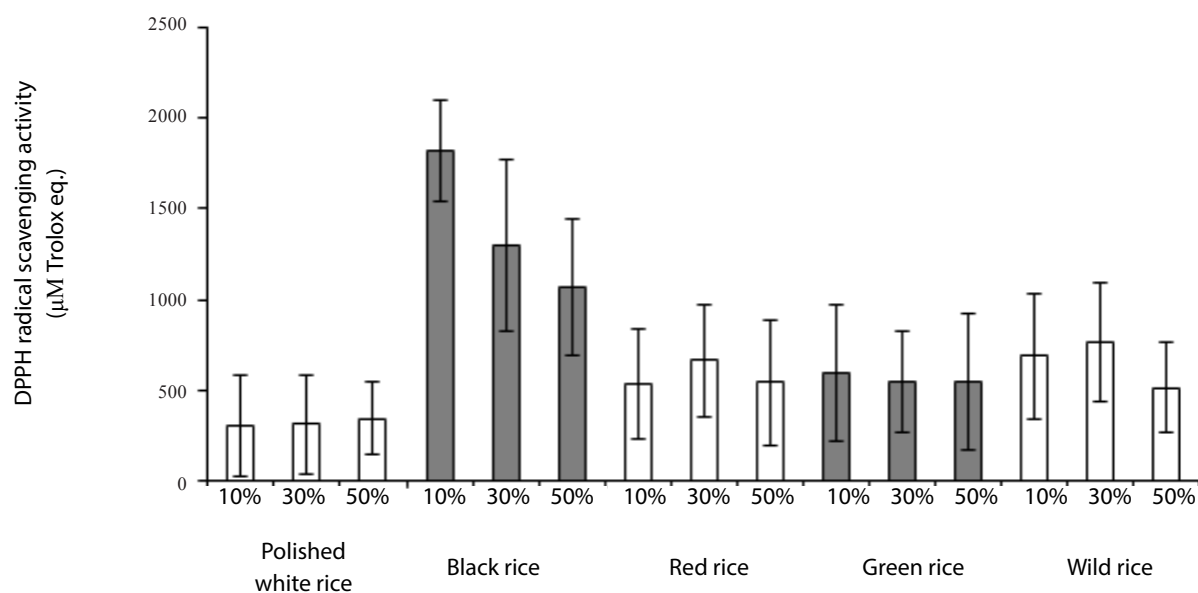


Figure 13 DPPH radical scavenging activity of alcoholic beverages made from various grains using *A. rouxii* YTH3 koji and K7 yeast. Each value is the mean $\pm$ S.D. (n=3).

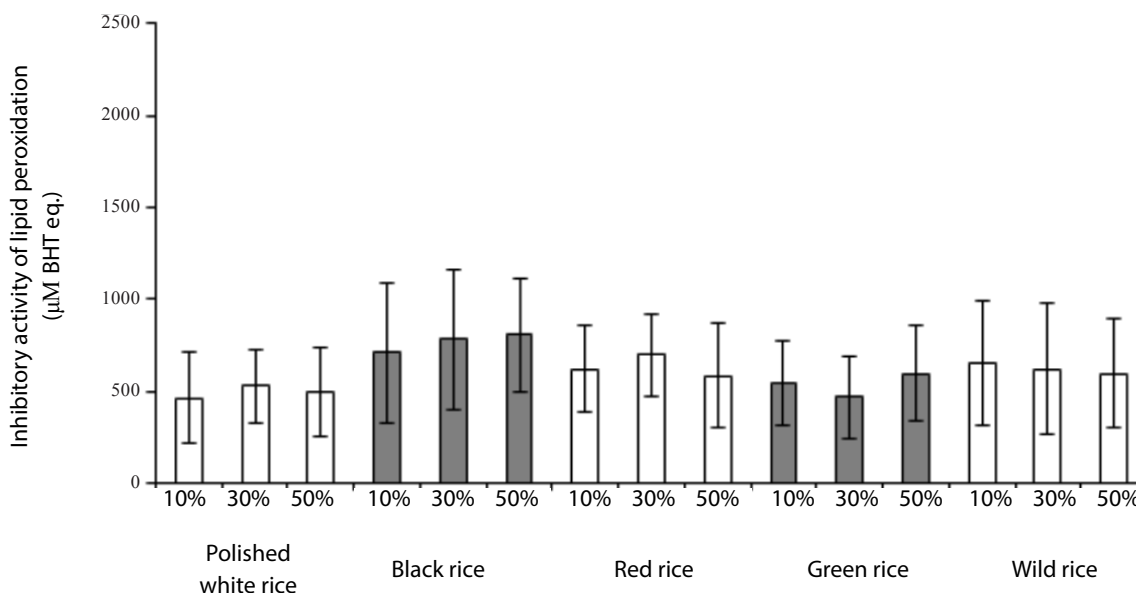


Figure 14 Inhibitory activity of lipid peroxidation of alcoholic beverages made from various grain using *A. rouxii* YTH3 *koji* and K7 yeast. Each value is the mean $\pm$ S.D. (n=3).

4 Conclusion

Amylomyces rouxii YTH3 isolated from Thai microbial starter, *loog pang* was applicable for preparation of *koji*. We investigated the possibility of new alcoholic beverage using *A. rouxii* widely used in fermented food of Thailand. Especially in this study, we tried using various colored rice as fermentation raw materials. A positive correlation has been reported between the phenolic compound content of colored rice and DPPH radical scavenging ability, but no positive correlation was found in this alcoholic beverage. One possible reason is that colored rice pigment was adsorbed to the fermentation residue and extraction of phenolic compound derived from colored rice did not work well. However, *A. rouxii* was applied to rice *koji* and expectation was given in the production of alcoholic beverage. From now on, it is necessary to investigate the production method which can efficiently extract the coloring matter of colored rice and can effectively use it for color and functionality of fermented wine. We would like to improve the quality of alcoholic beverage made by *A. rouxii* YTH3 *koji* rice to produce a novel alcoholic beverage having antioxidant activity.

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