Chapter 3

Methodology

This chapter mentioned materials and method of this work including preparation of starter culture and Thai fermented sausage. The microbiological, chemical and sensory analysis were described.

3.1 Materials and apparatus

Instrument and apparatus

Analytical balance: GF-3000, AND, Japan
: XS 204, Mettler-Tolede, Switzerland
Autopipette: Biopette, Labnet, U.S.A.
C18 cartridge: Sep-Pak C18, Waters Associates, Inc., Milford, Mass., U.S.A.
Centrifuge: CN180 nüve, Nkata, Turkey
GC: 14-A gas chromatograph, Shimadzu, Kyoto, Japan
: DB-624 capillary column 60 m, 0.32 mm i.d., film hickness 1.8 μm (J&W Scientific, California, U.S.A.)
: Flame ionization detector (FID) (Shimadzu, Kyoto, Japan)
GelScan software: BioSciTec, Frankfurt, Germany
HPLC: Agilent 1100 Series, Palo Alto, CA, U.S.A.
Analytical column: Aminex HPX-87H (BioRad, Richmond, California, U.S.A.)
: AA column 200×2.1 mm and guard column (Hypersil ODS, 20×2.1 mm, Agilent Technologies)
: DAD detector (Agilent 1200 Series, Palo Alto, CA, USA)
Incubator shaker: CH-4103, Bottmingen, Switzerland
: GLS 200, Grant, U.S.A.
Laminar flow cabinet: AILAS 2000, Flufrance, Cachan, France
pH meter: F-21 E; Horiba, Japan
Reactor: DRB 200, Hach, Loveland, U.S.A.

SDS–PAGE: v16-2 gel electrophoresis, Biometra, Goettingen, Germany

Spectrophotometer: U1201, Shimadzu, Tokyo, Japan

SPME: 75 μm carboxen/ poly(dimethylsiloxane) (CAR/PDMS) fiber (Supelco, Bellafonte, Pennsylvania, USA)

Vortex: Zx^3, VELP Scientifica, Italy

Chemical

Acetonitrile, HPLC grade, CARLO ERBA, Italy

Amino Acid separation kit, Agilent Technologies, USA

Agar agar, Scharlau, Barcelona

Beef extract, Scharlau, Barcelona

Coomassie Brilliant Blue R-250, Usb, Cleveland, U.S.A.

deMan Rogosa Sharpe (MRS), Merck, Germany

Glucoses HK enzymatic kit, Sigma, U.S.A.

High and low molecular weight calibration kit of SDS electrophoresis, Amercham Bioscience, U.S.A.

Hydrogen gas (H_2) 99.999%, Praxair (Thailand) Co., Ltd.

Mannitol Salt Agar (MSA), Merck, Germany

β-mercaptoethanol, Amercham Bioscience, U.S.A.

Methanol, HPLC grade, CARLO ERBA, Italy

3-Methyl-butanal, Aldrich, Germany

3-Methyl-butanoic acid, Fluka, Switzerland

3-Methyl-butanol, CARLO ERBA, Italy

Peptone, HIMEDIA, India

sodium dodecyl sulfate, Usb, Cleveland, U.S.A.

Total Nitrogen Test N Tube (0-150 mg/L), Hach, Loveland, U.S.A.

Trichloroacetic acid (TCA), CARLO ERBA, Italy

Tris, Usb, Cleveland, U.S.A.

Yeast malt agar (YM), HIMEDIA, India

Other common chemicals were obtained from Merck, Fluka, BDH or Sigma

3.2 Microbial strains

The selected lactobacillus strains used in this study for sausage fermentations are shown in Table 3.1. The six external starter cultures were mostly identified from traditional Thai fermented sausages (uninoculated sausages) (Phalakornkule and Tanasupawat, 2006). They were routinely grown in deMan Rogosa Sharpe (MRS) medium at 30 °C for 24 h and then stored at -80 °C in fresh medium containing 16% glycerol.

