Research Article

The Optimization of Aerobic Bacteria Inactivation in Tilapia (*Oreochromis niloticus*) Fillets using Micro-Nano Bubbles of Carbon Dioxide and Shelf-Life Extension

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Abstract
This study aims to examine the influence of NaOCl, NaCl and contact time on the inactivation of aerobic bacteria in tilapia fillets treated with micro-nano bubbles of CO_2 (CO_2 MNB) in a washing process of fish fillets, and compared to soaking with tap water and untreated fillets for their shelf-life extensions. Response surface methodology (RSM) with a central composite design was used to compare and predict of the inactivation effects. The fish fillets were soaked in a NaOCl solution before washing with a NaCl solution and CO_2 MNB produced from an MNB generator system, maintaining the liquid temperature in the range of 4–7 °C for all experiments. According to the regression analysis from the experimental design, aerobic bacteria inactivation was reduced by 1.509 log CFU/g at 100 mg/L NaOCl, 10%w/v NaCl, and a contact time of 32 min with CO_2 MNB. The experimental value of the reduction of aerobic bacteria by 1.503 ± 0.009 log CFU/g (before washing 5.623 log CFU/g; after washing 4.120 log CFU/g) was found after treatment under the aforementioned condition. The number of aerobic bacteria counted on the tilapia fillets treated with the upper condition after being stored at 4 ± 2 °C for 7 d was below the acceptable limits, but untreated and treated with tap water had bacteria counts exceeding the upper microbial limit (6 log CFU/g). The combined results showed that the NaOCl, NaCl solution and CO_2 MNB treatment could extend the storage time by more than 7 d.

Keywords: Aerobic bacteria, Carbon dioxide Micro-Nano bubble, Central composite design, Inactivation, Tilapia fillet, Response surface methodology

1 Introduction

In the past decades, the demand for fish and fish products has been growing worldwide because fish are a variety of sources of high-quality protein, various vitamins (A, D, E, and K), essential fatty acids, and minerals [1]. Tilapia (*Oreochromis niloticus*) is an economically important species of freshwater fish in Thailand. According to data collected in 2021, the aquaculture industry yielded approximately 200,000 tons of farm-produced tilapia, of which more than 5,000 tons were exported [2]. Domestic and international demand for fresh tilapia has increased over the past decade due to its high nutritional value and superior organic properties. As a result, there is an increasing demand in both the domestic and export markets because fresh fish is a highly perishable commodity, and consumers prefer fresh products rather than frozen products. However, shelf-life extension in order to retain its quality attributes as long as possible and to yield safe products is therefore essential for both producers and consumers [3]. Despite the availability of cooling facilities and transportation systems, the distribution of fresh fish remains a problem, particularly in tropical countries such as Thailand, due to the deteriorating quality of the fish. After the fish dies, its entire body's defense mechanisms fail, allowing bacteria or the enzymes secreted by them to attack fish tissues, resulting in spoilage bacteria and the deterioration of fish products [4]. For this reason, the shelf life of fish and other seafood products is limited for fresh products [5]. The

key point for retaining high quality products is to retard deterioration as much as possible, for example through modified atmosphere packaging, radiation, and chemical or organic compounds, combined with low temperature storage [6]. Although effective modern preservation techniques are available, the use of dipping, soaking, or washing in solutions is simple, including washing with disinfectant solution or washing with micro-nano bubbles (MNBs) before low temperature storage.

MNB technology has been applied in various areas such as science, engineering, medical, agricultural, and food sectors because MNBs are large specific surface areas, charged surfaces, high dissolved gas and long residence time in solution compared to macrobubbles (2–5 mm) [7]. MNBs can be produced from various types of generators, such as the rotary liquid flow type, the ejector type, the ultrasonic type, the venturi type and the decompression type [8]–[10]. Among them, the decompression type MNB generator has attracted great attention due to the good stability of its bubbles [10]. When the pressure is increased, the solubility of the gas increases, resulting in an increase in the amount of gas dissolved in the solution. Afterward, the pressure suddenly drops and the gas state in the solution changes from saturation to supersaturation, resulting in a large number of MNBs being generated [11]. In food research, the combination of MNBs with some type of gas can inactivate microorganisms after treatment, such as ozone [12] and carbon dioxide [13]. However, ozone is a highly reactive oxygen species, which might affect fish tissues [7]. Thus, carbon dioxide is more interesting than ozone in this study. In previous research, the inactivation of suspended *Escherichia coli* in distilled water was investigated using different types of gas bubbles, including carbon dioxide, nitrogen, and argon [14]. The results showed that carbon dioxide (CO_2) gas bubbles have better inactivation efficiency for *Escherichia coli* than other gas bubbles, and the number of surviving *Escherichia coli* was reduced by 0.76 log CFU/mL after CO_2 bubble treatment at 4–10 °C for 40 min. The inactivation capability of CO_2 depends on the dissolved CO_2 concentration, exposure time, and number of bacteria in the solution. The amount of gas in solution is represented in terms of gas solubility, which is affected by system temperature and pressure. For example, the solubility of CO_2 at 10 °C is around 1 mol/L, decreasing to 0.7 mol/L at 20 °C [15]. Consequently, the adhesion intensity attained is sufficient to attach many bacterial cells and directly helps the diffusion of CO_2 in the aqueous phase into the cell via a bacterial membrane, resulting in cell death. Not only the dissolved CO_2 has an effect on cell inactivation, but also the decompression type MNB generated from cavitation and shear forces can enhance cell inactivation by the contact between CO_2 and the cell membrane [13].

