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Antioxidant, Antibacterial, Tyrosinase Inhibitory, and Biofilm Inhibitory Activities of Fermented Rice Bran Broth with Effective Microorganisms

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Abstract The effective microorganism fermentation extract (EM-X) is a refreshment beverage widely consumed in East Asia. Due to the antioxidant property of the health beverage, EM-X has potential benefits for the human immune system and has been generally accepted in clinical practice. In search of new functions of EM-X, another version of EM-X, named EM-YU in this study, which is fermented from rice bran, seaweed, and kiwifruit with effective microorganisms demonstrates high antioxidant, antibacterial, tyrosinase inhibition activities, and biofilm inhibition activity of pathogenic bacteria. The antioxidant activity of 1.8% EM-YU was equivalent to that of 0.01 mg/mL vitamin C. Also, EM-YU clearly inhibited the cell growth of two pathogenic bacteria, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* PAO1; and EM-YU broth (6.7%) inhibited (55 ± 1%) the activity of mushroom tyrosinase. Moreover, the ethyl acetate extract of EM-YU, called EM-YU-EX (0.6 mg/mL), 7-fold reduced the biofilm formation of *E. coli* O157:H7 without affecting its cell growth. It is the first report that EM-X variant possess antibacterial, tyrosinase inhibitory, and biofilm inhibitory activities. These results support other beneficial properties of the natural health product EM-X variant (EM-YU). © KSBB

Keywords: rice bran, effective microorganisms, antioxidant, antibacterial, tyrosinase, biofilm

INTRODUCTION

Rice bran is one of the most abundant byproducts in the rice milling process. Rice bran contains lots of proteins, minerals, amino acids [1], lipase [2], and various antioxidant compounds, such as polyphenols [3], ferulic acids [3], γ-oryzanols [4], tocopherols, and tocotrienols [5]. Due to the antioxidant ability inhibiting the production of reactive oxygen species, rice bran has recently obtained much attention as a natural source of health food related to diabetes [6], atherosclerosis [7], and cancer [8].

Among rice bran derived health foods, the effective microorganism fermentation extract (EM-X) is a refreshment drink widely consumed in East Asia, especially Japan. EM-X is produced by fermentation of rice bran, papaya, and seaweed (kelp) with effective microorganisms of more than one hundred bacterial strains including lactobacillaceae, saccharomycetes, funguses, actinomyces, and photosynthetic bacteria [9]. Since EM-X contains many health beneficial compounds such as γ-tocopherol, lycopene, ubiquinone, saponin, and flavonoids, it has been reported that EM-X may enhance immune system related to inflammation [10], asthma [10], osteoporosis [9], Parkinson's disease [11], and cancer [12], and also there is no toxicity of EM-X in animal models [13].

In search of new functions of the fermented rice bran broth with effective microorganisms, we noticed that several fermented plant beverages showed antibacterial activities [14] and that the ethyl acetate extract from black rice bran contained tyrosinase inhibitors [15]. Tyrosinase (EC 1.14.18.1) is the key enzyme in melanin production [16] and a tyrosinase inhibitor has potential as a skin-whitening agent in the cosmetic industry [17].

Various pathogenic bacteria, such as *E. coli* O157:H7 and *P. aeruginosa* PAO1, can form biofilms (sessile communities), and pose serious problems to human health such as food borne diseases and lung diseases (cystic fibrosis). Currently, there is explosive amount of biofilm research, most of

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it with the ultimate aims of biofilm prevention and control [18]. Since plants have advanced defense mechanisms against pathogenic bacteria, we hypothesized that EM-X was a potential source of biofilm inhibitors.

In this study, we generated another version of EM-X, called EM-YU, using local plant sources with the same effective microorganisms due to the unavailability of active plant sources. It is investigated here that EM-YU has antioxidant, antibacterial, tyrosinase inhibitory, and biofilm inhibitory activities.

