Chapter 2

Materials and Methods

Materials

1. Raw material

Seabass (*Lates calcarifer*), a brackish water fish from Ko Yor, Songkhla Lake, Songkhla province was used throughout this experiment.

2. Media

All media used were purchased from Merk (Darmstadt, Germany) and Difco (France) such as Plate Count Agar (PCA), Brain Heart Infusion (BHI), Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), de Man Rogaso Sharpe (MRS) agar etc.

3. Chemicals

Chemicals used were analytical grade. The major chemicals were standard trans β-carotene, thiobarbituric acid (TBA), genistein and diazein purchased from Fluka (Buchs, Switzerland)

4. Modified atmosphere bag

The bags used for modified atmosphere packaging were Havel Vacuum bags, b.v. (Europac Co., Ltd) size 15 cm × 30 cm with O_2 transmission rate of 46.6 $cm^3m^{-2}day^{-1}$ at 38°C, 1 atm.

5. Instruments

5.1 Liquid Chromatograph consisted of C18 Suplex PKB-100 column size 25 cm x 4.6 nm, 5 μm, 120°A (SUPELCO, USA), a Thermoseparation pump (Thermo Finigan, San Jose, CA) and Absorbance detector (Isco, INC., Lincoln, NA)

5.2 Texture analyzer TA-XT2i (Stable Micro System, England)

5.3 Biological hazard hood (Hoten, Denmark)

5.4 Spectrophotometer (Spectronic, SP 21, USA)

5.5 Refrigerated centrifuge (Sorvall, RC, 5B Plus, USA)

5.6 Bench pH meter (Cyber Scan pH 2500, Eutech Instruments, Singapore)

5.7 Omni Mixer Homogenizer (Waterbury, CT, USA)

Methods

This experiment was divided into 4 main sections as follows:

1. **Determination of rigor process and sensory profile (aroma/odor, flavor and texture) of fresh fish and fish stored in ice**

1.1 Determination of rigor process

Sample preparation

Hooking or sieving method, 1day prior to experiment a cage was used to catch live seabass. Fish weighing 1.0-2.5 kg were immediately cold shocked in ice water slurry and transported in this condition for 45 min. After arriving at the laboratory, the fish were removed from the ice water slurry to avoid a cold shortening effect. Fish were divided by weight into 3 groups, 1: 1.1 - 1.2 kg, 2: 1.5 - 1.7 kg and 3: 2.0 - 2.5 kg.

Rigor index

The method of Bito *et al.* (1983) was slightly modified. The fish were placed on a stainless steel table with the flat side down and the convex side up. When rigor mortis started, the fish stiffened and the base of the tail (caudal peduncle) had a gap between the tail and table surface. The full rigor was recorded when the tail's gap was parallel with the table as a straight line. Rigor index was expressed as in the equation below:

Rigor index (R_i) = l_i/l_m

Where: l_i = gaping height at anytime,

l_m = maximum gaping height under rigor process

Mechanical analyses

The instrument used was a Texture Analyzer TA-XT2i (Stable Micro System, England) with a 5-kg load cell. The probe applied was a flat-ended cylinder with a diameter of 6 mm (type P/6). Measurements of the tail, middle and head parts were taken just above the central bone (Fig. 2). Scaled and filleted fish were compressed at least at 3 locations at 3 mm depth and the firmness was recorded (gram-unit). The speed of the probe was 2 mm/s (Morkore *et al.*, 2002).

