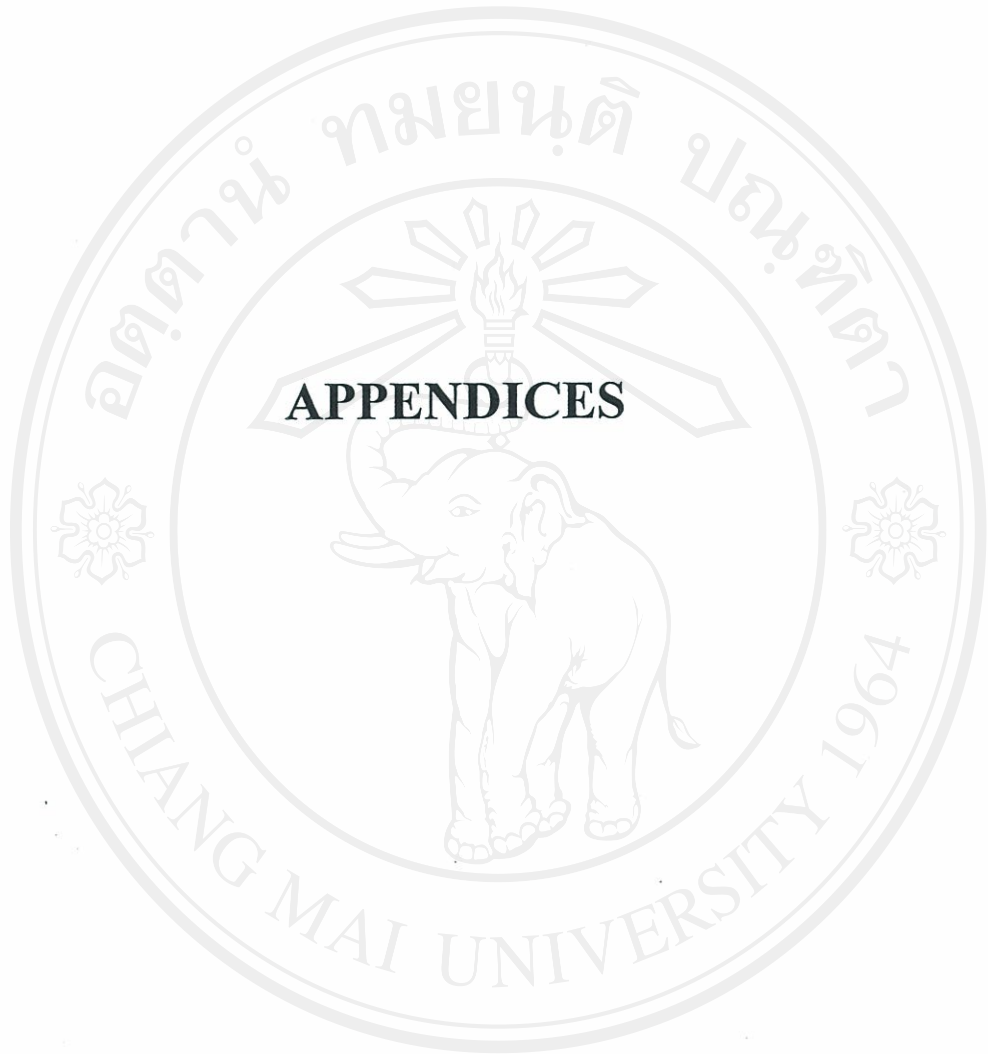

APPENDICES

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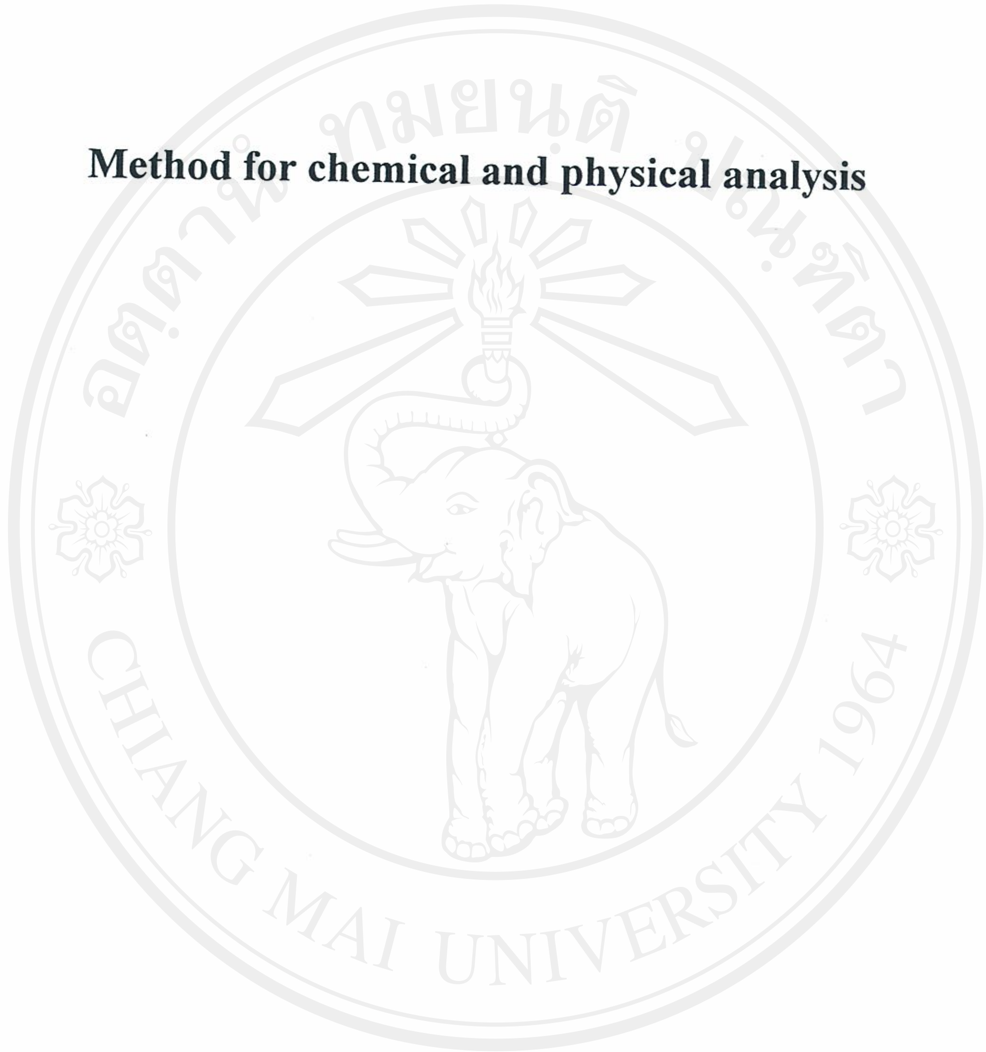
APPENDIX A

Method for chemical and physical analysis



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1. Total titratable acidity analysis (AOAC, 2000)

Pipette 10 ml of sample into a 125 ml flask. Drop phenolphthalein 2-3 drops and titrate the sample with 0.01 M NaOH until sample reach the end point (sample solution became pink that was persisted for 30 seconds).

$$\% \text{ lactic acid} = \frac{\text{ml of } 0.01 \text{ M NaOH} \times 0.01 \times 0.009 \times 100}{\text{volume of sample}}$$

2. Moisture and Total solid analysis (AOAC, 2000)

Heated an empty moisture dish in a hot air oven about 20-30 minutes. Cool in a desicator and weigh the dish. Into the cooled and weighed dish (provided with a cover), previously heated to 100 ± 3°C, accurately weigh 20 g of sample. Uncover the dish and dry the dish with its cover and contents for 3 hours in an oven provided with opening for ventilation and maintained at 100 ± 3°C. Cover the dish while it is still in the oven, transfer to a desicator and weigh the dish soon after it reached a room temperature. Dry the sample again for several times until the sample has a constant weight.

