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Antibacterial activity of coconut shell liquid smoke (CS-LS) and its application on fish ball preservation

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Abstract: Liquid smoking emerges as a potential substitution for traditional smoking method in preserving proteinaceous foods. The study is aimed to investigate antibacterial activity of coconut shell liquid smoke (CS-LS) and potential application of CS-LS on fish ball preservation. Minimum Inhibitory Concentration (MIC) of CS-LS is determined with broth dilution method against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. CS-LS was able to inhibit microbial activities of *P. aeruginosa* and *S. aureus*, giving 0.22% and 0.40% MIC of CS-LS, respectively. Shelf life of fish ball was increased from 16 to 32 hours, determined at 27–28°C, by applying 2.5% CS-LS. At the same concentration of CS-LS, fish ball can achieve 20 days of storage at 4±1°C, giving acceptable TPC count (1.80 log CFU/g) while maintaining stability of pH at 5.7-5.8 and slight moisture content reduction ($p<0.05$). The results indicated that CS-LS was an effective preservative agent for fish ball.

Keywords: Coconut shell liquid smoke, antibacterial activity, MIC value, fish ball preservation

Introduction

Food smoking has a long history in preservation. The use of traditional smoking methods, or recently liquid smoking, have proven that this means of preservation still being vastly practiced in community as well as in food industry. Liquid smoking in preserving protein-based foods, namely meat, fish, and cheese, has been increasingly utilized, owing to pleasant flavour and inhibitory effects on pathogens (Soldera *et al*., 2003). Preservative effect in food smoking is achieved due to the presence of antimicrobial and antioxidant compounds, such as aldehyde, carboxylic acids and phenols (Leroi and Joffraud, 2000; Rorvik, 2000). Liquid smoke has several advantages compared to traditional smoking techniques in terms of easiness of application, speed, uniformity of the product, good reproducibility of desired characteristics obtained in the final smoked food, and omission of harzardous polycyclic aromatic hydrocarbons (Hattula *et al*., 2001).

Antibacterial activities of liquid smoke have been reported in commercial application (Sunen *et al*., 2001; Milly *et al*., 2005). Satisfactory quality of liquid smoked salmon has been extensively studied (Stohr *et al*., 2001; Kolodziejska *et al*., 2002; Montero *et al*., 2003; Martinez *et al*., 2007). Similar advantages of liquid smoking have been published for swordfish (Muratore and Licciardello, 2005) and other cultured fish (Jittinandana *et al*., 2003; Sunen *et al*., 2003), However, little information is obtained for antibacterial activity of coconut shell liquid smoke (CS-LS) and its application on fish ball.

Coconut shell liquid smoke has been reported to contain phenolic compounds, such as phenol, 2-methoxylphenol (guaiacol), 3,4-dimethoxylphenols, and 2-methoxy-4-methylphenol. Dihydroxy benzoic acid, methoxybenzoic acid and hydroxy benzoic acid are present as minor acidic components in CS-LS. Neither benzo[a]pyrene nor other polycyclic aromatic compounds are reported in the CS-LS. Safety test indicated that coconut shell liquid smoke is not toxic and safe (LD_{50} value more than 15.000 mg/kg body weight of mice) (Budijanto, *et al*., 2008). Therefore, the CS-LS have a potential in increasing shelf life of proteinaceous food products.

Fish ball is a popular food in Asian countries, like Indonesia. Production of fish ball follows simple emulsification of fine flesh fish with starch, addition of salt and specific herbs to meet local tastes and finally formation of ball shape. Most fish ball is stored at low temperature. In addition, modified atmosphere packaging is used in order to extend shelf life of fish ball (Holley and Patel, 2005). Nonetheless, these steps do not eliminate or reduce undesirable or pathogenic microorganisms. Kok and Park (2007) have reported that fish ball has a relatively short shelf life of 4–5 days at around 5°C of storage. Fish ball that is distributed in uncovered bamboo baskets without refrigeration has a shelf life of only two days.

The aim of this study was to examine antibacterial activity of coconut shell liquid smoke and its application on fish ball preservation. CS-LS is expected to extend the shelf life of fish ball by reducing microbial growth rate or viability.

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Materials and Methods

Materials

Coconut shell liquid smoke (CS-LS) was obtained from small scale industry of CV. Wulung Prima (a foundation division of Faculty of Agriculture Technology, Bogor Agriculture University, Indonesia). Fish ball samples, made of Spanish mackerel, tapioca flour, spices (salt, shallot, garlic, ginger, and pepper), were obtained from traditional markets in Bogor, Indonesia. Pathogenic microorganisms of *Staphylococcus aureus* (BCC 1577) and *Pseudomonas aeruginosa* (BCC 2268) were obtained from Balitvet (Veterinary Research Station) Collection and Cultures, Bogor, Indonesia.