On the other hand, NaCl and NaOCl have been conventionally used in the preservation of food products for many years. NaCl alone without any other chemicals has been used to preserve fish in the past decades because the ability of NaCl to inhibit autolytic activity in fish products extends the shelf life of fresh fish [16]. However, McDermott *et al*., [17] found that immersion crab (*Cancer pagurus*) meat in a 5% NaCl solution for 30 s had no significant effect on mesophilic and psychrophilic bacteria. This indicates that bacterial inactivation does not only vary with NaCl concentration but also changes with contact time. Additionally, increasing NaCl concentration in the MNB solution increased bubble-solution surface tension, resulting in bubble coalescence inhibition [18]. In a comparison of the effects of adding NaCl concentrations to solutions with CO_2 gas bubbles, it was found that bubble coalescence was 87% for 0.0004% NaCl and 0% for 0.68% NaCl and the inactivation of bacteria in solutions with CO_2 gas bubbles was more effective than treatment with NaCl solution alone [19]. NaOCl solution is widely used in the washing process for microbial inactivation particularly in fresh seafood products [20] and fish products because of its highly powerful oxidizing property [21] in the form of chlorine. The amount of chlorine used in the washing process is normally 200 mg/L for whitefish and slaughtered salmon [22]. Moreover, gilthead seabream (*Sparus aurata*) fillets treated with 100 mg/L NaOCl for a dipping time of 10 min reduced the psychrotrophic bacteria slightly (0.3 log CFU/g) [22]. The use of NaOCl before NaCl combined with CO_2 MNB for washing in food has not been previously reported. However, the use of NaOCl combined with ozone was reported that mesophilic aerobic count of tilapia fillets treated with the 5 mg/L chlorine and 5 mg/L chlorine combined with 5 mg/L ozone had reductions of 0.49 and 0.56 log CFU/g, respectively [12], while ozone treatment alone had no significant difference in the reduction in microbial counts compared to control.

As mentioned above, the efficiency of inactivation depends on the types of additives, the concentration of additives, the contact time, and the pathogen strains. The objective of this work was to

investigate the inactivation of aerobic bacteria on tilapia fillets during a washing step using dissolved CO_2 from MNB treatments and the potential extension of the shelf life of the tilapia fillets. For this purpose, three factors, concentrations of two additives (NaCl and NaOCl) and contact time, were utilized in the central composite design (CCD) with response surface methodology (RSM). After the optimum condition from a prediction model was verified, the shelf life of the tilapia fillets was conducted to determine the microbial growth during storage at 4 ± 2 °C for 7 d. The results of the prediction model will be useful for commercial applications in the food industry by using new technology from the dissolved CO_2 from MNBs with food additives to maintain and control the microbes under food safety standards.

2 Materials and Methods

2.1 *Experimental design*

The response surface methodology was used to optimize the inactivation condition by implementing the central composite design (CCD). The CCD was employed in 2^3 factorial experiments (Table 1), and twenty experiments were performed to evaluate the effects of NaOCl, NaCl and contact time on aerobic bacteria inactivation on tilapia fillets. In the CCD, the central point was replicated six times in order to evaluate the experimental errors of the model by using Minitab 19 Statistical Software.

Table 1: Variables and levels used for the central composite design.

Variables	Levels				
	−1.68	**−1**	**0**	**+1**	**+1.68**
NaOCl (mg/L)	40	52.16	70	87.84	100
NaCl (%w/v)	4	5.22	7	8.78	10
Time (min)	16.6	20	25	30	33.4

2.2 *Fish preparation*

The tilapia for the present study with the weight in the range of 0.8 to 1 kg were purchased from a local fish market, the Suea Yai Uthit Market, in Bangkok, Thailand and were washed with tap water and cut into fillets. Next, the fish fillets were placed in storage at 4 ± 2 °C for 15–16 h after cutting. Then, they were laid on crushed ice with a fish/ice ratio of 1:3 (w/w) and transported to a laboratory within 1 h. Afterward, the fish fillets are washed with running tap water again, de-skinned and cut into fillets with a thickness of 0.8–1 cm and an average weight of 10 g.