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$$\% \text{ moisture content} = \frac{\text{Loss in the sample weight during drying} \times 100}{\text{Initial weight of the sample}}$$

$$\% \text{ total solid} = 100 - \% \text{ moisture content}$$

3. Fat analysis (AOAC, 2000)

Weight sample (0.5 – 12.0 g) and place into a separated funnel. Add 10 ml water and shake. Add 1.25 ml ammonia solution, 10 ml ethyl alcohol and 25 ml

diethyl ether, close with a stopper and shake vigorously for 1 minute. Carefully release the pressure of the funnel. Add 25 ml petroleum ether, close the stopper and shake vigorously for 1 minute. Carefully release the pressure. Let stand until an upper liquid is practically clear (~30 minutes). Pour the upper clear solution into a previously weighed beaker. Take the beaker to stand in a hood until diethyl ether and petroleum are evaporated and place the beaker in a hot air oven (T = 102 ± 2°C) for 2 hours. Cool in a desicator and weigh the sample.

$$\% \text{fat content} = \frac{(W_2 - W_1) \times 100}{W_1}$$

W_1 = Weight of sample

W_2 = Weight of beaker and fat

W_3 = Weight of beaker

4. Ash analysis (AOAC, 2000)

Weigh 30-40 g sample into an ashing dish that has been heated, cooled in a desicator and weighed soon after reaching room temperature. Before ashing the sample, heat the sample on a bunchen lamp until no more black smoke appeared. Then ash the sample in a muffle furnace at 550°C until light gray ash results or until it reaches a constant weight. Cool in a desicator and weigh soon after reaching room temperature.

$$\% \text{Ash} = \frac{\text{Weight of ash} \times 100}{\text{Weight of sample}}$$

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5. Fiber analysis (AOAC, 2000)

Weigh 1-17 g sample into a 500 ml beaker. Transfer 1.25 M sulfuric acid (200 ml) into the beaker. Boil the sample solution on a hot plate for 30 minutes. Filter the sample solution using a Whatman paper no. 4 until it dries by applying a vacuum pump and wash the residue with boiling water until the sample does not have acid (do a test using a litmus paper). Place 200 ml of 1.25% NaOH into a beaker and boil the beaker on a hot plate. Wash the residue on the filter paper with distilled water. Boil the sample again on the hot plate for 30 minutes. Filter the sample using a Whatman paper no. 4 and wash the residue with boiling water. Transfer the filter paper with the sample residue into a crucible and dry at 102 ± 2°C for 3 hours. Cool in a desicator and weigh. Then ash the residue for 2 hours at 550 ± 10°C, cool in the desicator and weigh.

$$\%\text{Crude fiber} = \frac{(W_4 - W_3 - W_2) + (W_5 - W_3)(100 - \%H_2O - \%\text{fat})}{W_1}$$

W_1 = Weight of sample

W_2 = Weight of filter paper

W_3 = Weight of crucible

W_4 = Weight of crucible + filter paper + sample after drying

W_5 = Weight of crucible + ash

$\%H_2O$ = Moisture content of sample

%fat = Fat of sample

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6. Carbohydrate content (AOAC, 2000)

Carbohydrate content was determined by measuring the difference of the original sample minus the moisture, protein, crude fat and mineral contents calculated at the same moisture level.

7. Protein analysis (AOAC, 2000)

Place weighed sample (15 ml) in a digestion flask. Add 8 g catalyst mixture and 20 ml H_2SO_4. Place the flask in an inclined position in a digestion machine and heat the machine gently until frothing ceases. Continue boil briskly until the solution clears (~2 hours).

Cool, add distilled water to dilute the mixture solution and pour into a distilling flask. Add 400 ml H_2O (ammonia-free water) and a few Zn granules to prevent bumping. Immediately immerse a condenser tip into a receiver that contains 50 ml of 2% boric acid solution in a 500 ml flask and 5-7 drops indicator. Add 75 ml of 50% sodium hydroxide using a funnel into the distilling equipment. Rotate the distilling flask to mix the contents thoroughly; then heat until all NH_3 has been distilled (≥ 150 ml distillate). Remove the receiver, wash the tip of the condenser and titrate excess standard acid in distillate with 0.05 M H_2SO_4. Do blank determination to correct any nitrogen content in reagents.

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$$\%N = \frac{(V_a - V_b) \times N.H_2SO_4 \times 1.4007}{W}$$

V_a = ml of standard acid for sample titration

V_b = ml of standard acid for blank titration

$N.H_2SO_4$ = normality acid

W = weight of sample (g)

% Protein = % N × factor (a factor value = 5.95 for rice)

8. Sugar analysis

Equipment

Microplate reader

Test tubes

Autopipette and piptte tips

8.1 Sugar analysis in rice milks

Reagents

1) Dinitrosalicylic acid (DNS) solution (นฤมล, 2549)

3, 5 Dinitrosalicylic acid	7.49 g
Sodium metabisulfite	5.86 g
Sodium hydroxide	13.98 g
Sodium potassium tartrate	216.1 g
Phenol	5.37 g

The composition were mixed then added distilled water to 1 liter.

2) Hydrochloric acid (concentrate) (HCl)

3) 5 M Potassium hydroxide (KOH)

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Preparing a standard solution of fructose

Preparing a standard solution of fructose with concentrated of 0, 50, 100, 200 and 400 μg/ml. Pipette 500 μl of fructose in a test tube, add with 500 μl of DNS and shake. Heat the mixture at 90^0C for 5 minutes. After cooling to room temperature in a cold water bath, add 1 ml of distilled water and shake. Take representative samples into 96 well plates. For each of the sample, take 4 replicates of 200 μl. Measure with a microplate reader at a wavelength of 546 nm.

8.1.1 Reducing sugar (Frost, 2004)

Procedure

1. Pipette 10 ml of rice milk sample into a 15 ml of centrifuge tube and centrifuge with a speed of 1,250 g at 4^OC for 15 minutes.
2. Take a supernatant to prepare appropriate concentration.
3. Add 500 μl of DNS reagent to 500 μl of dilute supernatant sample (2) in a lightly capped test tube (To avoid the loss of liquid due to evaporation).
4. Heat the mixture at 90^0C for 5 minutes to produce a red-brown color.
5. After cooling to room temperature in a cold waterbath, add 1 ml of distilled water then shake the test tube.
6. Take representative samples into 96 well plates. For each of the sample, take 4 replicates of 200 μl.
7. Record the absorbance of the sample with a microplate reader at a wavelength of 546 nm.