Determination of CS-LS antibacterial activity

Broth dilution method (Vigil *et al*., 2005) is employed to determine antibacterial activity of CS-LS against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Stock cultures of pathogens were grown in Nutrient Broth (NB; Oxoid) for 20 hours prior to inoculation.

CS-LS was serially diluted in NB. Tubes were then inoculated with 10^5 CFU/ml of a single pathogenic species. A negative control, not containing CS-LS, is prepared and inoculated with same amount of pathogen. Tubes were incubated for 24 hours at 37°C. 1 ml medium from all tubes showing no growth (no turbidity) were plated on Nutrient Agar (NA; Oxoid), used the pour plate method. The lowest concentration of liquid smoke that produced 90-91% reduction of both pathogens is defined as Minimum Inhibitory Concentration (MIC).

Application of coconut shell liquid smoke on fish ball

Flesh from Spanish mackerel was separated from bone and fibrous content. Fish ball was manufactured according to a formula: 1 kg of minced fish, 120 g of tapioca flour and 10 g of spices. All ingredients were mixed, battered and shaped into balls (2.5-2.8 cm of diameter) followed by cooking in boiling water (90–95°C) for about 15 minutes. During the cooking step, 2.5% of CS-LS was added. Preliminary study has shown that 2.5% of CS-LS was effective to fish ball based on inhibition the mucus formation and hedonic acceptability on aroma, color, taste and whole likeness parameters (data not published). Fish ball samples were packed in sterile HDPE (High Density Polyethylene) and stored at 27–28°C and 4±1°C. Untreated (0% of CS-LS) fish ball was used as negative control. Total Plate Count (TPC) (Harrigan, 1998), pH value (AOAC, 1995), and moisture content (AOAC, 1995) were determined (every eight hour at 27–28°C and four day at 4±1°C).

Total Plate Count

Five gram of each homogenated sample was diluted with 45 ml of NaCl 0.85%, followed by serial dilutions (1:10) until 10^{-7} g/ml of samples were obtained. One milliliter of diluted aliquots were placed on petri dishes and approximately 15 ml plate count agar (PCA; Himedia) was added. After agar had been solidified, all petri dishes were inverted and placed in an oven at 37°C for 48 h. Colony forming unit was counted based on ISO Standards for microbiological methods (Harrigan, 1998):

$$N = \frac{\sum c}{(n_1 + 0{,}1n_2)d}$$

Where:

$\sum c$: amount of whole bacteria colony in all dishes which contain 30 - 300 colonies

n_1 : amount of Petri dish which counted on the first dilution.

n_2 : amount of Petri dish which counted on the later dilution.

d : factor of the first dilution.

Determination of pH

A 10 g of fish ball sample was homogenized in 100 ml of distilled water and filtered (with Whatman paper no 4). pH of filtrate was measured with ORION 410A pH-meter at ambient temperature (AOAC, 1995).

Moisture content

Moisture content was determined by oven drying of 5 g of fish ball at 105°C until a constant weight was obtained (AOAC, 1995).

Statistical analysis

Determined parameters (TPC, pH, and moisture content) were obtained from two independent experiments, each replicated twice. Average values and standard deviations for TPC and pH were calculated, while moisture content of fish ball was subjected to analysis of variance (ANOVA). Duncan Multiple Range Test (DMRT) procedure was used to test for difference between means (significance was defined at $p<0.05$).

Results and Discussion

Antibacterial activity of coconut shell liquid smoke

Table 1. MIC values of coconut shell liquid smoke against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Organism	Concentration of liquid smoke (%)	Amount of initial bacteria (No, CFU/ml)	Amount of bacteria after 24 hour incubation (Nt, CFU/ml)	Inhibition (%) [100%-(Nt/No x 100%)]
Staphylococcus aureus BCC 1577	0.10	2.1×10^5	4.1×10^7	-
	0.20	2.1×10^5	2.1×10^6	-
	0.30	2.1×10^5	8.6×10^4	59.05
	0.40*	2.1×10^5	1.9×10^4	90.95
	0.50	2.1×10^5	3.0×10^2	99.86
Pseudomonas aeruginosa BCC 2268	0.14	2.1×10^5	4.7×10^7	-
	0.16	2.1×10^5	2.4×10^7	-
	0.18	2.1×10^5	2.0×10^6	-
	0.20	2.1×10^5	1.0×10^5	52.38
	0.22*	2.1×10^5	1.7×10^4	91.90
	0.24	2.1×10^5	1.1×10^4	94.76
	0.26	2.1×10^5	1.7×10^3	99.19

Results are the mean values of duplicate trials.
*MIC value is the lowest concentration which kill 90-91% of the test microorganism.

