APPENDICES

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
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Appendix A

Picture

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Figure A.1 Black glutinous rice milk

Figure A.2 Black glutinous rice yoghurt with adding full fat milk powder

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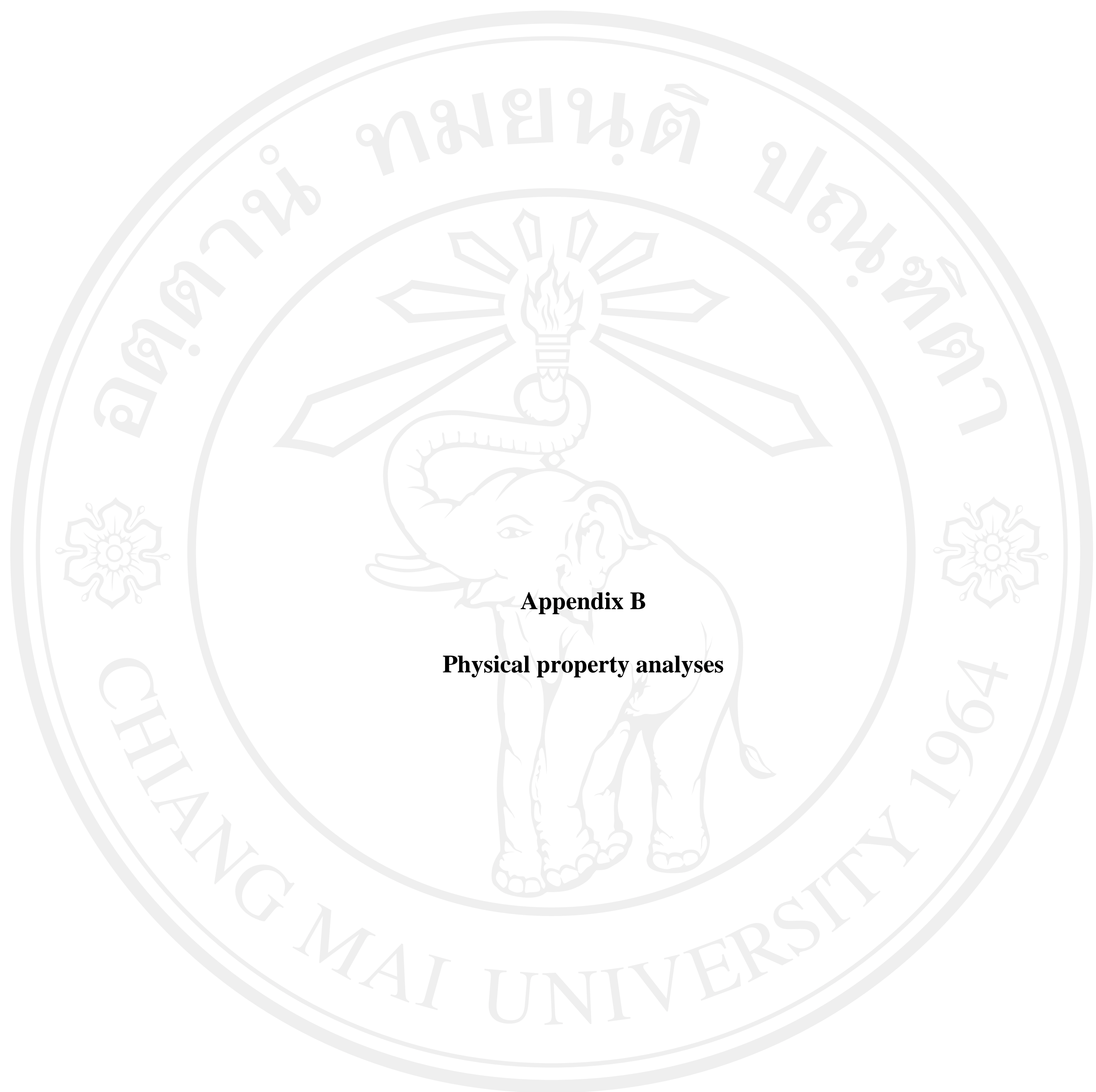

Appendix B

Physical property analyses

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1. Color (Hunter lab)

Color evaluation was performed on all rice milk and fermented rice samples using a colorimeter, Minolta Data Processor DP-301 Chroma meter. The instrument was calibrated with a white tile. A white standard tile with reflectance values of x = 82.51, y = 84.53, and z = 101.23 was used as a reference. The Hunter L*, a*, and b* scales give a measurement of color in units of approximate visual uniformity throughout a solid. L* value represented the lightness of color, a* value represented the greenness or 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.