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8.1.2 Inversion sugar (Wang, 2005)

Procedure

1. Pipette 10 ml of rice milk sample into a 15 ml of centrifuge tube and centrifuged with a speed of 1,250 g at $4^{O}C$ for 15 minutes.
2. Take 1 ml of supernatant into a test tube. Add 20 µl of HCl then shake.
3. Heat the mixture at $90^{0}C$ for 5 minutes, cooling in a cold water.
4. Add 50 µl of 5 M KOH solution to neutralize the acid, because the DNS method must be applied in an alkaline condition to develop the red brown color which represents the presence of reducing sugar.
5. Prepare appropriate concentration depending on the sucrose concentration in the sample.
6. Add 500 µl of DNS reagent to 500 µl of dilute supernatant sample (5) in a lightly capped test tube (To avoid the loss of liquid due to evaporation).
7. Heat the mixture at $90^{0}C$ for 5 minutes.
8. After cooling to room temperature in a cold waterbath, added 1 ml of distilled water then shake the test tube.
9. Take representative samples into 96 well plates. For each of the sample, take 4 replicates of 200 µl.
10. Record the absorbance of the sample with a microplate reader at a wavelength of 546 nm.

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Standard curve for fructose solution

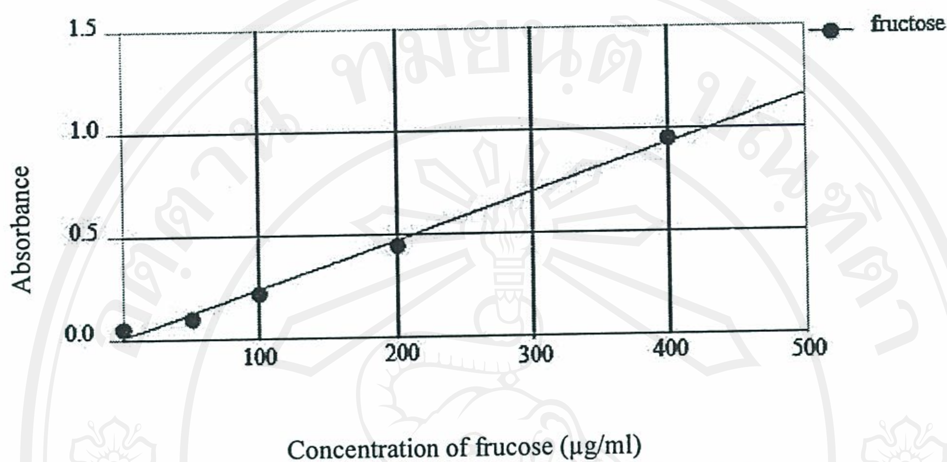

Figure A1 A standard curve of fructose solution

$Y = 0.0023X + 0.0111$ ($R^2 = 0.9925$)

8.2 Analysis of sugar in rice milks and rice grains

Reagents

1) Dinitrosalicylic acid (DNS) solution (นฤมล, 2549)

2) 1.5 M Sulphuric acid (H_2SO_4)

3) 10% Sodium hydroxide (NaOH)

Preparing a standard solution of glucose

Preparing a standard solution of glucose similar as for fructose (section 8.1). Measuring the microplate reader at a wavelength of 540 nm.

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8.2.1 Reducing sugar (James, 1995)

Procedure

1) Weigh 5 g of rice milk sample or rice grain that had be blended into a powder and add 50 ml of distilled water.
2) For the rice grain sample heat the sample at 50^0C for 10 minutes
3) The rice milks from 1) and rice grain power solution from 2) were filtrated by used a Whatman filter paper no. 40, wash residue with distilled water and adjust to 100 ml in a volumetric flask.
4) Add 500 µl of DNS reagent to 500 µl of sample (3) in a lightly capped test tube (To avoid the loss of liquid due to evaporation).
5) Heat the mixture at 90^0C for 5 minutes..
6) After cooling to room temperature in a cold waterbath, add 1 ml of distilled water then shake the test tube.
7) Take representative samples into 96 well plates. For each of the sample, take 4 replicates of 200 µl.
8) Record the absorbance of the sample with a microplate reader at a wavelength of 540 nm.

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8.2.2 Total sugar (James, 1995)

Procedure

1) Weigh 5 g of rice milk sample and rice grain that was previously blended into a powder and add 10 ml of distilled water. Add 10 ml of 1.5 M H_2SO_4.

2) For the sample, heat the sample at 100°C for 20 minutes and dip to cool water immediately.

3) Add 12 ml of 10% NaOH and shake.

4) Filter by using Watman filter paper no. 40, wash the residue with distilled water and adjust to 100 ml in a volumetric flask.

5) Add 500 µl of DNS reagent to 500 µl of sample (4) in a lightly capped test tube (To avoid the loss of liquid due to evaporation).

6) Heat the mixture at 90°C for 5 minutes.

7) After cooling to room temperature in a cold waterbath, add 1 ml of distilled water then shake the test tube.

8) Take representative samples into 96 well plates. For each of the sample, take 4 replicates of 200 µl

9) Record the absorbance of the sample with a microplate reader at a wavelength of 540 nm.

A standard curve for glucose solution

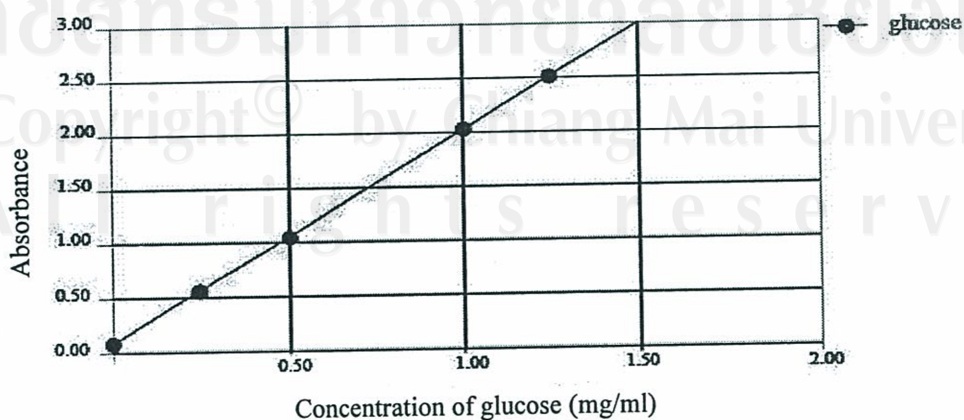

Figure A2 A standard curve of glucose solution

$Y = 1.9571X + 0.0719$ ($R^2 = 0.9999$)

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9. Viscosity by a Brookfield viscometer (Brookfield Engineering Laboratories, USA)

Viscosity of rice milks was measured using a Brookfield viscometer. The needle S18 was used in this measurement. An amount of 8 ml rice was poured into a special container and placed in the correct position under the viscometer. In the section 3.6.4, 10% (w/v) of white milk used 1 rpm viscometer speed and had 91.7-100% Torque, while 7% (w/v) of white milk used 60 rpm viscometer speed, and had 72.2-87% Torque. For 5% (w/v) white rice milk, brown and black glutinous rice milk used 200 rpm viscosity speed, and had 8.3-20.8% Torque. In the sections 3.6.5, 3.6.6 and 3.6.7, the viscosity speed at 200 rpm was used. The viscosity value was recorded 30 seconds after inserting the viscometer needle. Measurements were conducted every 7 days storage at 6°C.