MATERIALS AND METHODS

Materials and Chemicals

Rice bran from white rice (*Oryza sativa L. ssp. Japonica*) was obtained from a mill (Gyeongsan, Korea). Kiwifruit (*Actinidia deliciosa)* and seaweed (*Laminaria Japonica*) were obtained in a local market (Kyeongsan, Korea). EM was purchased from Korean EM Research Organization (Pusan, Korea). In order to concentrate EM-YU, ethyl acetate was used and purchased from Duksan Pharmaceutical (Ansan, Korea). Dimethyl sulfoxide (DMSO), sodium phosphate dibasic (Na_2HPO4), sodium phosphate monobasic ($NaH_2PO_4{\cdot}2H_2O$), and L-ascorbic acid (vitamin C) were purchased from Duksan Pure Chemical (Ansan, Korea). DL-α-tocopherol (vitamin E) was purchased from Kanto Chemical (Tokyo, Japan). 2,4,6-tris(2-pyridyl)-s-triazine was obtained from Acros Organics. (NJ, USA). L-tyrosine and mushroom tyrosinase were purchased from Sigma (St. Louis, MO, USA).

Production of New Version (EM-YU) of EM-X

The production of fermented rice bran broth with effective microorganisms was modified from the previous study [19] using rice bran, kiwifruit, seaweed, and EM. Rice bran, kiwifruit, and seaweed were grinded with an electric blender, filtered, and mixed with EM and water in the total volume of 1 L. In order to obtain the most active EM-YU for the antioxidant activity, the ratio of rice bran, kiwifruit, seaweed, and EM was determined by the design of orthogonal experiment [20]. After incubation for 7 days without shaking in an incubator at 35°C, the fermentation broth was filtered with a cellulose nitrate membrane (0.45 μm, Whatman International, Maidstone, England).

Concentration of EM-YU with Ethyl Acetate

In order to concentrate EM-YU, ethyl acetate extraction was used since the ethyl acetate fraction from black rice bran showed the highest tyrosinase inhibitory effect [15]. Ethyl acetate was mixed with EM-YU at equal volume and shaken in a separating funnel for 20 min. The procedure was repeated 3 times and the 3 extracts were mixed together, and concentrated with a rotary evaporator under vacuum. Brown oily extract was named as EM-YU-EX. The concentrated EM-YU-EX was diluted with DMSO to obtain a desired concentration.

Antioxidant Assay

For the antioxidant assay, the ferric reducing/antioxidant power (FRAP) assay was adapted [21]. Briefly, the acetate buffer (300 mM, pH 3.6), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (10 mM), HCl (40 mM), $FeCl_3{\cdot}6H_2O$ (20 mM), and water were mixed together in the volume ratio of 10:1:1:1.2, respectively, to make the FRAP reagent. Various samples of total 30 μL were added to the FRAP reagent (1 mL). After incubation for 4 min at 37°C, optical density was measured at 593 nm. As positive controls, vitamin C and E were used. As negative controls, water and DMSO were used. The relative activity of samples was compared with the activity of vitamin C and E. At least 2 independent experiments were performed.

Antibacterial Assay

The paper disc diffusion method [22] was used to test EM-YU and EM-YU-EX for the antibacterial activity. Two pathogenic bacteria, *E. coli* O157:H7 [23] and *P. aeruginosa* PAO1 [24] used in this study, were the sequenced strains. Overnight cultures were re-grown to an optical density at 600 nm of 1.0 and about 10^4cells/plate were spread on LB agar (Becton, Dickinson and Company, Sparks, USA). EM-YU (100 μL) and 30 mg/mL EM-YU-EX (100 μL) was placed on a paper disc (8 mm diameter, Tokyo Roshi Kaisha, Tokyo, Japan). Plates were incubated at 37°C for 18 h and the antibacterial activities of samples were evaluated by measuring a clear zone. Kanamycin (0.3 and 3 mg/mL) was used as a positive control, and HCl (pH 3.5) and DMSO were used as negative controls. Three independent experiments were performed.