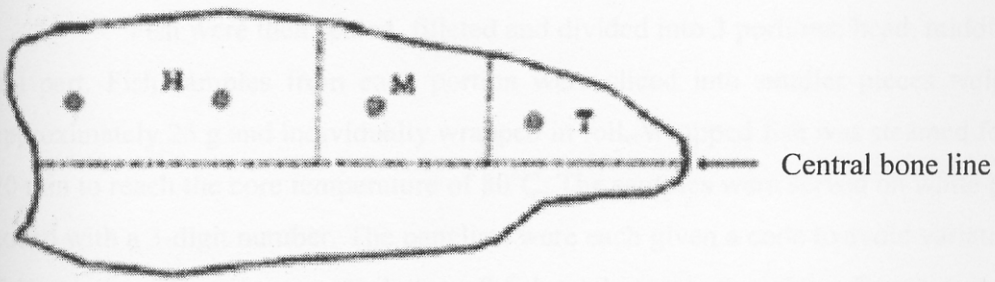

Fig. 2 schematic presentation of sections of a seabass for mechanical analysis

Statistical analysis

All data were statistically analyzed using Analysis of Variance. The difference between the means was evaluated using the Tukey multiple range test. Significance was established at $p<0.05$ unless otherwise indicated. The SPSS version 10 was used to perform the data analysis.

1.2 Determination of sensory profile (aroma/odor, flavor and texture) of fresh and ice stored fish

Sample preparation

Fish weighing 1.5-2.0 kg were killed and transported to the laboratory as described in section 1.1. Upon arriving they were kept in fresh ice immediately at the ratio of 3:1 (ice: fish) by adding ice in the polystylene box 3 inches in thickness before laying the fish on the ice. The box was kept in a chilled room at 4°C.

Sensory evaluation

Raw fish

Five fish samples were examined on days 0, 1, 2, 4, 6, 8, 10, 12 and 14 by 15-20 panelists who consisted of housewives and students who had studied in the fish quality course. Fish were examined for freshness by observing the parameters such as eyes, gill, body color, belly as shown in form I (Appendex 1).

Cooked fish

Fish were then scaled, filleted and divided into 3 portions: head, middle and tail part. Fish samples from each portion were sliced into smaller pieces weighing approximately 25 g and individually wrapped in foil. Wrapped fish was steamed for 15-20 min to reach the core temperature of 80°C. The samples were served on white plates coded with a 3-digit number. The panelists were each given a code to avoid variation of fish position. The sensory attributes of fish such as meat, colors, flavor, taste and texture were determined using form II. (Appendix 1)

Statistical analysis

All data were statistically analyzed using Analysis of Variance as described in section 1.1.

The result obtained from this section provided a firmness pattern of the rigor mortis stage for each sample position. In addition, sensory profile was used as a guideline for shelf-life of ice stored fish.

2. Effects of the killing method, icing delay and parts of the flesh fish on its shelf-life quality

2.1 Killing by immediate immersion in ice-water slurry

Sample preparation

Fish weighing 1.2-1.7 kg obtained as stated in section 1 were divided into 2 groups.

2.1.1 fish were kept in fresh ice immediately after arriving the laboratory in the ratio of ice to fish as described in section 1.2.

2.1.2 fish were left at room temperature (approx. 28 ± 2°C) for 45 min to stimulate the event occurring in industry before placing in ice as described in section 1.2.

Sampling

Two-three fish samples from each treatment were randomly taken from each group on days 0, 1, 3, 5, 7 and day 9. One side of each fish at the tail section was aseptically taken for microbiological determination. The rest was scaled and filleted for firmness measurements and chemical analyses.

Mechanical analysis

Fish firmness was prepared and measured as in section 1.

Chemical analyses

- pH

Ten grams of each part of the fish was well homogenised with 90 ml of distilled water and measured with the pH meter (Cyber Scan pH 2500, Eutech Instruments, Singapore).

- Total volatile base nitrogen (TVB) and Trimethylamine (TMA)

Due to limitation of sample size it was necessary to divide the sample into just 2 parts: dorsal and ventral meat unlike the procedures for mechanical and pH determination. Two-gram chopped samples were ground with 8 ml of 4% trichloroacetic acid (TCA) in mortar then filtered with Whatman no.1. The supernatant was adjusted to the volume of 10 ml and kept at –20°C until analysis using the method of Conway (1950).

