APPENDICES

APPENDIX A

Picture

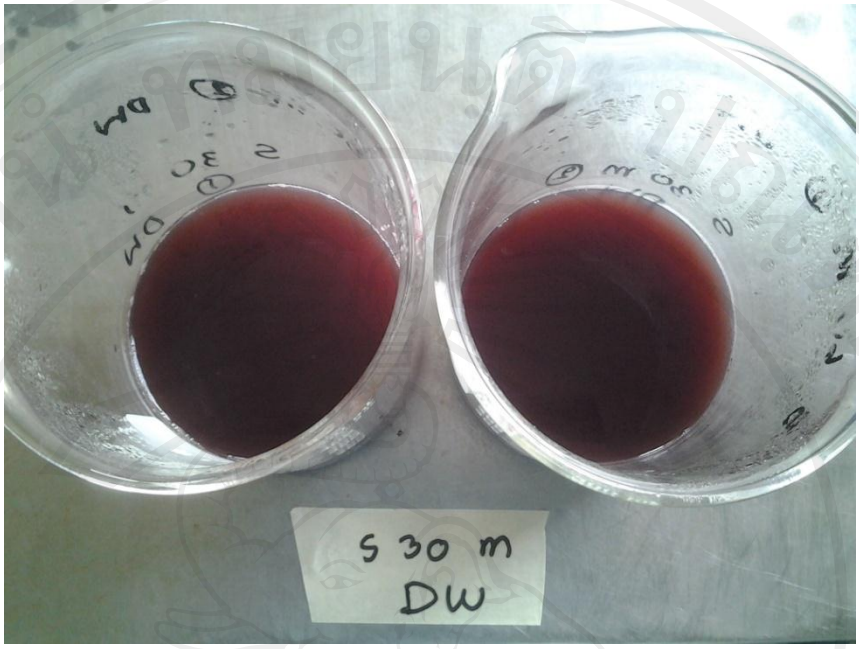

Figure A.1 Black glutinous rice solution from rice particles 595 µm before acetic acid addition

Figure A.2 Fermented black glutinous rice drink

APPENDIX B

Physical analysis

Viscosity analysis

Viscosity of black glutinous rice solution and fermented rice drink was measured using a viscometer. An amount of 500 ml black glutinous rice liquid was poured into a cylinder and placed in the correct position under the viscometer equipment. The black glutinous rice solution was measured using a viscometer speed at 50 rpm (10-70%T) and a probe head of number 6. Measurements were conducted at room temperature.

Color analysis

Color evaluation was performed on black glutinous rice solution and fermented rice drink using a colorimeter, Minolta Chroma Meter, Japan. The instrument was calibrated with a white tile. The Hunter L*, a*, and b* scales gave a measurement of color in units of approximate visual uniformity throughout a liquid. L* value represented the lightness of color, a* value represented the greenness and redness and b* value represented the blueness and yellowness.

- L* value measures lightness and varies from 100 for perfect white to zero for black.
- a* value measures redness when positive (+) and greenness when negative (-) with maximum values of 60.
- b* value measures yellowness when positive (+) and blueness when negative (-) with maximum values of 60.

Each value represented a mean value of three determinations for each sample.

APPENDIX C

Proximate analysis

Moisture content (a method from AOAC, 2000)

Weight 3 g of black glutinous rice solution into a moisture can with a tight-fit cover, which was known accurately the weight. Place loosely the covered can in a hot air oven at 100±2°C for 4 h. After the drying time, place the can cover tightly into the moisture can, then remove them from the oven, cool in an active dessicator and weight. Express the loss weight as a moisture content of black glutinous rice solution.

$$\%\textbf{Moisture content} = \frac{(a-b)}{c} \times 100$$

a = weight of can and sample before drying in an oven (g)

b = weight of can and sample after drying in an oven (g)

c = weight of sample (g)

Fat in black glutinous rice solution by Rose-Gottlieb method (a method from AOAC, 2000)

Weight 1 g black glutinous rice solution into a separatory funnel. Add 1 ml NH_4OH and mix thoroughly. Add with 10 ml ethyl alcohol 95% and mix well. Next, add with 25 ml diethyl ether that must be peroxide-free, and then close with a stopper, and shake very vigorously for 1 min. After that, add with 25 ml petroleum ether which has a boiling point range of 40-60°C and repeat vigorous shaking for 30 s. Let the funnel stand until the liquid is separated into two layers. Upper liquid is separated into a flask that is dried and known accurately its weight. Repeat the extraction of the remaining liquid in the separatory funnel twice, using 15 ml diethyl ether and 15 ml petroleum ether. The upper liquid that is extracted for 3 times is added together into the dried flask. Then, the flask is taken into a hot air oven at 100°C, and dried to a constant weight.