10. Color analysis by a colorimeter (Minolta, Japan)

CIE L*, a* and b* values of rice milks were measured by a colorimeter (Minolta CR-300, Japan). Samples of rice milks were prepared by pouring 30 ml of rice milk into a white plastic cup. The colorimeter probe was then dipped into the rice milk samples and the L*, a* and b* values that were shown by the colorimeter were recorded. The colorimeter was calibrated against a standard white tile prior to the rice milk measurement (Kritsawan, 2006).

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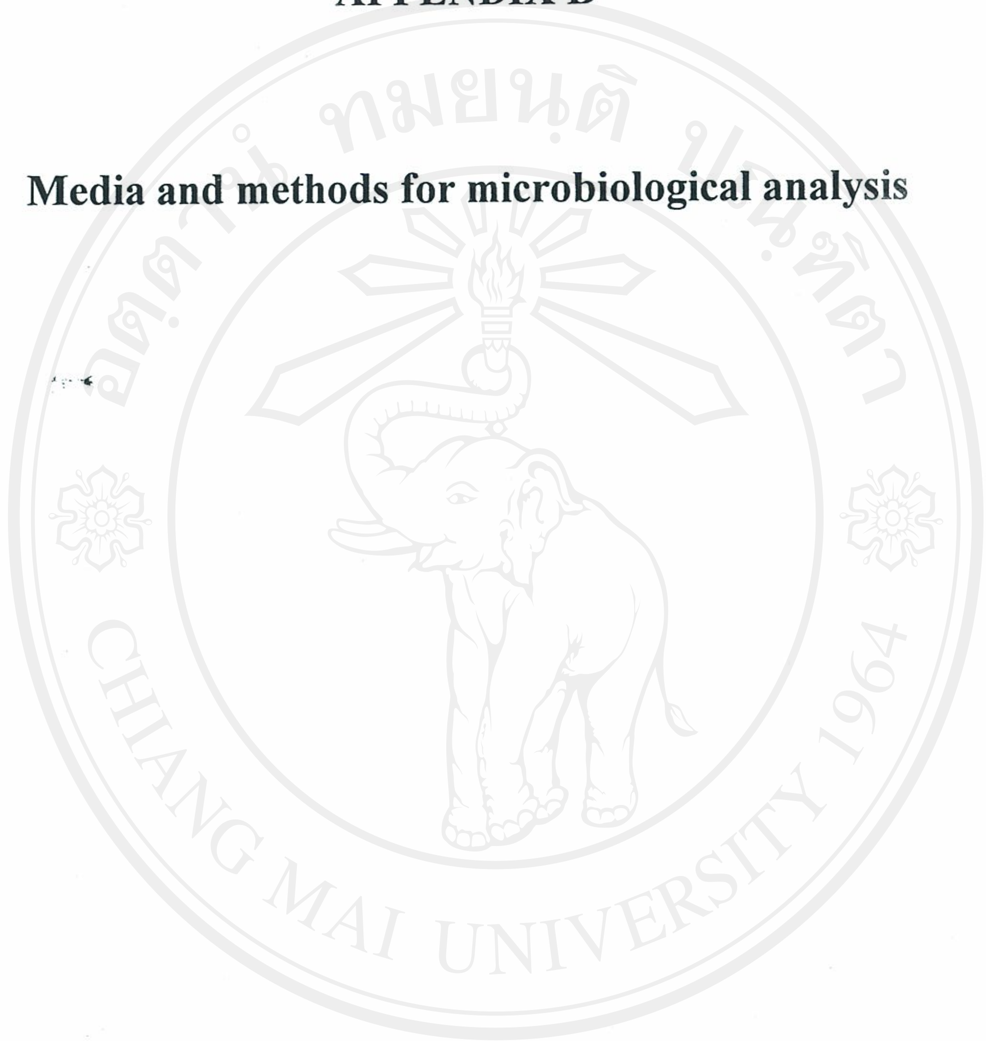
APPENDIX B

Media and methods for microbiological analysis



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1. Media

1.1 MRS-sorbitol (Atlas, 1993 and Dave and Shah, 1996)

Ingredient	Amount
Peptone	10.0 g
Agar	10.0 g
Beef extract	8.0 g
Sodium acetate.3H_2O	5.0 g
Yeast extract	4.0 g
K_2HPO_4	2.0 g
Triammonium citrate	2.0 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.05 g
Sorbitol	1% (w/v)

pH 6.2 ± 0.2 at 25 ^{O}C

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Preparation of medium

Add all ingredient, except sorbitol, in distilled water to make a volume of 990 ml. Mix thoroughly and bring to boil by a gentle heat. Distribute into bottles and autoclave for 15 minutes at 121°C (15 psi). Cool the medium to 40-45 ^{O}C and add 10 ml of sorbitol solution that is made from 10% (w/v) sorbitol in distilled water

and sterilized by a membrane filter, to make 1% (w/v) sorbitol in the final medium before using the agar medium.

1.2 Potato Dextrose Agar (PDA agar) (Atlas, 1993)

Composition per liter:

Potato infusion	200.0g
Glucose	20.0g
Agar	20.0g

pH 5.6 ±0.2 at 25 ^{O}C

Potatoes Infusion

Composition per 1.0 liter :

Potato unpeeled and sliced	200.0 g

Preparation of Potato Infusion :

Add potato sliced to 1.0 liter of distilled water. Gently heat and bring to boiling. Continue boiling for 30 min. Filter through a cheesecloth.

Preparation of medium:

Add ingredients composition with distilled water and bring to volume to 1.0 l. Mix thoroughly. Gently heat and bring to boiling. Distribute into bottle. Autoclave for 15 minutes at 121°C, 15 psi pressure.

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2. Gram strain (คณาจารย์จุลชีววิทยา, 2544)

2.1 Crystal violet stain

Crystal violet (Gentian violet)	0.5 g
Distilled water	100 ml

2.2 Gram iodine solution

Iodine	1.0 g
Potassium iodine	2.0 g
Distilled water	300 ml

2.3 Decolourizer

95 % Alcohols	250 ml
Acetone	250 ml

2.4 Sofranin O stain

Sofranin O	2.5 g
95 % Alcohols	100 ml

Dilute with distilled water to 5-10 fold before used.