Microbiological analyses

- Total viable count (Mesophilic bacteria)

Twenty-five grams of each tail sample were blended with 225 ml of 0.1% sterilized peptone water. Serial dilution was carried out and plated on the plate count agar (Merk) to determine the total viable aerobic count. The plates were incubated at 35±1°C for 24–48 hr. Microbial counts were expressed in colony forming unit per gram ($\log_{10}$ cfu/g) (Speck, 1976).

2.2 Killing by keeping in black bag (anoxia condition)

Refer to material in section 1. Fish weighing 1.5-2.0 kg were killed by keeping the fish in a black bag and transporting the bag to laboratory within 45 min. The fish were divided into 2 groups and the experiment was conducted as described in killing method 2.1.

Sampling

Five fish samples from each treatment were randomly taken from each group on days 0, 1, 3, 5, 7 and day 9. One side of each fish at the tail section was aseptically taken for microbiological determination. The rest was scaled and filleted for firmness measurements and chemical analyses.

Mechanical analysis

Results of the fish firmness measurements done in the previous killing method (2.1) showed that fish firmness was very high in the dorsal tail area. The properties of the dorsal tail area and the ventral meat need to be compared. Measurements of areas such as the tail, the middle and the head were taken both above (dorsal meat) and under (ventral meat) the central bone as shown in Fig. 3. Fish firmness was prepared and measured as in section 1.1.

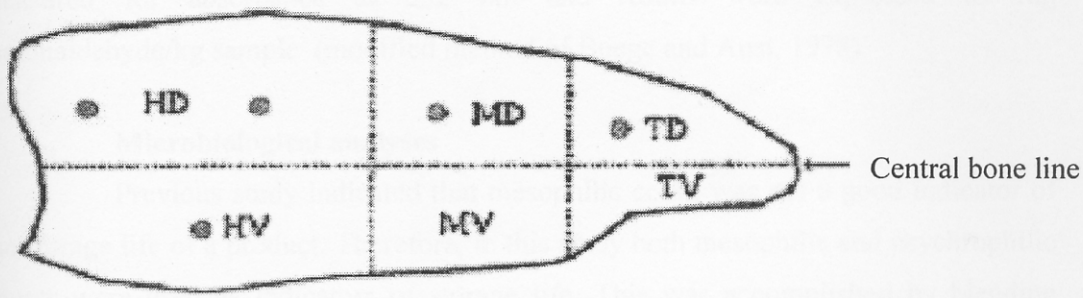

Fig. 3 Schematic presentation of sections of a seabass for mechanical analysis

TD : Tail dorsal part

TV : Tail ventral part

MD : Middle dorsal part

MV : Middle ventral part

HD : Head dorsal part

HV : Head ventral part

Chemical analyses

Measurements of fish samples from every area of the fish were conducted to determine the following parameters:

- pH and Lactic acid

pH was determined as described in section 2.1. After filtration, samples were measured for lactic acid according to the modified method of Lees (1975).

- Total volatile base nitrogen (TVB) and Trimethylamine (TMA)

TVB and TMA were determined as described in section 2.1.

- Thiobarbituric acid reactive substances (TBARS)

Two grams of chopped samples were homogenized with 10 ml of mixture of 0.375% TBA, 15% trichloroacetic acid and 0.25 N HCl (termed TBA reagent) for 2 min, then heated in boiling water for 10 min. The sample was cooled down with running tap water and centrifuged at 8,000 rpm for 20 min. Appropriate dilution was

measured for absorbance at 532 nm and results were expressed as mg malonaldehyde/kg sample (modified method of Buege and Aust, 1978).