Tyrosinase Inhibition Assay

Using the mushroom tyrosinase (Sigma), tyrosinase activity was determined spectrophotometrically [17]. Briefly, an assay reaction mixture was prepared by adding 60 μL sample (dissolved in DMSO), 120 μL L-tyrosine (1.5 mM), and 660 μL sodium phosphate buffer (pH 6.5), and finally adding 60 μL fresh mushroom tyrosinase (2.2 U/μL). The mixture was incubated for 10 min at 37°C and the absorbance was measured at 475 nm. DMSO was used as a control. The activity of tyrosinase with samples was compared to the control sample that contained no inhibitory compound and showed 100% enzyme activity. Vitamin C was used as a positive control. At least 2 independent experiments were performed.

Crystal-violet Biofilm Assay

A static biofilm formation assay was performed in 96-well polystyrene plates as previously reported [25]. Overnight cultures were inoculated with an initial turbidity at 600 nm of 0.05 for 8 h without shaking at 37°C. EM-YU-EX and

two controls (vitamin C and E) were dissolved with DMSO. DMSO (0.2 vol%) was added as a negative control. Cell growth and total biofilm were measured using crystal violet staining. Each data point was averaged from at least 12 replicate wells (6 wells from each of 2 independent cultures).

RESULTS AND DISCUSSION

The goal of this research was to identify new functions of fermented rice bran broth (EM-YU) that was generated in this study. Since the original EM-X has a high antioxidant activity, EM-YU was evaluated first with respect to the antioxidant activity. Then, antibacterial, tyrosinase inhibitory, and biofilm inhibitory activities were investigated.

Production of EM-YU and Concentrated EM-YU Extract

Since the active plant sources used in the production of EM-X [26] was not locally available, we generated a new version of EM-X. Fermentation conditions were optimized by evaluating the antioxidant activity of fermented rice bran broth. Initially, various amounts of rice bran (75, 100, 125, 150, and 175 g/L), different fermentation temperature (25, 30, 35, and 40°C), and culture time (0, 3, 7, and 14 days) were investigated using a single factor design. As a result, the highest antioxidant activity was obtained at 125 g/L rice bran, 35°C, and 7 days (data not shown). Unlike the previous study [26], since local papaya resulted in a low antioxidant activity, kiwifruit was used due to the abundance of vitamins, and since the seaweed (kelp, *Macrocystis*) was not locally available, *Laminaria Japonica* (another seaweed) was used in this study. Using the design of orthogonal experiment, the amounts of rice bran (125, 175, and 225 g/L), kiwifruit (125, 250, and 375 g/L), seaweed (25, 75, and 125 g/L), and EM (25, 75, and 125 mL/L) were evaluated at 3 variation levels. Out of total 9 combinatorial conditions, the condition with 125 g/L rice bran, 375 g/L kiwifruit, 25 g/L seaweed, and 75 mL/L EM led to the highest antioxidant activity (data not shown). After the extraction and evaporation, 8.15 g of ethyl acetate extract, called EM-YU-EX, was obtained from 1 L of EM-YU. As a control sample, same amount of raw materials without EM fermentation resulted in only 0.62 g of ethyl acetate extract. Therefore, fermentation with EM produced 13-fold more ethyl acetate extract than raw substrates.

Antioxidant Activity of EM-YU and EM-YU-EX

Antioxidant ability of EM-YU and EM-YU-EX was measured with the FRAP assay. As expected, EM-YU demonstrated a high antioxidant activity in dose response manner (0~3%) and 1.8% EM-YU almost reached at a maximal measurement of activity (Fig. 1A). Raw materials without EM fermentation also showed about 20% lower activity than that of EM-YU probably due to the abundance of vitamins in kiwifruit. Also, the ethyl acetate extract of EM-YU, called EM-YU-EX, showed a high antioxidant activity in dose response manner (0~0.4 mg/mL) (Fig. 1B). In comparison