The MIC values of CS-LS against *S. aureus* and *P. aeruginosa* are shown in Table 1. Inhibitory effects of CS-LS on *S. aureus* and *P. aeruginosa* have different profiles, giving MIC values of 0.40% and 0.22%, respectively. The inhibitory effect can be attributed to presence of phenolic compounds in liquid smoke (Holley and Patel, 2005).

Phenolic compounds, such as phenol, 2-methoxylphenol (guaiacol), 3,4 dimethoxylphenols, and 2-methoxy-4-methyl phenol are prominent in liquid smoke and play a major contribution to antibacterial activity (Vitt *et al*., 2001; Sunen *et al*., 2001; Sunen *et al*., 2003; Muratore and Licciardello, 2005; Milly *et al*., 2005; Gomez-Estaca *et al*., 2007; Kristinsson *et al*., 2007; Soldera *et al*., 2008). Acidic compounds of liquid smoke have also shown antibacterial activity, but concentration of acidic compounds is much lower than that of phenolic compounds (Budijanto, *et al*., 2008). Therefore, contribution of acidic compounds to antibacterial activity of liquid smoke can be neglected.

Phenols inhibit the growth of bacteria by dose-dependently prolonging lag phase of pathogenic microorganisms, whereas the growth rate in the exponential phase remains intact, unless concentration of phenols become toxic. Phenols interfere cellular metabolism through substrate complexing, membrane disruption, enzyme inactivation and metal chelation (Russell, 2005).

Antibacterial activity of CS-LS is higher for Gram-negative bacteria than Gram-positive as shown in *S. aureus* versus *P. aeruginosa* inhibitions. Tetrapeptida chain on Gram-positive bacteria containing (L-alanil-D-isoglutaminil-L-lisil-D-alanin) and interpeptide bridge of glisin produces strong structure and can restrain damage. In addition, muramic acid unit forms crosslink with tetrapeptida and covalent bond with interpeptide bridge that also help Gram-positive bacteria survival against phenol intrusion (Thorpe, 1995).

In comparison to *S. aureus*, *P. aeruginosa* is more sensitive to CS-LS. This could be caused by larger porin protein PAO1 on cell wall which has diameter of 2 nm, comparatively to porin protein of OmpF and OmpC with the diameter 1.2 nm in *E. coli* K-12 (Helander *et al*., 1998). Porin protein can create channels large enough to allow restricted passage of small molecular mass compounds (<200 Mw), like the phenolic compounds in CS-LS, giving better access to periplasmic space, glycoprotein layer and cytoplasmic membrane (Holley and Patel, 2005).

Total Plate Count

Figure 1 highlights TPC values of fish ball with 0% of CS-LS (untreated/FL-1) and 2.5% of CS-LS (liquid smoke/FL-2) during storage at 27–28°C and 4±1°C. After 16 hours of storage at 27– 28°C, significant increase in microbial count was observed in the untreated sample, giving 6.35 log CFU/g. This would indicate spoilage and possible rejection of the sample. It has been advised that microbiological threshold of ready to eat fish products is 6.00 log CFU/g (ICMSF, 1986). TPC of untreated sample was at very high concentration (8.48 log CFU/g) after 40 hours of incubation at 27– 28°C.

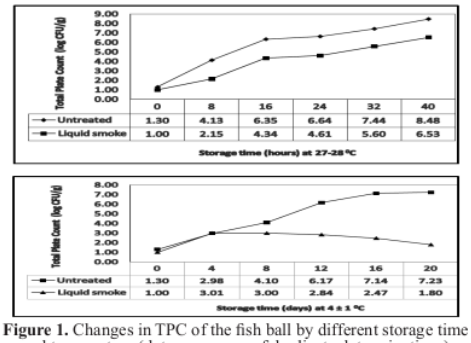

Figure 1. Changes in TPC of the fish ball by different storage time and temperature (data are means of duplicate determinations)

Inhibitory effect on microbial growth was shown in CS-LS treated fish ball, giving 6.53 log CFU/g after 40 hours of storage. It suggested two log reductions on CS-LS treated sample compared to control. CS-LS able to extend the shelf life of fish ball for 16 hours longer than control (untreated) which stored at

27–28°C.

Possible rejection on microbial quality (>6.00 log CFU/g) of untreated fish ball sample was observed after 12 days of storage at 4±1°C. On the other hand, 2.5% of CS-LS treatment was able to maintain microbial quality, giving highest recorded microbial population at 3.01 log CFU/g after 4 days of storage at 4±1°C. The value of CS-LS treated fish ball during the whole observable time-course passed the standard of ICMSF of lower than 6.00 log CFU/g. CS-LS continuedly gave unfavourable environment for microbial growth as indicated by final population of 1.80 log CFU/g at the end of experiment (20 days at 4±1°C).

