CHAPTER 3
MATERIALS AND METHODS

3.1 Raw Materials

1. Black glutinous rice (*Oryza sativa* L.) from The Royal Foundation, Doi Kham, Chiang Mai.
2. Distilled water from Polestar Demineralized Water, Chiang Mai Polestar (1992) Ltd., Chiang Mai.
3. Freeze dried cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (commercial code YC 380), Christian Hansen, Denmark).

3.2 Chemical Reagents and Equipment

3.2.1 Chemical reagents

Names of chemical reagent	**Production company**
(1) Sulfuric acid	Merck, Germany
(2) Ammonia Solution	Merck, Germany
(3) Ethyl alcohol	VWR International, UK
(4) Diethyl ether	Labscan Asia, Bangkok, Thailand
(5) Petroleum ether 40-60°C	Labscan Asia, Bangkok, Thailand
(6) Kjelblet/Coppper for protein analysis (ratio K_2SO_4: $CuSO_4 \cdot 5H_2O$ (9:1))	Oskon, Bangkok, Thailand
(7) Boric acid	Merck, Germany
(8) Mixed indicator or Screen methyl red (0.2(w/v) of methyl red and bromocresol green, 1:1)	BDH Laboratory supplies Poole and VWR International, UK
(9) Sodium hydroxide	Merck, Germany
(10) Filter papers No1.	Whatman, Germeny
(11) 3, 5-Dinitrosalicylic acid	Sigma-aldrich, Switzerland

(12) Potassium tartrate	VWR International, UK
(13) D-Glucose	Fisher Scientific, UK
(14) Phenolphthalein	Merck, Germany
(15) Hydrochloric acid	ACI Labscan, Bangkok, Thailand
(16) Folin-Ciocalteu-reagent	Merck, Germany
(17) Sodium carbonate	Merck, Germany
(18) Gallic acid	Sigma-aldrich, China
(19) Ferric solution	Fisher Scientific, UK
(20) 2, 2- bipyridine	Sigma-aldrich, India
(21) Plate Count Agar (PCA)	Himedia Laboratories, India
(22) Peptone	Himedia Laboratories, India
(23) MRS	Himedia Laboratories, India
(24) M17	Oxoid, England
(25) Acetic acid	Northern Chemical and Glasswares, Chiang Mai, Thailand
(26) Methanol	Merck, Germany

3.2.2 Equipment

Names of Equipment	**Production company**
(1) pH meter	Consort®; C830T, Belgium
(2) Shaking water bath	Memmert; SV2945, Germany
(3) Centrifudge	Hettich; EBA20, Germany
(4) Heating mantle	PNP
(5) Hot Air Oven	Memmert; 400, Germany
(6) Distillation unit for distilled protein	Foss kjeltec^{TM}8100, Sweden
(7) Digestion unit for distilled protein	Velp Scientification;1007Digestion, Italy
(8) Spectrophotometer	Genesys 10 UV scaning, USA
(9) Colorimeter	Minolta Data Processor DP-301 Chroma Meter, Japan
(10) Viscometer	Brookfield, USA
(11) Incubator	Stuart scientific, UK

(12) Autoclave	Hirayama, Japan
(13) Hammer mill	Armfield, UK
(14) Sieves	Endecotts, UK