2.3 *Apparatus and its preparation*

The MNB washing process with about 5–35 μm diameter bubbles (Figure 1) consisted of an MNB generator (3) (Fuan USR pump Co., Ltd., China model 25QYB-2, 1.1 kW), a CO_2 supply system, a coil (7), a washing tank (8), and a cooling tank (9). As well, the MNB washing process was fabricated from food-grade PVC pipes and valves, a washing tank, a cooling coil, and a pressure tank made from stainless steel. Next, the CO_2 gas (Bangkok Industrial Gas Co., Ltd., Thailand, with 99.5% food grade) and the liquid from the washing tank were introduced to the pump suction. The CO_2 gas flow rate was controlled by a flowmeter with a needle valve; the pump discharge pressure was administered by the valve (6). At the same time, the CO_2 gas under the pump discharge pressure in the pressure tank was dissolved into the liquid much better than it was under atmospheric pressure. After the pressure of this mixture of CO_2 gas and liquid was released via the valve (6), many MNBs were produced. The system was washed for 5 min with 6 L of tap water and 20 mL of dishwashing solution. Then it was rinsed twice with tap water. Finally, it was washed again with 100 mg/L of NaOCl solution for 15 min and then rinsed four times again with tap water.

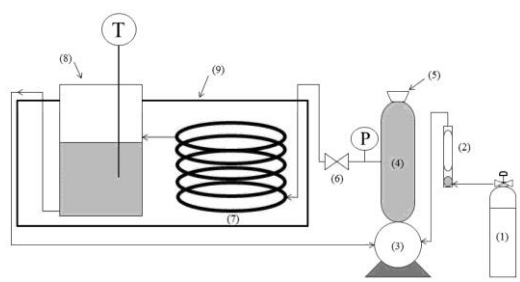

Figure 1: Flow diagram of the MNB washing process: (1) CO_2 gas tank, (2) flow meter, (3) MNB generator, (4) pressure tank, (5) gas release valve, (6) valve, (7) coil, (8) washing tank, (9) cooling tank, (P) pressure gauge and (T) thermometer.

2.4 *Experimental procedure*

First, the tilapia fillets were soaked in a NaOCl solution (Krungthpchemi Co., Thailand) in different concentrations (40, 52.16, 70, 87.84 and 100 mg/L) for 10 min before washing in the MNB tank (8). NaCl (Loba, India) was individually dissolved in the

distilled water to obtain a final concentration of 4%, 5.22%, 7%, 8.78% and 10% (w/v). Then, the system was filled with 6 L of NaCl solution, and the circulating liquid flowrate was controlled at a 15 L/min, CO_2 feed rate of 2 L/min, a discharged pressure of 0.47 MPa and a temperature in the range of 4–7 °C for all of the experiments. After 25 min of the washing process, the pH of the solution in the washing tank was measured using a portable pH meter (Mettler Toledo model FP20 pH meter) and the results were in the pH range of 3.95 ± 0.02. Moreover, the fish fillets were soaked in the solution inside the washing tank (8) for 16.6, 20, 25, 30 and 33.4 min and subsequently dipped in distilled water once. In this case, the ratio of the fish fillets and washing solution was 1:200 (30 g of 3 pieces of fish: 6000 mL of washing solution in the overall system). Finally, the fish fillets before and after the treatments were taken for pH measurement and aerobic bacteria determination.

2.5 *Shelf-life study of refrigerated tilapia fillets*

The tilapia fillets were immediately packed in sterilized 260 mL seal bags with a high density polyethylene double zipper 1 piece/1 bag after washing by the CO_2 MNB process. Other two sets of the tilapia fillets with different conditions, namely, the unwashed sample and the tap water washing for 2 min were also packed in the sterilized seal bags. Next, all of the samples were stored at 4 ± 2 °C for a period of 7 d, and then their pH and aerobic bacteria were measured on 0, 2, 5 and 7 d.

2.6 *Microbial analysis and enumeration*

Enumeration of aerobic bacteria was conducted in both a tilapia fillet and the washing solutions (before and after washing). To begin the microbial analysis, the tilapia fillet (10 g) was homogenized thoroughly with 90 mL of distilled water in a sterilized homogenizer (Otto model CP-390A) for 1 min, and the homogenate was subjected to pH measurement by a pH meter (Mettler Toledo model FP20 pH meter) and then used for aerobic bacteria determination. As 1 mL of the diluted solution was reduced into the serial dilution (1:10), 1 mL of each diluted sample was dropped onto a commercial count plate (3M Aerobic Petrifilm™ count plate). Next, all of the plates were incubated at 35 °C (+1 °C) for 48 ± 3 h in an incubator (Dae Yang ETS Co., Ltd., Korea). Colony counts were expressed as log CFU/g. By subtracting the log CFU/g for the corresponding treatments (log N) from the log CFU/g of the samples before washing (log N_0), the response variable, since log (N/N_0) = log (N) – log (N_0), was obtained. For the enumeration of aerobic bacteria in the washing solutions, each 1 mL of the washing solution from the beginning and the end of the washing process was also taken for aerobic bacteria determination.