Microbiological analyses

Previous study indicated that mesophilic count was not a good indicator of the storage life of a product. Therefore, in this study both mesophilic and psychrophilic counts were used as indicators of storage life. This was accomplished by blending twenty-five grams of each sample with 255 ml of 0.1% peptone water and a serial dilution was made. Appropriate dilution was plated on Plate Count Agar (PCA) (Merk, Germany). The plates were separately incubated at $35\pm1^{o}C$ for 24-48 hr for mesophilic bacteria and $7\pm2^{o}C$ for 7-10 days for psychrophilic bacteria. Microbial counts were expressed in logarithm colony forming units per gram ($\log_{10}$ cfu/g).

Statistical analysis

All data were statistically analyzed using Analysis of Variance as previously described in section 1.1.

The result obtained in this section showed that in the fish samples area of the fish body strongly affected its quality. Therefore, in the last section (section 4), fish were cut along the body length to avoid variation in each sample.

3. Natural preservative properties of Tom-Yum mix and its ingredients

3.1 Antibacterial and antioxidant properties of β-carotene and isoflavone in the spices of Tom–Yum mix.

Antibacterial activity

Bacterial culture

Two Gram–negative bacteria: *Pseudomonas fluorescens* ATCC 49839, *Escherichia coli* O157:H7 204P and two Gram-positive bacteria: *Staphylococcus aureus* ATCC 13565 and *Listeria monocytogenes* LCDC 81-861 were used as target bacteria. The Department of Food Science and Technology provided all bacteria tests except *P. fluorescens* ATCC 49839 was provided by the Department of Poultry Science,

University of Georgia. Individual test bacteria were maintained on beads and aseptically sub cultured three times on Tryptone Soy Agar (TSA; Difco), Tryptone Soy Broth (TSB; Difco) and BHI broth (Difco). Five loopfuls of each of the test bacteria from BHI were aseptically transferred into 5 ml of BHI and incubated at 32°C for 18 hr before use. These culturing conditions yielded approximately 10^9 cfu/ml except for *P. fluorescens* which yielded approximately 10^8 cfu / ml. Bacteria were plated on PCA and TSA for 18 hr at 32°C in a range of 10^5-10^9 cfu/ml for disk diffusion test (National Committee for Clinical Laboratory Standard, 2000).

Spices material and preparation

All fresh spices used (cultivar and origin unknown) were purchased from Oriental Farmer Market located in Atlanta, Georgia. Garlic and shallots were kept in chilled storage at 4±1°C and the rest were kept frozen at -40°C until use. Individual spice was washed with running tap water for 2 min and rinsed with sterilized distilled water in a ratio of spice and water of 1:2, then aseptically trimmed and cut into small pieces and ground. Sterilized disks were impregnated with each ground spice and kept for at least 12 hr at 10°C then dried in a biological hood for 15 min to remove the excess liquid before it was put on the PCA. It was then TSA inoculated with test bacteria as mentioned above and incubated at 32°C for 18 hr to determine the inhibition zones.

β-carotene content

The method of β-carotene analysis was slightly modified from that of Limrongrungrat (2001) by using HPLC technique. Trans β-carotene was purchased from Fluka Bio Chemika, St. Louis, MO. Stock solution of β-carotene was prepared by weighing 5 mg of trans β-carotene into a 25 ml volumetric flask and adjusting the volume with hexane. Absorbance was determined at 453 nm and the concentration of stock solution was calculated from a coefficient of β-carotene ($E^{1\%}$ 1 cm = 2592) (Bauernfeind, 1987 cited by Limrongrungrat, 2001). Six concentrations of standard solution (0.5-4.0 μg/ml) were used to plot the standard curve.