Table 3.1
Microorganisms

Microorganism	**Strain**	**Source**
Lactobacillus plantarum	BCC 4355	National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand (BCC)
Lactobacillus pentosus	BCC 5496	
Pediococcus pentosaceus	BCC 4312	
Lactobacillus sakei	TISTR 890	Thailand Institute of Scientific and Technological Research, Thailand (TISTR)
Pediococcus acidilactici	TISTR 425	
Weissella cibaria	JCM 12495	Japan Collection of Microorganisms, Japan (JCM)

3.3 Preparation of starter culture

Pure cultures of lactic acid bacteria were grown in MRS broth and then incubated at 30 °C, 160 rpm for 24 hours. Prior to inoculation the cell concentration was adjusted to give 2×10^7 CFU/g sausage with sterile deionized water. The use of saline in cell dilution to avoid cell lysis was neglected, because it would affect the taste of fermented sausages. The inoculum density of 2×10^7 CFU/g sausage was suggested by Acton and Dick (1977), because at high inoculum level of specific LAB strains in the range of 10^6 and 10^8cfu/g, it was able to inhibit growth of other microorganisms.

3.4 Preparation of fermented sausage and sampling

The sausage mixture comprised the following: ground pork (13%), streaky pork (55%), garlic (3%), salt (0.5%), pepper (0.5%) and soy sauce (3%). The ingredients were thoroughly mixed and divided into seven separated batches; (1) no addition of starter culture (control); inoculation with (2) *P. acidilactici*; (3) *P. pentosaceous*; (4) *W. cibaria*; (5) *L. plantarum*; (6) *L. pentosus*; and (7) *L. sakei*. The starter cultures in the amount of 2×10^7 CFU/g sausage were added to the mixture of sausage ingredients. Each sausage mixture was then stuffed into air-dried bovine small intestine and incubated at 30°C for 60 hours. Samples were taken at 0, 12, 24, 36, 48, and 60 hours following the start of incubation. After removing the outer casing, the samples were prepared for analysis by grinding in a grinder until a homogenous suspension sample was obtained. The extracts were filtered and supernatants were stored at -20 °C before analysis.

3.5 Microbiological analysis

For microbiological analysis, a 20-gram sample of fermented sausage was aseptically transferred to a sterile plastic bag containing 180 mL of sterile peptone (0.1% w/v) solution and agitated vigorously for one minute. Appropriate decimal dilutions of the sample solutions were prepared using sterile peptone solution and 0.1 mL of each dilution was spreaded on selective agar plates in triplicate. The total viable count (TVC) was determined on nutrient agar (NA) incubated at 30 °C for 48 hours; lactic acid bacteria on MRS agar at 30 °C for 48 h in anaerobic jar; *Micrococcus/Staphylococcus* on mannitol salt agar (MSA) at 30 °C for 48-72 hours; and yeast and mold on yeast malt agar (YM) incubated at 30 °C for 72-96 hours. Bacterial counts were expressed as colony-forming units per gram of sample (CFU/g). The mean ± standard deviation of the count was calculated by using three replicates for each culture time.

3.6 Chemical analysis

3.6.1 pH

Exactly 10 grams of sample was homogenized with 40 mL distilled water and then centrifuged at 4000×g for 15 min (CN180 nüve, Nkata,Turkey). The supernatant was incubated at 0 °C for 20 min and then filtered through Whatman no.1. The pH was measured by pH meter (F-21 E; Horiba, Japan).

3.6.2 Cured meat pigment

Formation of cured meat pigments was assessed by the modified method of Hornsey (1956) as follows:

Nitrosyl-heme pigments: A mixture of 2.00 ± 0.03g of finely ground sausage and 8 mL of acetone in a 15 mL centrifuge tube was stirred for 3 min. The sample was centrifuged at 4000×*g* for 15 min. The acetone phase was filtered through Whatman No.1 paper. The absorbance at 540 nm was measured with a UV-Spectrophotometer (U1201, Shimadzu, Tokyo, Japan).