2. Viscosity

Seven g of sample was poured into a special cylinder that was used to measure viscosity. After that, place the cylinder in a viscometer, put in a viscometer stirrer and turn on the equipment with details: % Torgue > 10 and speed 80 RPM.

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Appendix C

Chemical property analyses

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1. Moisture content (a method from an AOAC official method no. 955.04, 2000)

Weigh 2 g (c) of sample into moisture can with a tight-fit cover (a), which was known accurately the weight. Place loosely the covered can in a hot air oven at 100±2 ºC. Dry the samples until the weight is constant (about 4 h). Press the cover tightly into the moisture can, then removed them from the oven, cool in an active desiccator and weight (b). Express the loss weight as a moisture content of the sample.

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

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)

2. Ash content (a method from an AOAC official method no. 945.46, 2000)

Weight 2 g of sample 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 becomes an ash. Cool the dish in an active desiccator and weight it again. Calculate the percentage of ash in the sample.

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

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3. Fat content (a method from an AOAC official method no. 905.02**,** 2000)

Apparatus

1. Separatory funnels
2. Flasks

Reagents

1. Ammonium hydroxide (NH_4OH) (Merck, Germany)
2. Petroleum ether (Labscan Asia Co. Ltd., Bangkok, Thailand)
3. Diethyl ether (Labscan Asia Co. Ltd., Bangkok, Thailand)
4. Ethyl alcohol 95% (VWR International Ltd., England)

Determination

Weight 2 g of sample into 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 40-60ºC 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 100ºC, and dried to a constant weight.

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

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4. Protein content by the Kjeldahl method (a method from an AOAC official method no. 991.20, 2000)

Apparatus

1. Distillation unit for distilled protein (Foss kjeltec™ 8100, Denmark)
2. Digestion unit for distilled protein (Velp Scientification1007 Digestion, Italy)

Reagents

1. Kjelblet/Coppper for protein analysis (ratio K_2SO_4: $CuSO_4 \cdot 5H_2O$ (9:1)) (Oskon co., Ltd., Bangkok, Thailand)
2. 2% Boric acid (Merck, Germany)
3. Mixed indicator (Screen methyl red) of 0.2(w/v) methyl red and 0.2(w/v) bromocresol green, 1:1 (methyl red from BDH Laboratory supplies Poole and bromocresol green VWR International Ltd., England)
4. 50% Sodium hydroxide (Merck, Germany)
5. 95-98% Sulfuric acid (Merck, Germany)
6. 0.1 N Sulfuric acid (Merck, Germany)

Determination

Weight 5 g of sample into a Kjeldahl digestion flash. Eight g of catalyst mixture and 25 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 milk is looked clear and then cool it to room temperature. Distilled water was added into the cooled flask. Then, transfer the 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

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green indicator (mixed 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. And then 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. Last, Titrate the distillate with 0.1 N sulfuric acid.

Calculate the percentage of nitrogen in the sample. The crude protein can be calculated using an appropriate factor of 5.70

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

%Protein = %N×5.70

5. Fiber content (a method from AOAC, 2000 no 985.29)

Chemical

1. 1.25 % Sulfuric acid (Merck, Germany)

2. 1.25 % Sodium hydroxide (Merck, Germany)

Determination

The rice milk sample is transferred into a flask/beaker and 200 ml of pre-heated 1.25% sulfuric acid is added and the solution is gently boiled for about 30 min, maintaining constant volume of acid by the addition of hot water. The buchner flask funnel fitted with whatman filter is pre-heated by pouring hot water into the funnel. The boiled acid sample mixture is then filtered hot through the funnel under sufficient suction. The residue is then washed several times with boiling water (until the residue is neutral using a litmus paper) and transferred back into the beaker. Then 200 ml of pre-heated 1.25% sodium hydroxide is added and boiled for another 30 min. Filter under suction and

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wash thoroughly with hot water and twice with ethanol. The residue is dried at 65ºC for about 24 h and weighed. The residue is transferred into a crucible and placed in muffle furnace (400-600ºC) and ash for 4 h, then cool in desiccator and weigh.

$$\%\text{Crude fibre} = \frac{\text{Dry wt of residue before ashing-wt of residue after ashing x100}}{\text{wt of sample}}$$

6. Carbohydrate (a method from AOAC, 2000)

$$\%\text{Carbohydrate} = 100 - (\%\text{Moisture} + \%\text{Fat} + \%\text{Protein} + \%\text{Ash})$$

7. Total acidity (a method from Park et al., 2005)

Weight 10 ml of black glutinous rice milk 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;

$$\%\ \text{total acidity} = \frac{(\text{amount of 0.1 M NaOH(ml)} \times 100) \times 0.0009}{\text{Amount of sample (ml)}}$$

8. Total soluble solid by hand refractometer (a method from AOAC, 2000)

Drop the black glutinous rice milk on the refrectometer. Look the scale on refrectometer. The scale of refrectometer was 0-32 ºBrix.