Liquid chromatographic system and condition for β-carotene determination

The chromatograph consisted of C18 SUPLEX PKB -100 column (25 cm x 4.6 mm, 5 μm, 120°A) (SUPELCO, USA), a Thermoseparation pump (Thermo Finigan, San Jose, CA), and Isoco V4 Absorbance detector (Isco, Inc., Lincoln, NA) set at 450 nm as a detector. The isocratic separation was performed and the flow rate of mobile phase was adjusted to 1 ml/min. The peak area was determined by using ChromJet integrator Model SP4400 (Thermo Separation Products, San Jose, CA). Two- ten grams (depending on the strength of yellow or red color) of edible part of test samples were ground three times with isopropanol and hexane containing 0.1% BHT (w/v) in the ratio of 15:25, 10:15 and 10:15 ml by using a homogenizer (Omni Mixer Homogenizer, Waterbury, CT) for 2, 1 and 1 min respectively. To remove water from the sample, 5-7 g of magnesium sulfate was added during the first homogenization. The homogenised sample was vacuum filtered, and the filtrate was transferred to a volumetric flask. The volume was then adjusted with hexane containing 0.1% BHT (w/v) in a 100 ml volumetric flask. One to three milliliters of each sample was dried with N_2 and dissolved in 1 ml of mobile phase, actonitrile/methanol/ tetrahydrofuran (25/28/2, v/v/v).

Isoflavone content

The isoflavone content determination method was a modification of Hutabarat *et al.* (1998) by using HPLC technique and the results were confirmed by using the LC-ESI-MS technique operated by the Department of Chemistry, University of Georgia. The edible part of the test sample was chopped and ground with a Handy chopper (Black and Decker (U.S.) Inc, Shelton, CT). A one- to three-gram sample was mixed with 10 ml of HCl and 40 ml of EtOH containing 0.05% BHT (w/v) in a boiling flat bottom flask, then sonicated for 25 min before refluxing at 80 °C for 9 hr. The extracted sample was cooled down to room temperature and then vacuum filtered.

The filtrate was transferred to a volumetric flask and the volume was adjusted with EtOH containing 0.05% BHT (w/v) in a 50 ml volumetric flask. One to three milliliters of each sample was dried with N_2 and dissolved in 1 ml of mobile phase, acetonitrile/water + acetic acid (99/1) (33/67, v/v).

Statistical analysis

This experiment, which was repeated 3 times, was conducted on separate lots of materials in different weeks. The study was designed as a randomized complete block design and the separate lot served as the blocking variable. Mean comparisons were performed using HSD the Tukey test to examine differences between treatments. All analyses were performed using the SPSS statistical software package.

3.2 Effects of Tom-Yum mix on microbial growth and consumer preferences

3.2.1 Effects of Tom-Yum mix on survival of target bacteria

Bacterial culture

Same types of bacteria used in reaction 3.1 were cultured to yield approximately 10^9 cfu/ml. Serial dilutions of each bacterium in the range of 10^2-10^9 cfu/ml were prepared for bacterial survival determination.

Spice materials and preparation

Individual spice was obtained and prepared as described in section 3.1. Tom-Yum mix, consisting of garlic, shallot, kaffir lime leaves, chili, galangal and lemon grass, was ground and then kept in a stomacher bag at 4±1°C. One-gram of Tom–Yum mix was added to 9 ml of serial dilution of each bacterium as mentioned in the range of 10^2-10^9 cfu/ml. The cultured sample was kept in chilled storage at 4±1°C and then investigated every day for 8 days by plating on Plate Count Agar (Merk, Germany). The plates were incubated at 35±1°C for 24-48 hr. In the meantime, bacterial load in fresh Tom-Yum mix was also determined.

3.2.2 Effects of Tom-Yum formulas on consumer preference

Results in section 3.1 showed that the garlic has the highest antibacterial effect in the Tom-Yum recipe. However, changing the formulation might affect consumer preference. To confirm consumer acceptability a suitable Tom-Yum formulation was validated.

Formulas preparation

The basic formula (FA) was formulated as in section 3.2.1. The other formulas were changed only in garlic content by adding garlic at 2 (FB), 3 (FC) and 4 (FD) times the basic formula.

Tom-Yum soup preparation

One and half liters water was boiled with one teaspoon of table salt. Tom-Yum paste weighting 100 g from each formulation was added into the brine water and heated. The soup was left to boil before adding fish in the amount of 312 g to each formulation.