Total heme pigment: A sausage sample 2.00 ± 0.03g was extracted with 2% HCl in acetone (v/v), centrifuged and filtered. The absorbance at 640 nm was measured. The results, reported as percent conversion, are the percentage of total heme pigments converted to the nitrosyl-heme pigment as expressed in Eq. (3.1).

$$\begin{aligned}\text{Percent conversion to cured meat pigments} &= \frac{\text{nitrosyl-heme pigments (ppm)}}{\text{total heme pigment (ppm)}} \times 100 \\ &= \frac{A_{540\text{nm}} \times 290}{A_{640\text{nm}} \times 680} \times 100\end{aligned} \tag{3.1}$$

3.6.3 Glucose concentration

Glucose concentration was determined by using the glucoses HK enzymatic kit (Sigma, U.S.A.). Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase as shown in Eq. (3.2). Glucose-6-phosphate (G6P) is then oxidized nicotinamide adenine dinucleotide (NAD) catalyzed by Glucose-6-phosphate dehydrogenase (G6PDH) as shown in Eq. (3.3). An equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-Phosphate (G6P)} + \text{ADP} \qquad (3.2)$$

$$\text{G6P} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate} + \text{NADH} \qquad (3.3)$$

Glucose assay reagent contained NAD, ATP, hexokinase and G6PDH. Sausage samples were filtered and diluted with deionized water to 0.05-5 mg glucose/ mL before analysis. Glucose concentration of sample solution was determined by adding the solutions into the marked test tubes as shown in Table 3.1. The solution in each tube was mixed and incubated for 15 min at room temperature. The absorbance at 340 nm was then measured. Glucose concentration was calculated as expressed in Eq. (3.4).

$$\text{mg glucose/mL} = \frac{(\Delta A)(TV)(F)(0.029)}{SV} \qquad (3.4)$$

when $\Delta A = A_{Test} - \left(A_{\text{Sample blank}} + A_{\text{Reagent blank}}\right)$

TV = Total assay volume (mL)

SV = Sample volume (mL)

F = Dilution factor from sample preparation

Table 3.2

Preparation of sausage sample for determination of glucose concentration

Test tube	Volume of glucose assay reagent (mL)	Sample volume (μL)	Volume of deionized water (mL)
Sample blank	-	200	1.0
Reagent blank	1.0	-	0.2
Test sample	1.0	200	-

3.6.4 Total carbohydrate determination

The phenol-sulfuric acid method (Duobis et al., 1956) was applied to determine total carbohydrate in sausage samples. The calibration curve of glucose standards was prepared in the range of 20-100 mg/mL of glucose concentration. 1.0 mL of 100g/L phenol solution and 5 mL concentrated H_2SO_4 were added into 1 mL of diluted sample and 1 mL of glucose standard solutions as shown in Table 3.2. The solution was mixed and incubated for 15 min in a fumed hood at room temperature. The absorbance at 488 nm was then measured. The relation between glucose concentration and absorbance was shown in Eq. (3.5).

$$Y = 0.01X \quad (3.5)$$

when Y = Absorbance

X = Glucose concentration (mg/L)

3.6.5 Organic acids

To determine the concentration of organic acids, high molecular weight compounds in sausage sample solution were eliminated by filtration through a C18 molecular sieve cartridge before analysis. The concentrations of organic acids

including lactic acid, acetic acid and formic acid were determined by High Performance Liquid Chromatography (HPLC) (Agilent 1100 Series, USA), equipped with a DAD detector (Agilent 1200 Series, USA). Chromatographic separation was performed using an ion-exchange column, Aminex HPX-87H (BioRad, Richmond, California, USA) under the following conditions: mobile phase; 0.02 M H_2SO_4, flow rate; 0.6 mL/min, and column temperature; 60 °C. The detection wavelength was 210 nm.

3.6.6 Extraction of sarcoplasmic and myofibrillar muscle proteins

The protein components in fermented sausage were fractionated according to the method of Visessanguan et al. (2006). Five grams of sausage sample was extracted with 40 mL of the mixture of 15.6 mM Na_2HPO_4 and 3.5 mM KH_2PO_4, pH 7.5 using a Moulinex homogenizer for 1 min. The precipitate was removed by centrifugation at 4000×g for 20 min and the clear supernatant was used for analysis as sarcoplasmic fraction. The pellet was extracted with 40 mL of the mixture of 0.45 M KCl, 15.6 mM Na_2HPO_4, 3.5 mM KH_2PO_4, pH 7.5 and centrifuged at 4000×g for 20 min. The supernatant was the myofibrillar fraction.