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9. Reducing sugar by DNS method (a method from Rattanapanone, 2011)

DNS solution reagent:

- 3,5-Dinitrosalicylic acid (Sigma-aldrich, Switzerland) 1 g
- Sodium hydroxide 2 M (Merck, Germany) 20 ml
- Potassium Sodium Tartrate (VWR International, England) 30 g
- D-Glucose (Fisher Scientific, UK)
- Distilled water

Determination

Weight 5 g of sample in beaker and add 50 ml distilled water. Heating that at 50°C for 10 min. Then filtrate of sample in volumetric flask. Pipette 0.5 of sample in tube and add DNS reagent 1 ml and 2.50 ml of distilled water, heating in water bath at 100°C for 5 min. Next quick cool down of sample and measure at A_{540} by Spectrophotometer (D1) and measure with curve standard glucose.

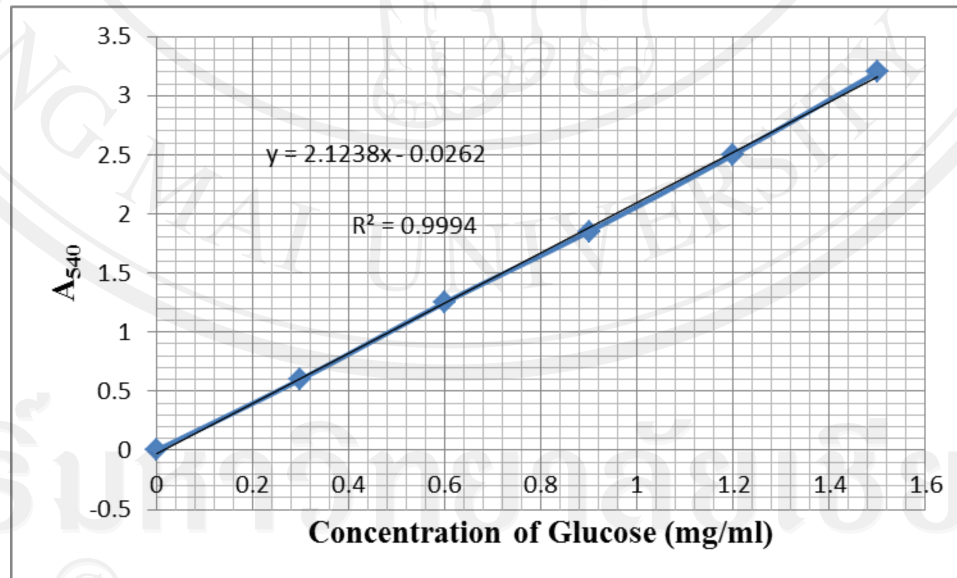

Figure C.1 Standard curve of glucose (mg/ml)

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The calculation to find out the amount of reducing sugar in sample

A correlation between the absorbance values and glucose standard solution at λ = 450 nm was figured out in the Figure C.1 and expressed in a linear equation which was:

$$Y = 2.1238x - 0.0262$$

where Y = absorbance values of reducing sugar of sample

x = the amounts of reducing sugar of sample

10. Total sugar (a method from James, 1995)

Weight 3 g of sample into 100 ml beaker. Add 10 ml of 1.5 M sulfuric acid and boiling at 100ºC in water bath after that, cool down. Then add 12 ml of 10% NaOH and filtrate with filter paper no.1. Adjust volume of sample from filtrate to 100 ml in a volumetric flask. Next pipette 0.5 ml of sample from volumetric flask to tube and add 1 ml of DNS reagent and 2.5 ml of distilled water. After that, boil at 100ºC in water bath and place in cool down. Last, measure absorbance at A_{540} by spectrophotometer (D2) and calculate with formula;

% sucrose (S) = % (D2-D1) $\times$ 0.95

% total sugar = D1 + S

11. 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. 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

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solution was then immediately diluted to volume (100 ml) with distilled water and mixed thoroughly. After 90 minutes at room temperature, the absorbance was read against the prepared blank at 765 nm. The standard curve for total phenolics was made using gallic acid standard solution (100-800 mg/l) under the same procedure as above. Total phenolics in black glutinous rice milk were expressed as ml of gallic acid equivalents per 100 g of sample. All samples were analyzed in 3 replications.