3.3 Methods

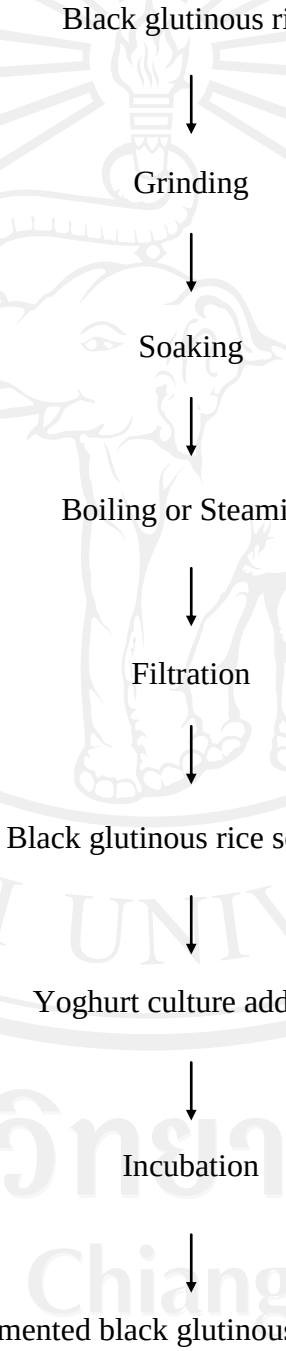

Figure 3.1 A basic production process of fermented black glutinous rice drink

3.3.1 Preparation of black sticky rice powder

The rice powder was prepared by grinding the black glutinous rice (*Oryza sativa L.*) sample using a dry miller (Hammer Mill) to get rice powder. The black glutinous rice powders were sieved through a metal screen with size either 30, 50 or 70 mesh (595, 297and 210 µm, respectively), packed in polyethylene (PE) bags and kept at 4°C (Iwaki and Kitada, 2007) to be used as a raw material in this research.

3.3.2 Optimization of rice particle size and type of soaking solution in the production of black glutinous rice solution

To produce rice solution, different sizes of black glutinous rice powder were soaked in distilled water with and without acetic acid at a ratio of 1:5 for 24 h. The addition of acetic acid was done to lower the pH of the soaking solution 1 unit than the control treatment (without acetic acid addition). The supplementation of acid was aimed to help the rice starch gelatinization (Ohishi et al., 2003; 2007). The rice particles (powder) were then boiled (at 95-100ºC) for 30 min in distilled water using a ratio similar to the soaking water. To get the rice solution, the blended liquid was filtered with a double cleaned white cloth. The final black glutinous rice solution was kept at 4ºC for physicochemical analyses. Each treatment was prepared in triplicate.

Analysis

The black glutinous rice solution was analyzed as followed;

a) Physical properties, including viscosity by a viscometer (Steffe, 1996), and color by a colorimeter (Pomeranz and Meloan, 1994).
b) Chemical properties, including pH values using a pH meter (AOAC, 2000), moisture contents by a drying method (Pomeranz and Meloan, 1994), protein contents (AOAC, 2000), carbohydrate contents (Pomeranz and Meloan, 1994), fat contents (Pomeranz and Meloan, 1994), crude fiber (AOAC, 2000), ash contents (Pomeranz and Meloan, 1994), reducing sugar (AOAC, 2000), total sugars (AOAC, 2000), total soluble solid contents using a hand refractometer (AOAC, 2000) and phytic acid (Kong and Lee, 2010).

c) Antioxidant components including total phenolic content (Tananuwong and Tewaruth, 2010), and anthocyanin content (Sompong et al., 2011).
d) Statistical analysis was analyzed by Factorial in Completely Randomized Design (CRD) using SPSS program (version 16). The Duncan's New Multiple Range Test was used to determine differences between treatment means.

3.3.3 Optimization for the soaking water ratio to black glutinous rice and soaking time to produce black glutinous rice solution

Based on the results in the section 3.3.2, one rice particle and one soaking medium were further investigated in this section. The selection was carried out for the rice solution that contained the highest reducing sugar, total sugar and/or antioxidant components. The black glutinous rice powder was soaked in distilled water at ratios of 1:2.5, 1:5 or 1:10 for either 0.5 h, 12 h or 24 h at 4°C. The rice was boiled (at 95-100ºC) for 30 min in distilled water (at a ratio similar to the soaking) to increase the moisture content of the rice powder. At the end of the boiling time, the mixed liquid was then filtered using a double cleaned white cloth to produce rice solution. The rice solution was stored at 4ºC for several analyses.