3.6.7 Extraction of non-Protein nitrogen

Five grams of sausage sample was extracted with 40 mL of the mixture of 15.6 mM Na_2HPO_4 and 3.5 mM KH_2PO_4, pH 7.5 using a Moulinex homogenizer for 1 min. The homogenate was centrifuged at 4000×g for 20 min. The supernatant was then mixed with cold 50% (w/w) trichloroacetic acid (TCA) to a final concentration of 10% (w/w). The filtrate was the non-protein nitrogen (NPN) fraction. The precipitate was removed by centrifugation at 4000×g for 20 min and the clear supernatant was non-protein nitrogen fraction.

3.6.8 Nitrogen content determination

The nitrogen content of sarcoplasmic, myfibrillar protein fractions and non-protein fraction was analyzed by persulfate digestion method (Hach, Loveland, U.S.A.). An alkaline persulfate digestion converts all forms of nitrogen to nitrate. Sodium metabisulfite is added after the digestion to eliminate halogen oxide interferences. Nitrate then reacts with chromotropic acid under strongly acidic conditions to form a yellow complex with an absorbance maximum at 410 nm.

Nitrogen standard concentration of 6.25, 12.5, 25 and 50 g/L were prepared for calibration curve. Total nitrogen persulfate reagent powder pillow was added into each total nitrogen hydroxide reagent vial. 2 mL of nitrogen standard and sample solutions were then added to the vials, shook vigorously and incubated at 105 °C for 30 min. After heating vial, the vials were cooled to room temperature. Total Nitrogen (TN) reagent A powder pillow was added to each vial, mixed and left for 3 min. TN reagent B powder pillow was then added into these vials and mixed. After 2 min for reaction, 2 mL of digested, treated nitrogen standard and sample were finally added into one vial of TN reagent C (acid reagent) and inverted 10 times to mix and incubated for 5 min. The absorbance was measured at 410 nm. Deionized water was used as blank. The relation between nitrogen concentration and absorbance was shown in Eq. (3.6).

$$Y = 0.0899X \tag{3.6}$$

when Y = Nitrogen concentration (g/L)

X = Absorbance

3.6.9 Protein analysis

Protein degradation was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Myofibrillar protein and sarcoplasmic protein were determined by the method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard protein to establish a standard curve.

The amount of protein injected into the electrophoresis gels was 25 μg in each lane. The protein concentration of the both proteins was mixed with Tris–HCl buffer pH 8.0 containing 5% (w/v) SDS, 14.5% (v/v) β-mercaptoethanol, 31% (v/v) glycerol and 0.03% (v/v) bromophenol blue. Samples were heated at 100 °C for 4 min prior to electrophoresis. Proteins were performed by horizontal SDS–PAGE using a model v16-2 gel electrophoresis apparatus (Biometra, Goettingen, Germany). SDS-PAGE was performed in gels containing 3% stacking and 12% separating gel. After electrophoresis, the gels were stained using 0.1% of Coomassie Brilliant Blue R-250 in methanol:water:acetic acid (45:45:10) and leave overnight in the shaker at room temperature. Gels were destained with water:methanol:acetic acid (65:25:10). The molecular weight of products from proteolysis was estimated by reference to the relative mobilities of standard protein markers. The markers (Amersham Biosciences, Biotech) consisted of the following proteins: myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), transferrin (76 kDa), albumin (66 kDa), glutamic dehydrogenase (53 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.2 kDa).

3.6.10 Quantification of SDS-PAGE

Quantification of the electrophoretic bands was performed by scanning the gels using the GelScan software (BioSciTec, Frankfurt, Germany). The density of each band with in SDS-PAGE was calculated. The differential integrated density was calibrated and normalized as expressed in percent of relative density.

3.6.11 Free amino acid analysis

To determined free amino acids concentration, samples were extracted and deproteinized with C18 cartridge. Sausage samples were derivatized before injection. The derivatization was performed using ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC). Derivatized samples were analysed by reversed phase high-performance liquid chromatography (RP-HPLC) (Agilent 1000 Series, Palo Alto, CA, USA) equipped with a DAD detector (Agilent 1200 Series,

Palo Alto, CA, USA). The separation was performed using an amino acid column 200×2.1 mm (Agilent Technologies) and guard column (Hypersil ODS, 20×2.1 mm, Agilent Technologies). The oven temperature was 40 °C. The injection program is shown in Table 3.2.