$$\%\textbf{Fat} = \frac{\textbf{weight of fat (g)}}{\textbf{weight of sample (g)}} \times 100$$

Protein in black glutinous rice solution by the Kjeldahl method (a method from AOAC, 2000)

Weight 2 g of black glutinous rice solution into a Kjeldahl digestion flask. Eight g of catalyst mixture and 20 ml sulfuric acid are added into the digestion flask. Place the flask in an incline position in a digestion machine. Next, increase the burner setting and boil until the black glutinous rice solution is looked clear and then cool it to room temperature. Distilled water was added into the cooled flask. Then, transfer the black glutinous rice sample that has been digested into a distilling flask. Distilled water of 400 ml is added into the distilled flask and swirl to mix. Three or four boiling chips are added into the flask too and a methyl red/bromocresol green indicator is also added. Connect up the distillation with a deliver tube dipping below a boric solution. Make a diluted digest alkaline with 50% sodium hydroxide solution in amount of 75 ml. Close the tap and distilled the ammonia into the boric acid solution. After about 30 ml the distillation is over, open the tap and wash down a condenser and the delivery tube into the receiver. Titrate the distillate with 0.05 M sulfuric acid.

Calculate the percentage of nitrogen in the sample (1 ml 0.05 M sulfuric acid equal to 0.014 g nitrogen). The crude protein can be calculated using an appropriate factor of 5.7.

$$\%N = \frac{[(\text{ml of } H_2SO_4 - \text{ml of } H_2SO_4\text{blank}) \times 0.05 \times 0.014]}{\text{weight of sample (g)}} \times 100$$

%Protein = %N×5.7

Ash (a method from AOAC, 2000)

Weight 3 g of black glutinous rice solution and place on a ceramic dish that is known accurately for its weight. Heat the dish on a steam bath. Afterwards, transfer the dish into a hot air oven at 500°C until the sample become an ash. Cool the dish in an active desiccator and weight it again. Calculate the percentage of ash in the black glutinous rice solution.

$$\%\text{Ash} = \frac{\text{weight of ash (g)}}{\text{weight of sample (g)}} \times 100$$

Carbohydrate (AOAC, 2000)

%Carbohydrate = 100 – (%Moisture + %Fat + %Protein + %Ash)

APPENDIX D

Chemical analysis

Total acidity of fermented black glutinous rice drink by titrimetric method (a method from AOAC, 2000)

Weight 10 ml of fermented black glutinous rice drink into a 250 ml flask and dilute the sample with distilled water for 2 times of its weight. Add 2 ml phenolphthalein indicator and titrate against 0.1 M NaOH until the first persistent pink appears. Calculate the total acidity by following an equation below;

$$\text{Lactic acid (\%)} = \frac{\text{amount of 0.1 M NaOH (ml)} \times 100 \times 0.009}{\text{amount of sample (ml)}}$$

Total soluble solid content of black glutinous rice solution by hand refractometer (a method from AOAC, 2000)

Drop the black glutinous rice solution on the refractometer. Look the scale on refractometer. The scale of refractometer should be in the range of 0-32 %Brix.

Reducing sugar of black glutinous rice solution by DNS method (a method from AOAC, 2000)

DNS solution preparation :

- 3,5-Dinitrosalicylic acid 1 g
- Sodium hydroxide 2 M 20 ml
- Potassium Sodium Tartrate 30 g
- Distilled water

Analysis :

Weight 5 g of black glutinous rice solution in 50 ml distilled water. Heating at 50°C for 10 min. Then filter the sample in a volumetric flask using filter paper. Pipette 0.5 ml of sample in a tube and add DNS reagent 1 ml and 2.50 ml of distilled water, heating in water bath at 100°C 5 min. Next quick cool down the sample and measure at A_{540} by a spectrophotometer.