Determination of pH

Coconut shell liquid smoke has initial pH of 2.0., thus we would expect reduction of pH on treated fish ball sample (Figure 2). Martinez *et al*. (2007) and Muratore *et al*. (2007) also observed reduction of pH on smoke treated fish fillets. The pH of FL-1 at 0 hour was 6.21, then decreased to 6.15 at 24 hours, and increased to 6.23 after 40 hours of storage. Stohr *et al*. (2001) reported that lowering the pH might have been caused by the metabolism of lactic acid bacteria, which previous studies have indicated as the major cause of deterioration of smoked fish.

In general, the pH value of FL-1 and FL-2 at 4±1°C increased during storage. The pH of FL-1 at day 0 was 6,26, while FL-2 was 5.75, than increased after 20 days of storage to 6.33 and 5.80, respectively. The increase of pH may be attributed to the production of volatile basic components such as ammonia, trimethylamine and other volatile compounds by fish spoiling bacteria (Ruiz-Capillas and Moral, 2001).

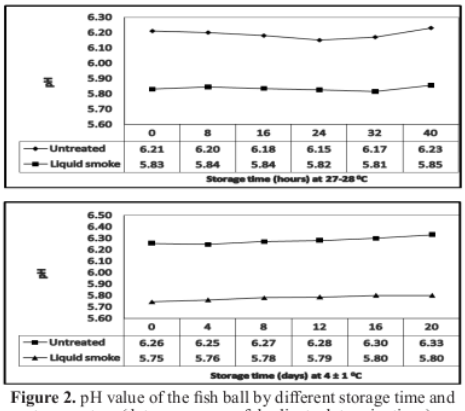

Figure 2. pH value of the fish ball by different storage time and temperature (data are means of duplicate determinations)

Moisture content

CS-LS treatment significantly reduced ($p<0.05$) initial moisture content of fish ball from 74.56% to 74.27% (Figure 3). This corresponds to the fact that liquid smoke can induce dehydration on food products (Leroi and Joffraud, 2000; Rorvik, 2000). In coherence to our report, Gomez-Guillen *et al.* (2000) showed that smoking leads to connective tissue insolubility and moisture loss, resulting texture hardening.

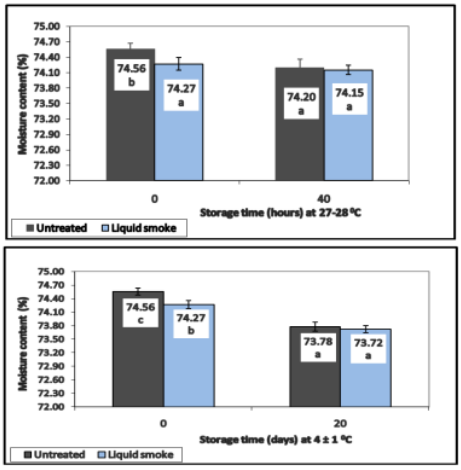

Figure 3. Moisture content of the fish ball by different storage time and temperature. Values followed by different letter are significantly different ($p<0.05$)

Significant reduction ($p<0.05$) of moisture content was also obtained on untreated fish ball that was incubated for 40 hours of storage at 27– 28°C, giving 74.20% of moisture content compared to 74.56% of initial moisture content. Similar pattern of reduction also occurred on moisture content of 40 hours incubated CS-LS treated fish ball compared to initial moisture content, but not statistically significant ($p>0.05$). Initial moisture content of untreated and CS-LS treated fish ball at 4±1°C were 74.56% and 74.27%, respectively. Prolong storage, 20 days, significantly reduce ($p<0.05$) moisture content of both fish ball samples (Figure 3). The decrease of moisture content was presumably indicated protein deterioration (Siskos *et al*., 2007).

Conclusion

CS-LS has a better inhibitory effect on *P. aeruginosa* in comparison to *S. aureus*, giving MIC value of 0.22% and 0.40%, respectively. Shelf life at 27–28°C is increased from 16 to 32 hours by applying 2.5% of CS-LS on fish ball. Storing in lower temperature, 4±1°C, extends the shelf life of CS-LS treated fish ball up to 20 days based on microbial quality. CS-LS was able to reduce two log CFU/g of microbial population on fish ball after 16 hours of

incubation at 27-28°C, while giving six log CFU/g reduction after 20 days of storage at 4±1°C. During storage at 27–28°C and 4±1°C, pH of fish ball was slightly increased but moisture content was slightly decreased.

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