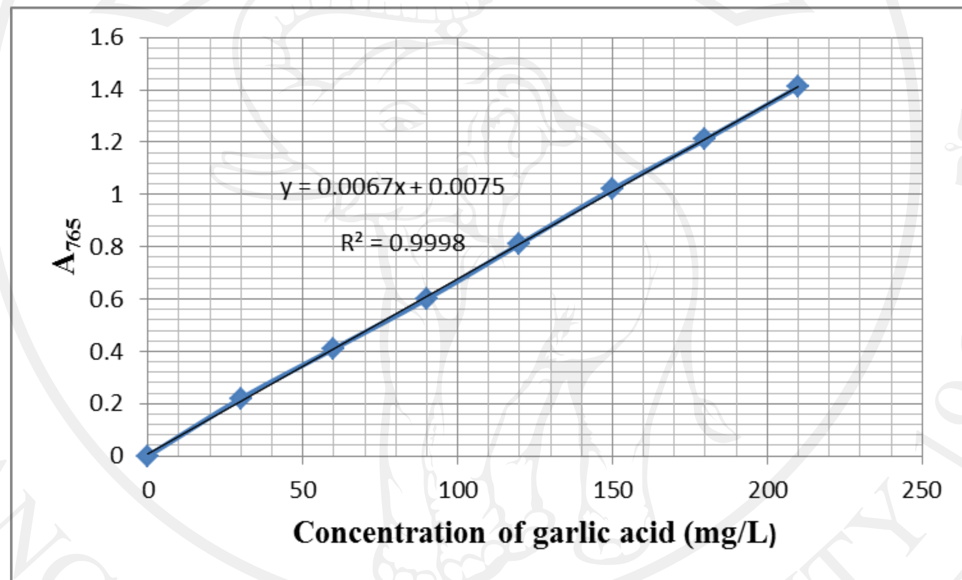

Figure C.2 Standard curve of garlic acid (mg/L)

The calculation to find out the amount of total phenolic content in sample

A correlation between the absorbance values and garlic acid standard solution at λ = 764 nm was figured out in the Figure C.2 and expressed in a linear equation which was:

$$Y = 0.0067x - 0.0075$$

where Y = absorbance values of total phenolic content of sample

x = the amounts of total phenolic content of sample

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12. 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 2,20-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.

13. 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. Filtrate of sample by filter paper no. 1. Absorbance was measured at 535 nm by spectophotometer.

Calculation the amount of anthocyanin

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

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

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Appendix D

Microbiological property analyses

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1. Plate Count Agar (PCA) (a method from Wohlsen et al., 2006)

Chemical

1. Peptone (Himedia Laboratories Pvt. Ltd., India)
2. Plate Count Agar (PCA) (Himedia Laboratories Pvt. Ltd., India)

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

1. Prepare 10^0, 10^{-1} and 10^{-2} dilution of sample. Mix the sample throughly by shaking to ensure uniformity of the solution

2. Pipette 1 ml representative sample from each dilution into a petri dish. Two petri dishes for each dilution.

3. 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

4. Incubate invert the petri dishes for 48 h at 35ºC. 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.

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2. Lactic acid bacteria and *Lactobacillus bulgaricus* (MRS) (a method from Marshall, 2006)

Chemical

1. MRS broth (Himedia Laboratories Pvt. Ltd., India)

2. Bacteriological Agar (Himedia Laboratories Pvt. Ltd., India)

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. 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 total microorganism

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

2.Pipette 1 ml representative sample from each dilution into a petri dish. Two petri dishes for each dilution.

3. 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

4. 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.

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The lactic acid bacteria were enumerated on de Man Rogosa Sharpe (MRS) agar. The incubation for lactic acid bacteria counts was done at 37 °C for 48 h.

3. *Streptococcus thermophilus* (M17) (a method from Ashraf and Shah, 2011).
Chemical

1. M-17 broth (Oxoid Ltd., England)

2. Lactose (Himedia Laboratories Pvt. Ltd., India)

3. Bacteriological Agar (Himedia Laboratories Pvt. Ltd., India)

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 total microorganism

1. Prepare 10^{-2}, 10^{-3} and 10^{-4} dilution of sample. Mix the sample throughly by shaking to ensure uniformity of the solution

2. Pipette 1 ml representative sample from each dilution into a petri dish. Two petri dishes for each dilution.

3. 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

4. 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.

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