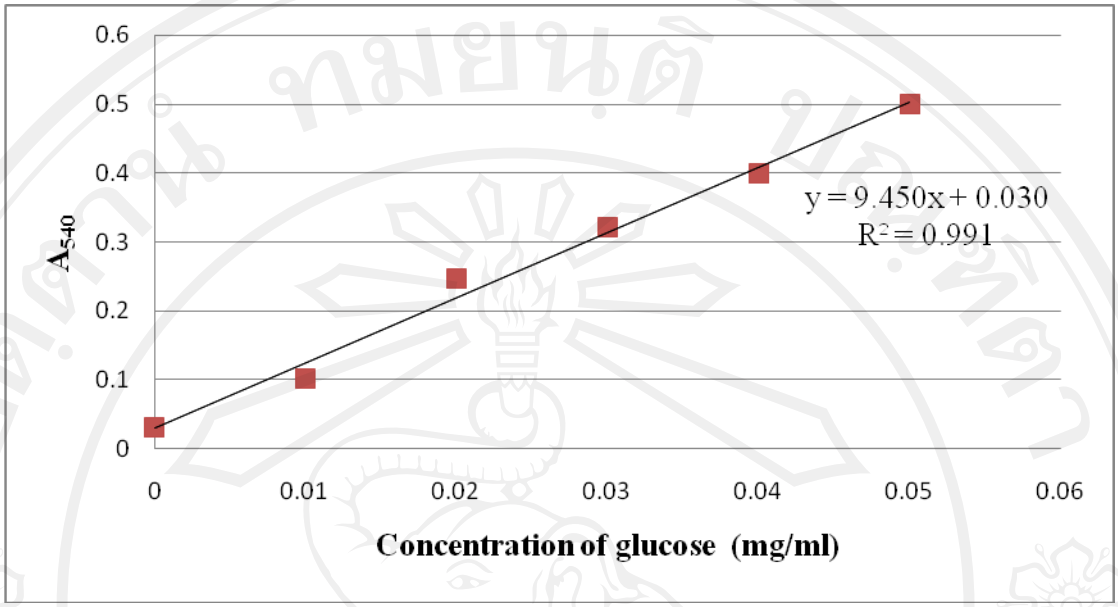

Figure D.1 Standard curve of glucose (mg/ml)

Calculation the amount of reducing sugar

For example

Rice solution with 30 mesh (595 µm) particle size in distilled water had an absorbance at 540 nm = 0.2441

$y = 9.450X + 0.030$

where, y = absorbance at 540 nm

x = amount of reducing sugar

$0.2441 = 9.450X + 0.030$

x = amount of reducing sugar = 0.02

1 ml of black glutinous rice solution had sugar 0.02 mg

50 ml of black glutinous rice solution had sugar 0.02×50 mg

5 g of sample had reducing sugar 0.02×50 mg

100 g of sample had reducing sugar $\frac{0.02\times50\times100}{5\times1000}$ g

In 100 g of sample, the reducing sugar was 0.02 g or 0.02%

Total sugar of black glutinous rice solution by DNS method (a method from AOAC, 2000)

Weight 3 g of black glutinous rice solution in a beaker 100 ml, add 1.5 M sulfuric acid 10 ml, heat at 100°C 20 min and cool down. Add 10% sodium hydroxide 12 ml and filter the sample in a volumetric flask 100 ml using filter paper. Transfer 0.5 ml of sample in a tube to measure total sugar, add 1 ml DNS reagent and 2.5 ml distilled water. Heating at 100°C 5 min, cool down and do measurement at A_{540} by spectrophotometer.

Calculation the amount of total sugar

For example

Rice solution with 30 mesh (595 µm) particle size in distilled water had an absorbance at 540 nm = 0.5140

$y = 9.450X + 0.030$

where, y = absorbance at 540 nm

x = amount of reducing sugar

$0.5140 = 9.450X + 0.030$

x = amount of reducing sugar = 0.05

1 ml of black glutinous rice solution had sugar 0.05 mg

100 ml of black glutinous rice solution had sugar 0.05 ×100 mg

3 g of sample had reducing sugar 0.05×100 mg

100 g of sample had reducing sugar $\frac{0.05\times100\times100}{3\times1000}$ g

In 100 g of sample, the reducing sugar was 0.17 g/100 g or 0.17%

Percent of sucrose = (0.17 – 0.02)×0.95 = 0.14%

Percent of total sugar = 0.14 + 0.02 = 0.16%

Determination of Phytic acid (a modified method from Kong and Lee, 2010)

Phytic acid was extracted with 20 ml of 0.2 N HCl by shaking 150 mg of sample at 200 rpm for 4 h at room temperature, and centrifugation at 3000 rpm (2300g) for 20 min. The supernatant was used for analysis. Supernatant (500 µl) was precipitated with 1 ml of ferric solution. The mixture was boiled at 100°C for 30 min in a water bath. After cooling, samples were transferred into Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. The 1 ml of supernatant was used to determine the phytic acid content estimated using 2,2-bipyridine solution (1.25 ml). After incubation for 1 min at room temperature, the absorbance was measured at 519 nm. The results were expressed as mg phytic acid equivalents per 1 g of sample.