Analysis

The black glutinous rice solution was analyzed as followed;

a) Physical properties were determined by following the methods stated in the section 3.3.2.
b) Chemical properties, including pH values using a pH meter (AOAC, 2000), moisture contents by a drying method (AOAC, 2000), reducing sugar (AOAC, 2000), total sugars (AOAC, 2000), total soluble solid contents using a hand refractometer (AOAC, 2000) and phytic acid (Kong and Lee, 2010).
c) Antioxidant components were established by doing the methods written in the section 3.3.2.
d) Statistical analysis was analyzed by Factorial in Completely Randomized Design (CRD) using SPSS program (version 16). The Duncan's New Multiple Range Test was used to determine differences between treatment means.

3.3.4 Optimization of heating time, heating method and heating medium to produce black glutinous rice solution

The optimum rice particle size and type of soaking medium from the section 3.3.2 continued to be utilized in this section. From the section 3.3.3, one ratio of rice powder and soaking water (distilled water) together with one soaking time were selected. The selection was based on the rice solution that contained the highest reducing sugar, total sugar and/or antioxidant components. After soaking, the rice powder was heated by boiling (at 95-100ºC) for 30 and 60 min or steaming for 30 and 60 min (Adhikaritanayake and Noomhorm, 1997; Bello et al. 2004). The medium to heat the rice was distilled water with and without acetic acid (the ratio of the water to the rice was maintained as in the soaking condition). The addition of acetic acid was done to lower the pH of the soaking solution 1 unit than the control treatment (without acetic acid addition). The studied of the heating time would be correlated with the amount of reducing sugar, total sugar and antioxidants. At the end of heating period, the rice solution was collected after filtering the blended slurry with a double cleaned white cloth. The black glutinous rice solution was stored at 4ºC until analyzed.

Analysis

The black glutinous rice solution was analyzed as followed;

a) Physical properties were determined by following the methods stated in the section 3.3.3.
b) Chemical properties were determined by following the methods stated in the section 3.3.3.
c) Antioxidant components were determined by following the methods stated in the section 3.3.2.
d) Microbial analysis, including total microbial numbers (Marshall, 2006), and the number of lactic acid bacteria (Marshall, 2006).
e) Statistical analysis was analyzed by Factorial in Completely Randomized Design (CRD) using SPSS program (version 16). The Duncan's New Multiple Range Test was used to determine differences between treatment means.

3.3.5 The effect of incubation times on fermented black glutinous rice drink

Black glutinous rice solution was prepared based on the results of the sections 3.3.2-3.3.4. The selection was carried out for the rice solution that contained the highest reducing sugar, total sugar and/or antioxidant components. Into the warm rice liquid, 0.02% (w/w) starter cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) was aseptically inoculated and mixed thoroughly. The rice solution was incubated at 40-42ºC until the pH of the milk reached a value of 4.2, 4.6 or 5.0. The incubation time of the rice solution would be recorded. In the case that the viscosity of the rice solution was high (similar to stirred yogurt), the fermented rice solution would be diluted with 35% (v/v) drinking (distilled) water (Marshall, 2006) to produce a product that was similar to drinking yogurt. The final fermented milk drink was stored at 4ºC for further analyses.

Analysis

The fermented rice drink was analyzed as followed;

a) Physical properties were determined by following the methods stated in the section 3.3.3.
b) Chemical properties were determined by following the methods stated in the section 3.3.3 and total acidity (AOAC, 2000).
c) Antioxidant components were determined by following the methods stated in the section 3.3.2.
d) Microbial analysis, including the number of *S. thermophilus* on M-17 media (Ashraf and Shah, 2011), and the number of *L. bulgaricus* on MRS media at pH 5.4 (Ashraf and Shah, 2011).
e) Statistical analysis was analyzed by Factorial in Completely Randomized Design (CRD) using SPSS program (version 16). The Duncan's New Multiple Range Test was used to determine differences between treatment means.