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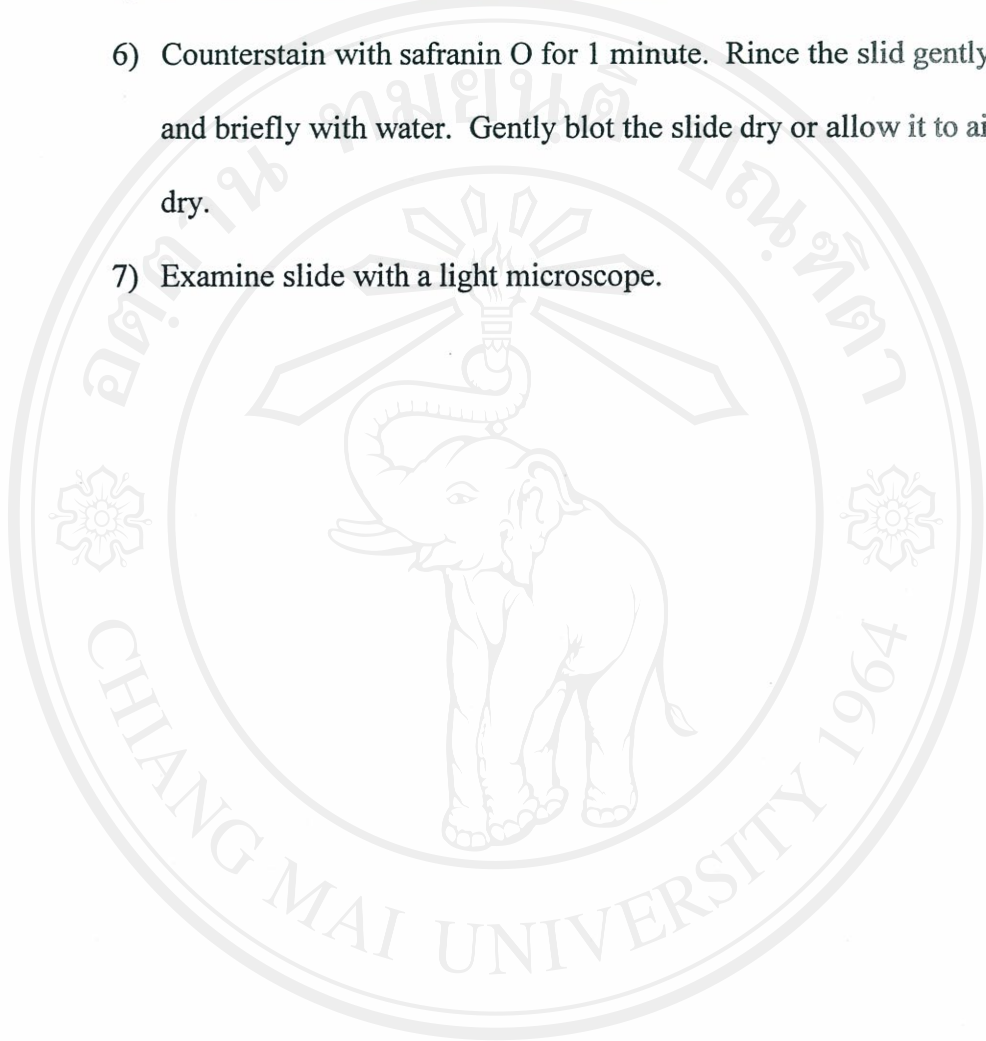
Gram stain procedure (Barnett *et al.*, 1997)

1) Use only thoroughly dried and heat-fixed smears for the Gram stain. Cover a cooled slide with crystal violet and stain for 1 minute.
2) Quickly and gently rinse off the dye with water.
3) Apply Gram iodine. Leave it on the smear for 1 minute.
4) Quickly and gently rinse off the mordant with water.

5) Decolorize the smear for less 1 second.
6) Counterstain with safranin O for 1 minute. Rince the slid gently and briefly with water. Gently blot the slide dry or allow it to air dry.
7) Examine slide with a light microscope.



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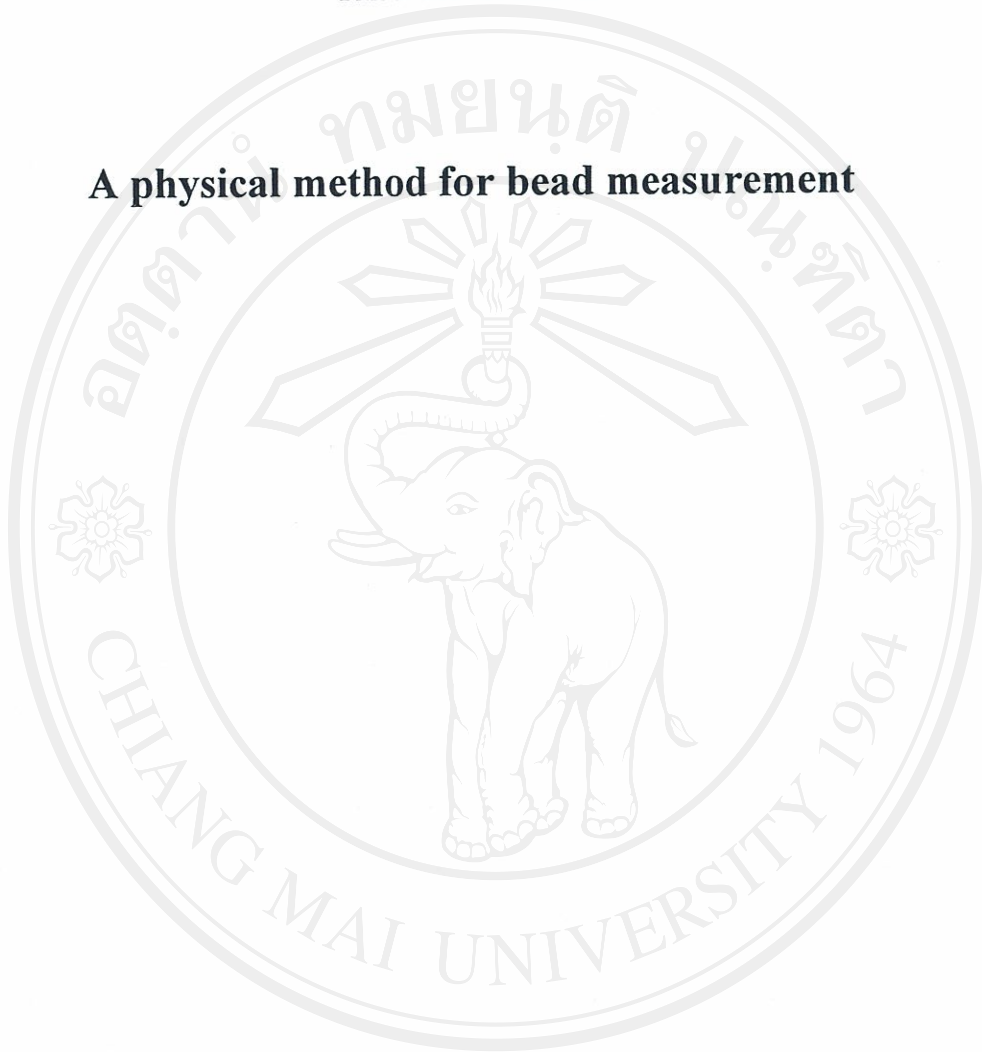
APPENDIX C

A physical method for bead measurement



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A measurement: An Ocular and Stage Micrometer

I. USE OF THE OCULAR MICROMETER:

A. Procedure:

1. Place the ocular lens containing a micrometer disc on the microscope.
2. Focus on the object to be measured and determine the size in ocular units.
3. Multiply the ocular units by the calibration factor for that specific microscope, objective and ocular micrometer. The units of the micrometer disc are arbitrary and a calibration procedure must be done to determine the calibration factor for each different objective and each different microscope.