Calculation of phytic acid

1 g of sample had phytic acid 0.0079 mg or 7.9 µg

APPENDIX E

Antioxidant analysis

Determination of Total Phenolic content (a method from Tananuwong and Tewaruth, 2010)

Total phenolics were evaluated using the spectrophotometric analysis with Folin-Ciocalteu's phenol reagent. In brief, a 20 g of sample was mixed with 20 ml of methanol/HCl 2% (95:5 v/v) in beaker (250 ml) and hold for 60 min. The solution was centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a whatman filter paper no.4 and diluted to volume 50 ml with methanol/HCl 2% in 50 ml volumetric flask. An aliquot (1 ml) of appropriately diluted extracts was added to a 100 ml volumetric flask containing 9 ml of distilled water. A reagent blank using distilled water was prepared. Five ml of Folin-Coicalteu's phenol reagent was added to the mixture and then shaken. After 5 min, 10 ml of a 7% Na_2CO_3 solution was added with mixing. The solution was then immediately diluted to volume (100 ml) with distilled water and mixed thoroughly. After 90 min at room temperature, the absorbance was read against the prepared blank (the solution not add sample) at 765 nm. The standard curve for total phenolics was made using gallic acid standard solution (100-800 mg/ml) under the same procedure as above. Total phenolics in black glutinous rice solution were expressed as mg of gallic acid equivalents per 100 ml of sample. All sample were analyzed in 3 replications.

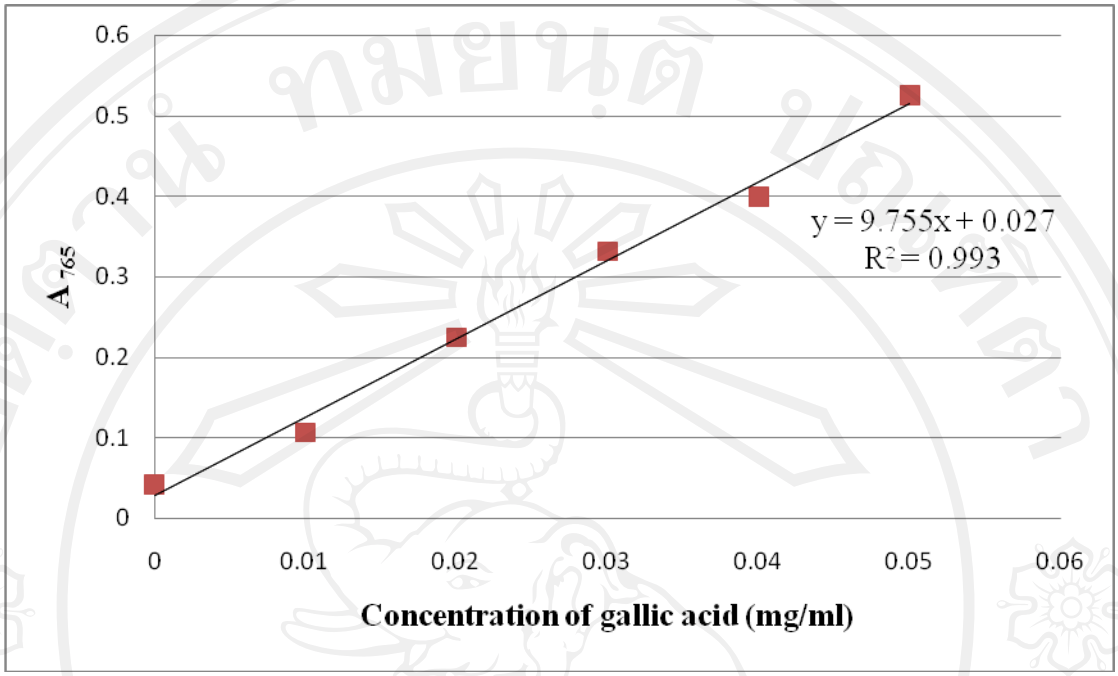

Figure E.1 Standard curve of gallic acid (mg/ml)

Calculation the amount of total phenolic

For example

Rice solution with 30 mesh (595 µm) particle size in distilled water had an absorbance at 765 nm = 0.224

y = 9.755X + 0.027

where, y = absorbance at 765 nm

x = amount of gallic acid

0.224 = 9.755X + 0.027

x = amount of gallic acid = 0.02 mg

1 ml of sample had total phenolic 0.02 mg or 20 µg

Determination of Anthocyanin (a method from Sompong et al., 2011)

Anthocyanins were extracted with acidified methanol (methanol and 1 M HCl, 85:15, v/v) with a solvent to sample ratio of 1:10. Keep at 4°C for 24 h. Filter the sample by No.1 paper. Absorbance was measured at 535 nm by a spectophotometer.