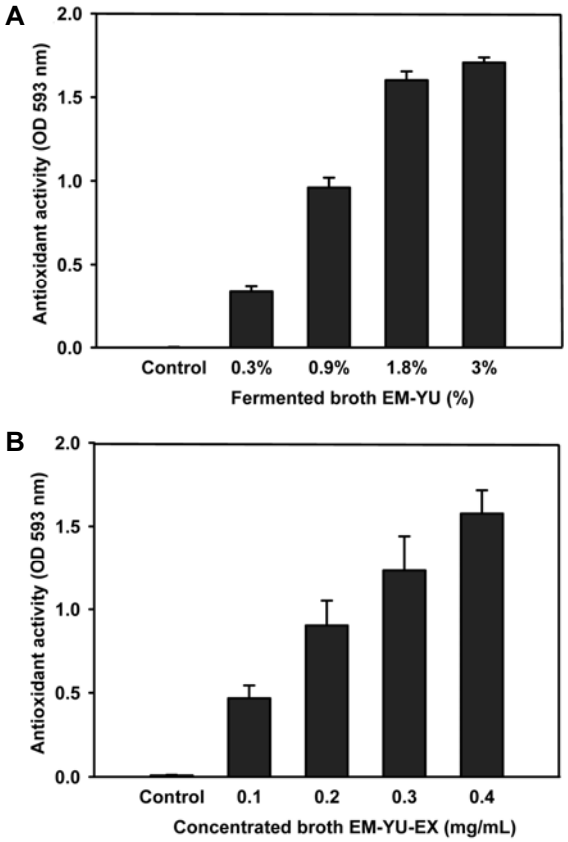

Fig. 1. (A) Antioxidant activity of fermented rice bran broth EM-YU and (B) concentrated broth EM-YU-EX. Assay of ferric reducing/antioxidant power (FRAP) was performed and activity was measured at 593 nm. EM-YU was diluted with water to make a final concentrations of 0, 0.3, 0.9, 1.8, and 3% (v/v) in the FRAP reagent. EM-YU-EX was diluted with DMSO and DMSO (100% v/v) was used as a negative control. The experiments were repeated at least twice and error bars represent one standard deviation.

with two positive controls, the activity of 1.8% EM-YU was equivalent to that of 0.01 mg/mL vitamin C and 0.017 mg/mL vitamin E, respectively. Also the antioxidant activity of 0.3 mg/mL EM-YU-EX was equivalent to the activity of 0.007 mg/mL vitamin C. Our results support well the previous studies on the antioxidant activity of EM-X [26,27]. Since the antioxidant activity of EM-X was associated to flavonoids, saponins, ubiquinones, lycopene, and vitamin E in EM-X [26,27], the antioxidant ability of EM-YU was probably due to the presence of these compounds. Hence, in agreement of previous results of EM-X [26,27], EM-YU has a potential as an antioxidant drink.

Antibacterial Activity of EM-YU and EM-YU-EX

In order to assess the antibacterial activity of EM-YU and EM-YU-EX, the paper disc diffusion method [22] was used.

Table 1. Antibacterial activity of fermented rice bran broth EM-YU and EM-YU-EX

Samples	Clear zone with *E. coli* O157:H7	Clear zone with *P. aeruginosa* PAO1
HCl (pH 3.5)	0	0
DMSO (10% v/v)	0	0
Kanamycin (0.3 mg/mL)	24 ± 2 mm	0
Kanamycin (3.0 mg/mL)	34 ± 2 mm	25 ± 1 mm
Substrates without EM fermentation (pH 4.7)	0	0
EM-YU (pH 3.5)	23 ± 2 mm	25 ± 1 mm
EM-YU-EX (30 mg/mL)	24 ± 1 mm	26 ± 2 mm

EM-YU-EX was diluted with DMSO (10 vol%). DMSO (10 vol%) and kanamycin (0.3 and 3 mg/mL) were used as a negative control and a positive control. Hundred microliter of substrates, EM-YU and EM-YU-EX (30 mg/mL) were placed on the paper disc. LB agar plates were incubated at 37°C for 18 h. The experiments were repeated 3 times and errors represent one standard deviation.