B. Example:

A bead was measured using an ocular micrometer in the eye piece of a phase contrast scope and its 40X objective. The bead was 10 ocular micrometer units wide. The calibrationfactor for that specific micrometer used on the phase scope with the 40X objective is 2.5µm.

10 ocular micrometer units x 2.5 µm = 25 µm ocular micrometer

The bead is 25 µm wide.

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II. CALIBRATION OF THE OCULAR MICROMETER:

Ocular micrometers are calibrated by comparing the ocular micrometer scale with a calibrated stage micrometer. The stage micrometer is a microscope slide that has a carefully calibrated scale which is divided into 0.1 mm and 0.01 mm units.

A. Procedure:

1. Install the 10X ocular containing the ocular micrometer disc in the microscope.

2. Place the calibrated stage micrometer slide on the stage and focus on the scale.

3. Adjust the field so that the zero line of the ocular disc scale is exactly superimposed upon the zero line of the stage micrometer scale.

4. Without moving the stage micrometer, locate the point as far to the extreme right as possible where any two lines are exactly superimposed upon each other.

5. Count the number of divisions (mm) on the stage micrometer between the zero line and the superimposed line to the far right.

6. Count the number of ocular divisions between the zero line and the superimposed line to the far right.

7. Divide the distance determined in step 5 by the number of ocular divisions in step 6 and multiply by 1000 to give the ocular micrometer units in µm.

Stage micrometer divisions (mm) x 1000 µm = µm per ocular unit

8. Repeat steps 3 through 7 for each objective on the microscope. If the ocular micrometer is moved to a different scope, the calibration procedure must be repeated. If a new objective is added to the microscope, the calibration procedure must be done for the objective.

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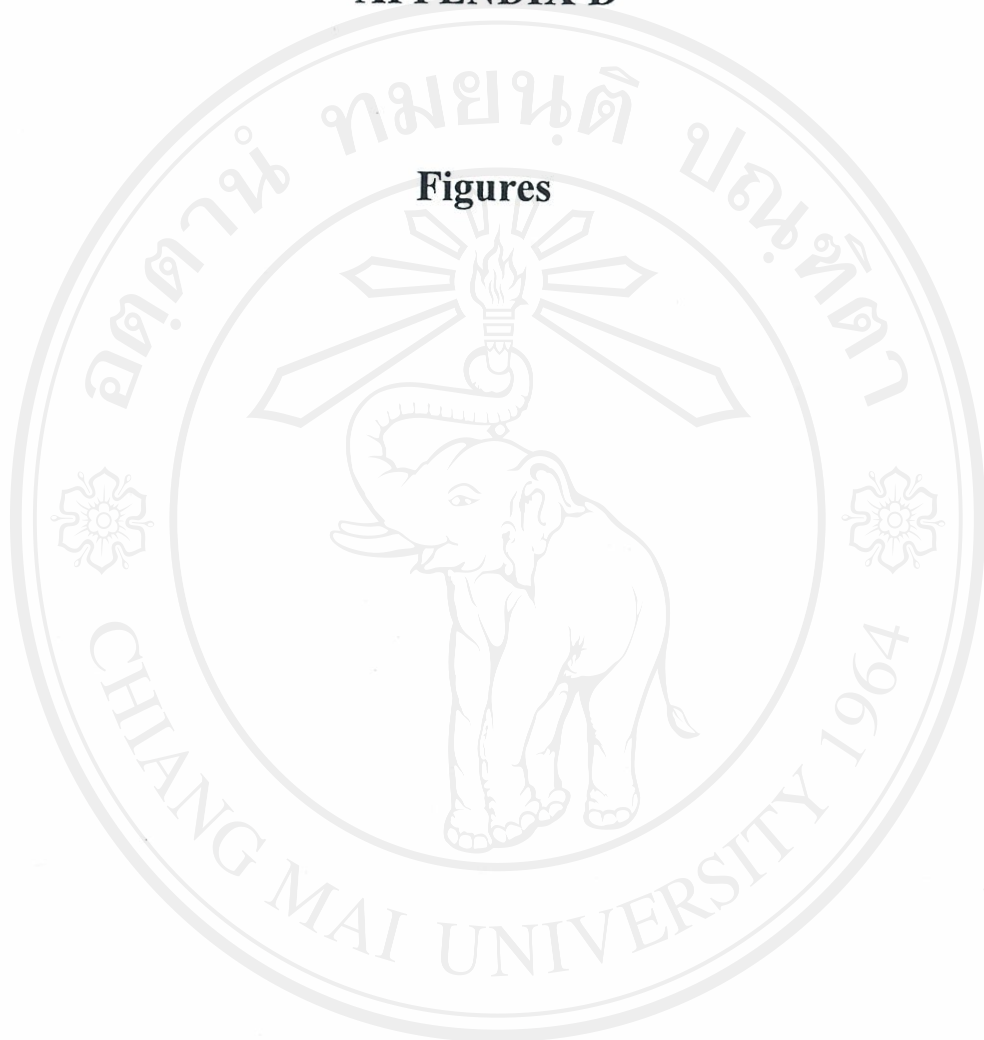
APPENDIX D