Sensory evaluation

The warm soup at temperatures of around 45-50^{o}C was served to the panelists who were familiar with Tom-Yum soup. The flavor intensity was analyzed by a scoring test using a 5-point scale for flavor intensity, 5: very strong, 4: strong, 3: just right, 2: mild and 1: very mild. Consumer preference was analyzed by using a 5-piont hedonic scale, 5: like much, 4: like, 3: neither dislike nor like, 2: dislike and 1: dislike much.

4. Shelf-life extension of cut fish marinated with Tom-Yum mix and packaged under various modified atmospheres

4.1 Screening for suitable gas mixture for cut fish marinated with selected Tom-Yum mix formulas

Sample preparation

Live fish weighing 1.5-2.0 kg were obtained and killed in ice water slurry as described in section 2.1. After arriving at the laboratory, the fish were filleted. Each fillet was divided into 5 pieces along the body length. Cut fish was then marinated with selected Tom-Yum mix formulation in the ratio 3 parts fish to 1 part Tom-Yum mix (3:1 w/w) were then packaged in Havel Vacuum bags b.v (Europac Co., Ltd). Gas ratios of CO_2: N_2: O_2 are shown in Table 4 and kept at 4±1°C for 21 days. Samples were taken for chemical and microbiological analyses on days 1, 3, 6, 9, 12, 15, 18 and 21.

Table 4 Composition of gas mixture for marinated cut fish

Treatment	CO_2	N_2	O_2
1 (with Tom-Yum mix)	30	65	5
2 (with Tom-Yum mix)	40	55	5
3 (with Tom-Yum mix)	50	45	5
4 (with Tom-Yum mix)	60	35	5
5 (with Tom-Yum mix)	70	25	5
6 (with Tom-Yum mix)	80	15	5
7 (without Tom-Yum mix)	air pack served as control		

Chemical analyses

- **pH and Lactic acid, TVB, TMA and TBARS** were determined as previously described

Microbiological analyses

- **Mesophilic bacteria** were determined as previously described in section 2.1.

Statistical analysis

All data were statistically analyzed using Analysis of Variance as previously described in section 1.1.

4.2 Effects of Tom-Yum mix on shelf-life of fish and packaged under 70 to 90 % CO_2 atmosphere

It was found in previous experiments that the higher the concentration of CO_2 the lower the bacterial count. Therefore, in this experiment three levels of CO_2 concentrations were used to evaluate the proper gas mixture. However, there was an inadequate supply of fish. Therefore, storage time was cut down to 15 days instead of 21 days as in previous experiments.

Sample preparation

Fish preparation was as in section 4.1. Marinated cut fish was subjected to flushing with gas mixtures as follows: (1) 70% CO_2: 25% N_2: 5% O_2, (2) 80% CO_2: 15% N_2: 5% O_2, (3) 70% CO_2: 25% N_2: 5% O_2 and air packaging served as control.

Chemical analyses

- pH and Lactic acid, TVB, TMA and TBARS were determined as previously described. However, it was found that TBARS value at 532 nm had high variation. Therefore, TBARS at 450 nm was conducted in the later sections.

Microbiological analyses

- **Mesophilic bacteria and psychrophilic bacteria** were measured as previously described in section 2.2.

- **Lactic acid bacteria** were determined as for mesophilc and psychrophilic bacteria counts, but using de Man Rogaso Sharpe (MRS) agar in double layers instead of Plate Count Agar (PCA). The plates were incubated at 35±2°C for 72 days (Ordonez *et al.*, 1991).

- ***S. aureus*** determination was carried out by using dilutions of 10^{-1} to 10^{-3} from mesophilic and psychrophilic bacterial samples, and then plated on BP agar. The typical colonies of black and clear zone were picked to perform further coagulate test.

Statistical analysis was carried out as previously described in section 1.1.