Table 3.3

Injector program for derivatized sausage sample

Action	Reagent	Volume (μL)
Draw	Borate buffer	5.0
Draw	OPA	1.0
Draw	Water	0.0
Draw	Sample	1.0
Draw	Water	0.0
Mix in air, max speed, six times		
Draw	FMOC	1.0
Draw	Water	0.0
Mix in air, max speed, three times		
Inject		

The gradient was used between two solvents: (A) 20 mM sodium acetate pH 7.2 containing 0.018% triethylamine and (B) 20% of 100 mM sodium acetate pH 7.2 containing 40% acetonitrile and 40% methanol. The gradient program was shown in Table 3.3. The detection wavelength was 338 nm.

Table 3.4
Gradient used for analysis of amino acids

Elution time (min)	% Solvent A	% Solvent B	Flow rate (mL/min)
17	100	Auto	0.45
18	40	60	0.45
18.1	0	100	0.45
18.5			0.8
23.9			0.8
24		100	0.45
25	100		0.45

3.6.12 Volatile compound analysis

The volatile compounds of Thai fermented sausage were evaluated by gas chromatography. 3-methyl-butanal, 3-methyl-butanol and 3-methyl-butanoic acid were used as standard volatile compounds.

Two milliliters of sausage sample solutions were placed in a 20 mL headspace vials and sealed with PTFE-silicone septum (Supelco, Bellafonte, Pennsylvania, USA) and then left at 40 °C for 10 min. Subsequently, the volatile compounds in samples were extracted by Solid Phase Micro-Extraction technique (SPME) using 75 μm carboxen/ poly(dimethylsiloxane) (CAR/PDMS) fiber (Supelco, Bellafonte, Pennsylvania, USA). Prior to collection of volatile compounds, the fiber was preconditioned at 240 °C for 60 min at GC injection port. The fiber was then exposed to the headspace of sample solution at 40 °C for 15 min. After adsorption, the volatile compounds were desorbed in the injection port of GC. The SPME fiber was placed in the injection port at 220 °C (splitless mode) during the entire chromatographic run.

A Shimadzu GC 14-A gas chromatograph equipped with a flame ionization detector (FID) (Shimadzu, Kyoto, Japan) was used. The compounds were separated in a DB-624 capillary column (J&W Scientific, 60 m, 0.32 mm i.d., film thickness 1.8 μm) and nitrogen was used as carrier gas with a linear velocity of 20.1 cm/s. The GC

oven temperature program began after the fiber was inserted. The temperature was held at 38 °C for 6 min, then ramped to 105 °C at a rate of 6 °C/min and finally raised to 220 °C at a rate of 15 °C/min and held for 5 min. The detector temperature was set at 240 °C.

3.7 Sensory evaluation

The prepared Thai fermented sausages were submitted to sensory evaluation to assess the differences between control sample and samples inoculated with starter cultures. Thai fermented sausages were presented to untrained 30-member panels for acceptability testing. Sausage samples were cut and halved into 1.5 cm. thick pieces. Samples were coded with three digit random numbers. The samples were assessed for appearance, flavor, color, sourness, saltiness, texture and overall acceptance in a scale of 1-7; with 1 being disliked extremely, 4 is neutral and 7 is liked best.

First, the sensory evaluation was to find the suitable fermentation time period amongst 30, 60, and 70 h of fermentation. The best score for acceptability yielded from these experiments was then investigated to find the favorite sausage amongst seven batches. Finally, the experiment was to test sensory evaluation between Thai fermented sausage inoculated with starter culture obtained the highest score and commercial Thai fermented sausage, under the brand name of S. Khonkhan.

3.8 Statistical analysis

Analyses of each parameter for the sausage treatments were carried out in triplicate at various sampling times. The data obtained for microbial counts, pH, concentrations of cured meat pigment, glucose and organic acids concentration and nitrogen content of myofibrillar, sarcoplasmic protein fractions and non-protein nitrogen were shown as mean values ± standard deviation. To study significant differences between the control and starter culture fermentation batches, one-way ANOVA was performed, with a confidence interval of 95% ($p < 0.05$). Means were compared by the least squares difference (LSD) test using the SPSS v.16 software for Windows (SPSS, Chicago, IL, USA).