Figures



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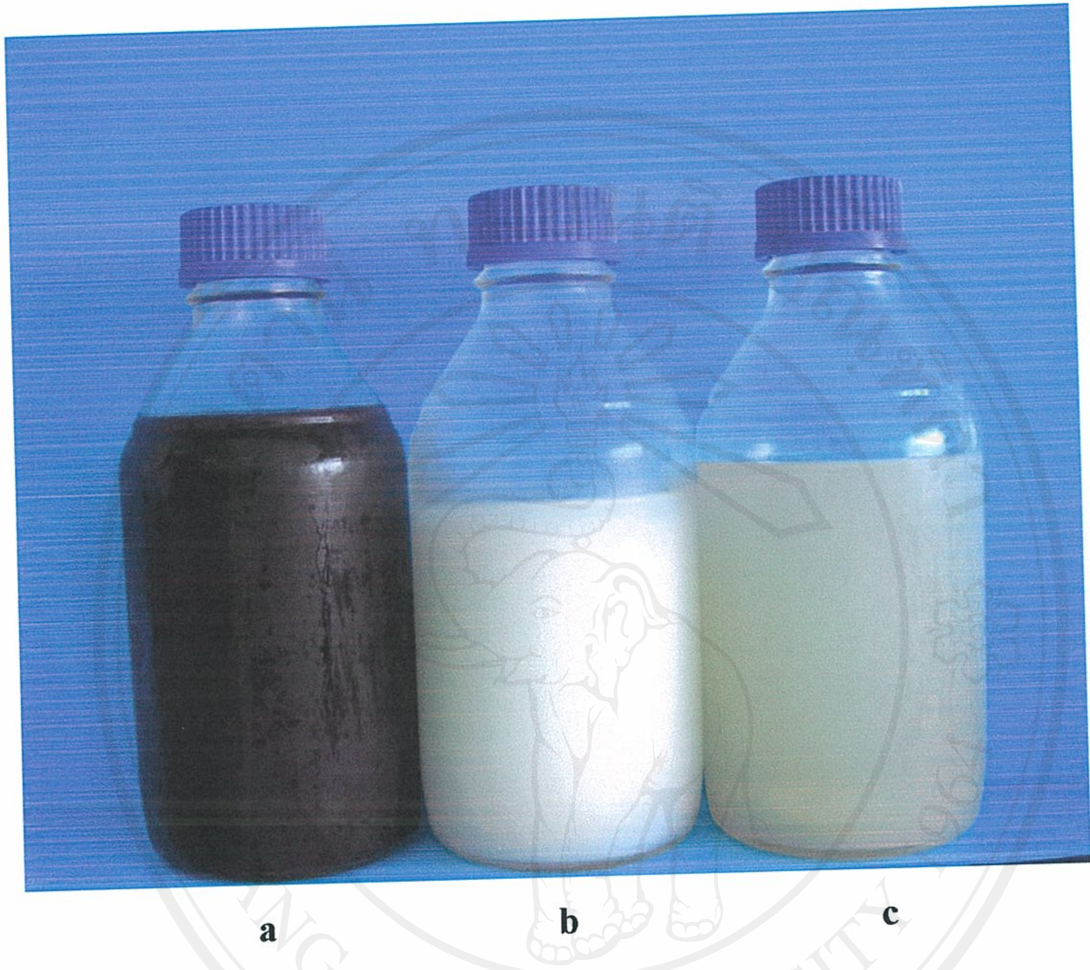

Figure D1 Rice milks, including a) black glutinous rice milk, b) white rice milk and c) brown rice milk

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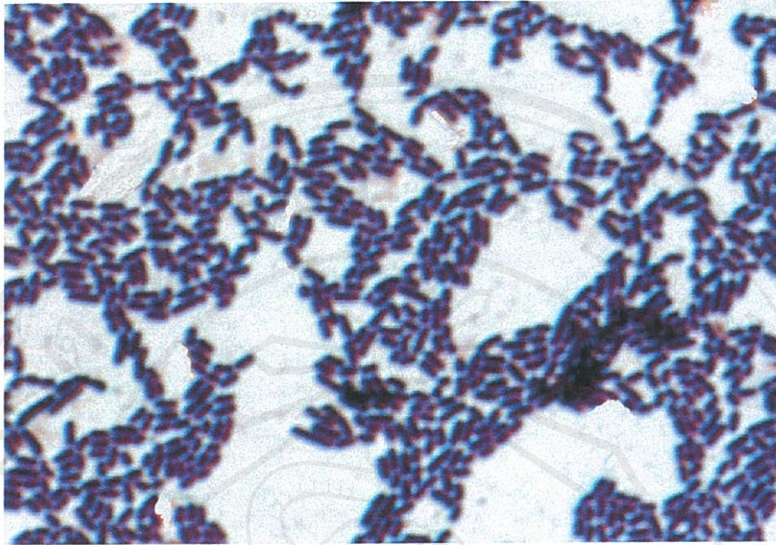
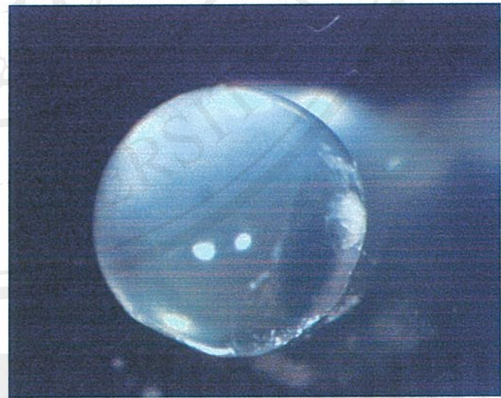


Figure D2 Gram staining of *Lactobacillus acidophilus* TISTR 450

(a)



(b)

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Figure D3 A calcium-alginate bead contained *Lactobacillus acidophilus* TISTR 450 cells with microscope magnifications of a) 32X10 b) 25X10