Since the effective microorganisms do not include pathogenic bacteria, *E. coli* O157:H7 [12], and *P. aeruginosa* PAO1 were chosen. The growth of *E. coli* O157:H7 was clearly inhibited by EM-YU broth (100 μL) and EM-YU-EX (100 μL of 30 mg/mL) with 23 ± 2 and 24 ± 1 mm clear zone for 18 h at 37°C, respectively, as the positive control (3 mg/mL kanamycin) resulted in 34 ± 2 mm clear zone (Table 1). Also, the growth of *P. aeruginosa* PAO1 was inhibited by EM-YU broth (100 μL) and EM-YU-EX (100 μL of 30 mg/mL) with 25 ± 1 and 26 ± 2 mm clear zone for 18 h at 37°C, respectively, and the control (3 mg/mL kanamycin) resulted in 25 ± 1 mm clear zone (Table 1). Results suggest that *P. aeruginosa* PAO1 is more sensitive to EM-YU than *E. coli* O157:H7.

It has been suggested that main contributing factors of antibacterial activity of fermented plant beverages are low pH due to organic acidic compounds and the production of ethanol and bacteriocins from the fermentation with lactic acid bacteria [28]. Since the effective microorganisms include lactic acid bacteria, pH of EM-YU was measured and pH of EM-YU was 3.5. However, a solution at pH 3.5 adjusted with HCl did not show antibacterial activity of *E. coli* O157:H7 and *P. aeruginosa* PAO1 on agar disc plate (Table 1) although cell growth was delayed in the LB liquid medium at low pH (data not shown), which indicated that pH was not a sole factor of antibacterial activity of EM-YU. Additionally, the raw substrates without EM fermentation did not show any antibacterial activity (Table 1). It was reported that the antibacterial activity of polyphenols produced from seaweeds (*G. acerosa* and *Haligra sps*.) [29]. Taken together, the antibacterial activity of EM-YU was likely due to the combination of organic acids, polyphenols, and other unknown compounds extracted from the fermentation with EM.

Table 2. Tyrosinase inhibition of fermented rice bran broth EM-YU and EM-YU-EX

Samples	Inhibition of tyrosinase (%)
Vitamin C (0.052 mg/mL)	50
Vitamin E (1.5 mg/mL)	0
Substrates without EM fermentation (6.7 vol%)	0
EM-YU (6.7 vol%)	55 ± 1
EM-YU-EX (0.99 mg/mL)	9 ± 2
EM-YU-EX (1.65 mg/mL)	34 ± 3
EM-YU-EX (1.98 mg/mL)	61 ± 2

Mushroom tyrosinase was used to determine the inhibition of tyrosinase activity. EM-YU-EX was diluted with DMSO (100 vol%) and DMSO was used as the control. The activity of tyrosinase without inhibition compound was defined as 100% enzyme activity. The experiments were repeated at least twice and errors represent one standard deviation.

Tyrosinase Inhibition Activity of EM-YU and EM-YU-EX

Tyrosinase inhibitory compounds have a commercial potential as skin-whitening agents [30]. In this study, the tyrosinase inhibition of EM-YU and EM-YU-EX was examined using the mushroom tyrosinase as previously utilized [17]. As a result, EM-YU broth (6.7 vol%) inhibited (55 ± 1%) the activity of tyrosinase, while raw substrates without EM fermentation showed no inhibition activity at all (Table 2). Also, EM-YU-EX inhibited the activity of tyrosinase in dose response manner (0~1.98 mg/mL) and IC_{50} of EM-YU-EX was estimated at 1.49 mg/mL (Table 2). As the positive control, IC_{50} of vitamin C was 0.052 mg/mL, which value was similar to the previous result [17] while vitamin E (1.5 mg/mL) had no activity of tyrosinase inhibition (Table 2). It is notable that EM-YU itself has a high inhibition activity and IC_{50} of EM-YU-EX is relatively high compared to vitamin C, which indicates that the ethyl acetate extract (EM-YU-EX) lost a large portion of tyrosinase inhibitors during the extraction process.