Calculation the amount of anthocyanin

$$\text{Total absorbance} = \frac{\text{absorbance} \times \text{final volume} \times 100}{\text{weight}}$$

$$\text{Total anthocyanin content (mg/100 g)} = \frac{\text{total absorbance}}{98.2}$$

APPENDIX F

Microbiological analysis

Total microorganisms : PCA (a method from Ashraf and Shah, 2011)

Preparation of culture media and reagents

1. Peptone diluent. Dissolved 0.1 g peptone in 100 ml distilled water and then autoclaving for 15 min at 121ºC
2. PCA. Dissolved 23.5 g PCA in 1 L distilled water and then autoclaving for 15 min at 121ºC

Measurement of total microorganism

Prepare 10^0, 10^{-1},10^{-2} and 10^{-3} dilution of sample. Mix the sample throughly by shaking to ensure uniformity of the solution. Pipette 1 ml representative sample from each dilution into a petri dish. Two petri dishes for each dilution. Pour 15-20 ml PCA into a petri dish from 2 and mix the sample and the media throughtly by gently shaking into the left and right combined with up and down. Let the petri dishes stand on a bench until the PCA media was harden with a smooth even surface on the top. Incubate invert the petri dishes for 48 h at 35°C. Count all colonies that growth in the petri dishes in the range of 30-300 colonies. The average number of colonies for each dilution was calculated and used in the report.

Lactic acid bacteria and *Lactobacillus bulgaricus* : MRS (a method from Ashraf and Shah, 2011)

Preparation of culture media and reagents

Dissolve 68 g of MRS in 1 liter of distilled water with adding 15% agar, mix until a homogenous suspension is obtained. The addition of acetic acid was done to lower the pH 5.4 of media (for analyse *Lactobacillus bulgaricus*). Heat gently, swirling frequently, then bring to the boil until completely dissolved and sterilize in autoclave at 121°C ± 1°C for 15 minutes.

Measurement of microorganism

Prepare 10^0, 10^{-1},10^{-2} and 10^{-3} dilution of sample for lactic acid bacteria and dilution 10^{-2}, 10^{-3} and 10^{-4} for *Lactobacillus bulgaricus*. Mix the sample throughly by shaking to ensure uniformity of the solution. Pipette 1 ml representative sample from

each dilution into a petri dish. Two petri dishes for each dilution. Pour 15-20 ml MRS into a petri dish from 2 and mix the sample and the media throughtly by gently shaking into the left and right combined with up and down. Let the petri dishes stand on a bench until the MRS media was harden with a smooth even surface on the top. Incubate invert the petri dishes for 24-48 h at 37ºC. Count all colonies that growth in the petri dishes in the range of 30-300 colonies. The average number of colonies for each dilution was calculated and used in the report.

***Streptococcus thermophilus* : M17 (a method from Ashraf and Shah, 2011)**

Preparation of culture media and reagents

Weight M-17 broth 48.25 g and dissolve it in 950 ml purified water upon boiling with adding 15% agar. Solution is light-medium to medium amber, very slightly too slightly opalescent. After that, autoclaved at 121°C for 15 min and cooled to 50°C. Add 50 mL sterile 10% lactose solution and mix well before pour on the petridishes.

Measurement of microorganism

Prepare 10^{-2}, 10^{-3} and 10^{-4} dilution of sample. Mix the sample throughly by shaking to ensure uniformity of the solution**.** Pipette 1 ml representative sample from each dilution into a petri dish. Two petri dishes for each dilution. Pour 15-20 ml M17 agar into a petri dish from 2 and mix the sample and the media throughtly by gently shaking into the left and right combined with up and down. Let the petri dishes stand on a bench until the M17 media was hardened with a smooth even surface on the top. Incubate invert the petri dishes for 48 h at 37°C. Count all colonies that growth in the petri dishes in the range of 30-300 colonies. The average number of colonies for each dilution was calculated and used in the report.

CURRICULUM VITAE

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