APPENDIX E

Viable counts of yeasts and molds

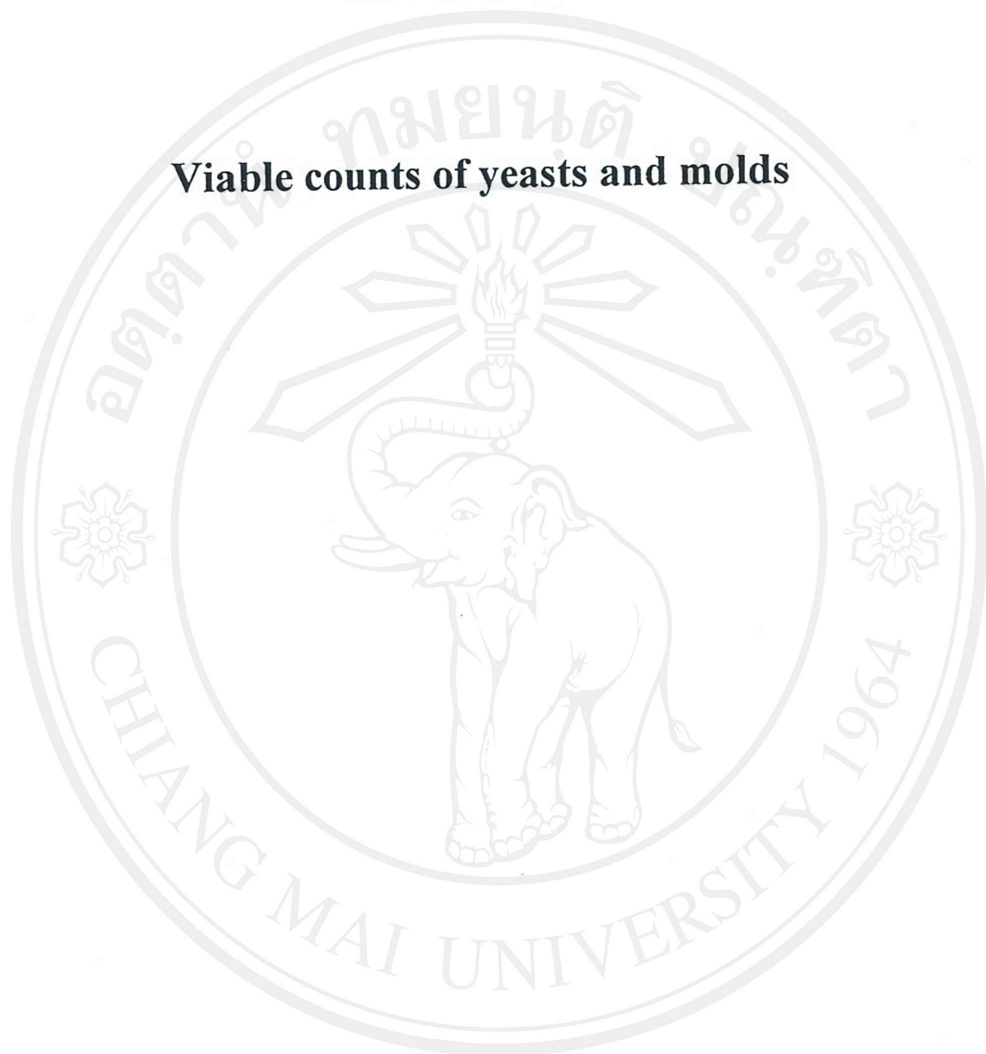

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Table E1 The viable count of yeast and mold (log CFU/ml) in different types and concentrations of rice milks during storage at $6^{O}C$ for 15 days

Different types and concentrations of rice milks	Storage time (days)		
	0	7	14
5%(w/v)white rice milks	7.69 ± 0.00^{Ca}	8.04 ± 0.12^{Aa}	$8.78\pm 0.89^{BCa*}$
7%(w/v)white rice milks	7.50 ± 0.00^{Ca}	7.72 ± 0.19^{Aa}	$8.85\pm1.25^{Ca*}$
10%(w/v)white rice milks	6.86 ± 0.22^{Ba}	7.61 ± 0.26^{Aa}	$7.73\pm0.46B^{Ca*}$
5%(w/v)brown rice milks	7.26 ± 0.00^{Ca}	8.02 ± 0.60^{Aa}	$7.58\pm0.39^{Ba*}$
7%(w/v)brown rice milks	7.89 ± 0.00^{Ca}	7.39 ± 0.76^{Aa}	$7.83\pm0.33B^{Ca*}$
10%(w/v)brown rice milks	7.77 ± 0.00^{Ca}	7.42 ± 0.67^{Aa}	$7.85\pm0.26B^{Ca*}$
5%(w/v) black glutinous rice milks	7.75 ± 0.00^{Ca}	$7.48\pm1.61^{Aa*}$	$8.17\pm0.59B^{Ca}$
7%(w/v) black glutinous rice milks	7.54 ± 0.00^{Ca}	7.39 ± 0.63^{Aa}	7.60 ± 0.59^{Ba}
10%(w/v) black glutinous rice milks	6.33 ± 0.08^{Ab}	5.47 ± 0.10^{Aa}	6.28 ± 0.28^{Aab}

Values are means of three determination (n=3) with the standard deviation (±SD) calculated with 95% confidence. Capital letters within a column and common letters within a row are not significantly difference.

*) Mold growth was observed in these treatment.

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Table E2 The viable count of yeast and mold (log CFU/ml) in 5% (w/v) brown rice milks affected by different initial pH values and initial population of *L. acidophilus* during storage at 6°C for 15 days

Initial pH values	Initial population of *L. acidophilus*	Storage period (days)		
		0	7	14
4.5	6 log CFU/ml	6.08±1.20^{A}	-	5.85±0.61^{A}
	8 log CFU/ml	6.77±0.56^{Aa}*	8.96±0.23^{Bb}	6.50±0.33^{ABCa}
5.5	6 log CFU/ml	5.36±0.35^{Aa}	6.85±0.15^{Ab}	6.82±0.07^{Cb}
	8 log CFU/ml	6.30±0.00^{Aa}	8.85±0.30^{Bb}	5.97±0.52^{Aa}
6.5	6 log CFU/ml	5.71±0.58^{Aa}	6.80±0.10^{Ab}	6.97±0.10^{BCb}
	8 log CFU/ml	6.31±0.47^{Aa}	7.48±0.00^{Ab}	7.16±0.34^{Cb}

Values are means of three determination (n=3) with the standard deviation (±SD) calculated with 95% confidence. Capital letters within a column and common letters within a row are not significantly difference.

*) Mold growth was observed in these treatment.

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Table E3 The viable count of yeast and mold (log CFU/ml) in 5% (w/v) brown rice milks affected by different types and levels of carbohydrate sources during storage at 6°C for 15 days

Carbohydrate source		Storage time (days)		
Honey (%)(w/v)	Sugar (%)(w/v)	0	7	14
0	0	5.99±0.99^{Aa}	6.83±0.05^{BCa}	6.29±0.61^{Aa}
	4	4.99±0.30^{Aa}	6.93±0.10^{Cb*}	7.24±0.35^{Bb*}
	7	5.77±0.83^{Aa}	6.94±0.08^{Cb}	7.21±0.31^{Bb}
4	0	6.47±0.48^{Aa}	6.80±0.13^{Ba}	6.86±0.12^{Ba}
	4	6.66±0.31^{Aa}	6.66±0.07^{Aa}	6.94±0.20^{Ba}
	7	6.73±0.15^{Aa*}	6.8±0.04^{Bab*}	7.03±0.20^{Bb}
7	0	6.70±0.07^{Aa}	6.78±0.06^{ABab*}	7.12±0.29^{Bb*}
	4	6.85±0.16^{Aab}	6.73±0.07^{ABa}	7.16±0.23^{Bb}
	7	6.26±0.41^{Aa}	6.82±0.07^{BCb}	6.88±0.00^{Bb}

Values are means of three determination (n=3) with the standard deviation (±SD) calculated with 95% confidence. Capital letters within a column and common letters within a row are not significantly difference.

*) Mold growth was observed in these treatment.

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Table E4 The viable count of yeast and mold (log CFU/ml) in 5% (w/v) brown rice milks affected by encapsulated or free cells of *L. acidophilus* during storage at 6°C for 15 days

Treatments of *L. acidophilus*	Storage period (days)		
	0	7	14
Free cells	4.92±0.32^{a}*	7.07±0.08^{b}*	7.08±0.08^{b}
Encapsulated cells ^{ns}	6.57±0.51	6.81±0.06*	6.53±0.10*

Values are means of three determination (n=3) with the standard deviation (±SD). Common letters within a row are not significantly difference. ns = not significant difference

*) Mold growth was observed in these treatm

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Curriculum Vitae

Name: Miss Kreuawan Thonglem

Date of birth: 09/11/1975

Academic background:

- Uttaraditdarunee School Uttaradit, 1988-1994
- Faculty of Science Chiang Mai University, Chiang Mai , B.S. (Microbiology) 1994-1998
- Faculty of Education Chiang Mai University Chiang Mai , Diploma (Teaching) 1998-1999

Work Experiences

- The scientist at Department of Packaging Technology Faculty of Agro-Industrial, Kasetsart University Bangkok, Thailand (July, 1999-June, 2000).
- The scientist at Department of Biology Faculty of Science, Chiang Mai University Chiang Mai, Thailand (July, 2000- at present).

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