Rice bran contained the tyrosinase inhibitory compounds, protocatechuic acid [15] and phenolic compounds [31], and EM-X included kaempferol, a kind of flavonoids [27] and it was confirmed that kaempferol showed the tyrosinase inhibitory activity [32]. Therefore, the tyrosinase inhibitory activity of EM-YU is possibly due to the presence of protocatechuic acid, phenolic compounds, flavonoids, and unknown compounds from the fermentation with effective microorganisms.

Biofilm Inhibition Activity of EM-YU-EX

Since EM-YU broth and EM-YU-EX (30 mg/mL) showed the antibacterial activity (Table 1), it was required first to test the toxicity of EM-YU in order to demonstrate that the biofilm inhibitory activity of EM-YU was not due to the antibacterial activity of EM-YU. Using the concentrated

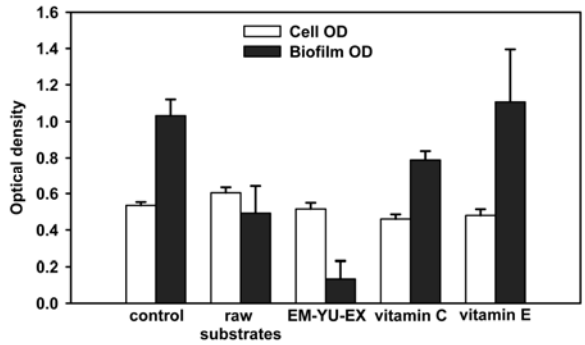

Fig. 2. Biofilm formation of *E. coli* O157:H7 in LB at 37°C after 8 h in 96-well plates with raw substrates (extract of raw materials without EM fermentation), EM-YU-EX, vitamin C, and vitamin E at 0.6 mg/mL. Each experiment was repeated 2 times with 6 wells each and one standard deviation is shown. EM-YU-EX was diluted with DMSO and DMSO (0.2 vol%) was used as the control.

EM-YU-EX, cell growth was measured during biofilm formation in 96-well plates. Like vitamin C and E (0.6 mg/mL), EM-YU-EX (0.6 mg/mL) did not decrease the cell growth of *E. coli* O157:H7 (Fig. 2). However, biofilm formation of *E. coli* O157:H7 was clearly inhibited (7-fold) by EM-YU-EX, whereas biofilm formation was only 2-fold decreased by the extract of raw substrates (note 13-fold less extraction yield compared to EM-YU-EX) and not affected by vitamin C and E (Fig. 2). Unlike the biofilm formation of *E. coli* O157:H7, the biofilm formation of *P. aeruginosa* PAO1 was not decreased by EM-YU and EM-YU-EX (data not shown). The results suggest that EM-YU-EX contains some biofilm inhibitor(s) that does not closely relate to the antibacterial activity. It has been reported that the furanone from the red micro-algae (seaweed), *Delisea pulchra*, inhibits biofilm formation of *E. coli* [33] and plants have developed many advanced defense mechanisms against bacteria. Hence, it is possible that EM-YU-EX contains furanone-like chemical and its derivatives during the fermentation since EM-YU was originated from the fermentation with seaweed *Laminaria Japonica*.

CONCLUSION

The potential of refreshment drink EM-X for clinical application has been validated by several studies and the active mechanism of EM-X has been started to be revealed [10,12]. In this study, a new version of EM-X (EM-YU) was generated with local plant sources. It is the first report that EM-X variant (EM-YU) possess other important properties, such as antibacterial, tyrosinase inhibitory, and biofilm inhibitory activities, in addition to the antioxidant activity. Additionally, the fermentation with EM produced 13-fold more ethyl acetate extract (EM-YU-EX) than raw substrates without fermentation. The result clearly indicates another advantage of fermentation with EM. Although the molecular mechanisms on these functions remain unknown due to the complexity of EM-YU, the preliminary results in this study indicate additional benefits of EM-YU. Taken together, EM-YU could be used as an antioxidant drink, a food additive for milk, a skin-whitening agent, and an antibacterial agent for the prevention of pathogenic bacteria contamination. Additionally, it is interesting to identify, which compounds are responsible for these properties and how the compounds work to the pathogenic bacteria.

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