4.3 Effects of Tom-Yum mix on shelf-life extension of fish packaged under selected gas mixtures

4.3.1 Shelf-life extension of cut fish marinated with Tom-Yum mix and packaged under selected atmospheres

Previous studies showed that 90%CO_2: 5%N_2: 5%O_2 had the highest microbiological effect, particularly on *Staphyloccus* spp. Therefore, that gas mixture was used further in the consumer test, and the survival rate of inoculated *S. aureus* and *E. coli* was carried out to confirm the natural preservative properties of Tom-Yum mix in a real food system.

Sample preparation

Live fish was obtained and prepared as mentioned in section 4.1. The treatments were designed as follows: (1) marinated with Tom-Yum mix and packaged under 90% CO_2: 5%N_2: 5%O_2 (T_1), (2) marinated with Tom-Yum mix but kept in normal air (T_2), (3) without marinating with Tom-Yum mix but packaged under the same gas mixture as in T_1 (C_1) and (4) without marinating with Tom-Yum mix and packaged in normal air (C_2). All samples were kept at 4 ±1°C and taken on days 1, 3, 6, 9, 12, 15, 18, and 21. for chemical, microbiological and sensory analyses.

Chemical analyses

- pH, Lactic acid, TVB, TMA and TBARS at 532 and 450 nm were determined as previously described.

Microbiological analyses

- Mesophilic, phycrophilic, lactic acid bacteria, *S. aureus* were determined as previously described.

- Coliforms and *E. coli* One-ml of serial dilution at 10^{-1}, 10^{-2} and 10^{-3} was carried out according to the method of Speck (1976). The results were expressed in most probable number (MPN) using three tubes at each dilution.

Sensory analysis

The marinated fish with and without Tom-Yum mix under various packaging regimes from days 1, 6, 12 and 18 were taken. The marinated fish with Tom-Yum mix was removed from the mix before the fish was sliced in to small pieces weighing approximate 10 g. Individually wrapped fish in foil was steamed for 12-15 min to reach the core temperature of 80°C. The samples were served on white plate coded with a 3-digit number. Thirty panelists consisting of housewives and students who had been trained in a fish quality course were asked to identify the sensory intensity of the attributes and consumer preference by using the 9-point scale, where 9 was extremely strong while 1 was extremely mild consumer preference was analyzed by using a 9-point hedonic scale, when 9 was like extremely while 1 was dislike extremely.

Statistical analysis

Data was computed as previously described.

4.3.2 Survival of inoculated *S. aureus* and *E. coli* in cut fish marinated with Tom-Yum mix and packaged under selected atmospheres

Bacterial culture

Stock pure cultures of *S. aureus* and *E. coli* from the Department of Food Technology, Prince of Songkla University were transferred 3 times into BHI broth yielding 10^8-10^9 cfu/ml. Each bacterium was then cultured in 150 ml of BHI broth yielding 10^8-10^9 cfu/ml, centrifuged and washed 2 times with 0.85% normal saline solution to obtain the bacterial count of about 10^9 cfu/ml. Two-hundred milliliters of each bacterium was inoculated in 25 g of fish with and without Tom-Yum mix to yield the bacterial count of 10^7 cfu/g. Samples were divided in various modified atmospheres as mentioned in section 4.3.1. The control treatment, without bacterial inoculation, was calculated from section 4.3.1. All samples were stored at 4°C for 21 days and taken every two days for analyses.

***S. aureus* analysis**

Serial dilutions were made up to 10^{-6} by using 0.1% peptone water then plated on BP agar. Typical colonies, black colonies and clear zones were picked to check for plasma clots.

***E. coli* analysis**

Serial dilutions were made up to 10^{-7} by using 0.1% peptone water then 1 ml of dilution 10^{-5},10^{-6} and 10^{-7} was inoculated into 3 tubes of LST broth following the method of Speck (1976). Positive gas producing tubes were transferred to EC broth before testing on the EMB agar. Biochemical characteristics were randomly checked. The bacterial count was reported in MPN.