2.7 *Experimental design and statistical analysis*

Initially, the polynomial model in Equation (1) was investigated in order to describe the effect of the independent variables (NaOCl, NaCl and time) on the inactivation of the aerobic bacteria using multiple regression analysis, performed by using commercial software (Minitab 19 Statistical Software) with a 95% confidence level. All of the experiments were conducted with three replicates. Analysis of variance (ANOVA), goodness of fit tests, mean square error (MSE) and lack of fit were used to analyze the effect of the different variables on microbial inactivation. Experimental data was analyzed using a response surface regression fitted to a second-order polynomial model:

$$\text{Log}\,(N/N_0) = B_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j>i}^{3} B_{ij} X_i X_j + \varepsilon \quad (1)$$

where
N_0 is the initial number of aerobic bacteria before washing
N is the final number of aerobic bacteria after washing
X is the variable (NaOCl NaCl and time)
B is the regression coefficient, and
ε is the experimental error.

3 Results and Discussion

3.1 *Aerobic bacteria inactivation*

Response surface methodology was applied to study the relationships between the inactivation (response, log N/N_0) of the aerobic bacteria on tilapia fillets using CO_2 MNB treatments at 4–7 °C for each condition and the three inactivating parameters, including concentration of NaOCl, NaCl and contact time. The parameters and experimental responses are presented in Table 2. The most effective result of aerobic bacteria reduction (1.286 log CFU/g reduction) was obtained at 70 mg/L NaOCl, 10% NaCl and 25 minutes, while the worst reduction (0.322 log CFU/g reduction) was conducted at 70 mg/L NaOCl, 7% NaCl and 16.6 min. This implies that CO_2 MNB treatment can effectively reduce the aerobic bacteria

following soaking in NaOCl solution. The details will be explained next.

The aerobic bacteria were significantly reduced with an increase in the concentration of NaOCl. For example, trials 11 and 12 showed that, with increasing concentrations of the NaOCl solution from 40 to 100 mg/L at 25 min and 7% NaCl, inactivation was a 0.597 and a 0.916 log CFU/g reduction, respectively. According to the hydrolysis reaction with water after adding NaOCl to the solution, hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) were formed. Upon inactivation by OCl^-, the rupture or disintegration of the microbial cell wall and membrane appeared to occur, and then the OCl^- would inactivate the functional proteins localized in the plasma membrane. Moreover, HOCl can pass through the lipid bilayer of the plasma membrane by passive diffusion, thereby increasing the inactivation activity [23]. The experimental results are similar to those of Loan *et al*., who studied gilthead seabream (*Sparus aurata*) fillets treated with 100 mg/L NaOCl for a dipping time of 10 min and found that psychrotrophic bacteria were reduced slightly by 0.3 log CFU/g [23].

The total number of aerobic bacteria was significantly reduced in the treatment groups at all concentrations of NaCl, and the highest level of bacterial inactivation was achieved at the concentration of 10% NaCl. At a high concentration of NaCl (10%) with 70 mg/L of NaOCl solution (trial 10), the aerobic bacteria inactivation was at its highest value at a 1.286 log CFU/g reduction at 25 min for this experiment. In addition, adding the concentrations of 4% NaCl (trial 9) and 7% NaCl (trial 15) at 25 min and 70 mg/L of NaOCl, there were 0.402 and 0.746 log CFU/g reductions, respectively. In comparison with soaking at 1% NaCl solution for tilapia fillets [24], 3% and 9% NaCl solution for mackerel fillets [25], 10% NaCl solution has a higher concentration. However, all samples from experiments were subsequently dipped in distilled water once before being packed in the sterilized seal bags. The disadvantage of higher sodium contents in fish fillets is a risk factor for several health problems [26], but the advantage obtained from the solution containing NaCl is bacterial inhibition.

Table 2: Central composite design arrangements and experimental results of the inactivation of aerobic bacteria on tilapia fillets after CO_2 MNB treatments at 4–7 °C for each condition.

Trial	NaOCl (mg/L)	NaCl (%w/v)	Time (min)	Inactivation (log N/N_0)*
1	52.16	5.22	20	–0.333 ± 0.001
2	52.16	8.78	20	–0.756 ± 0.001
3	87.84	5.22	20	–0.502 ± 0.004
4	87.84	8.78	20	–0.908 ± 0.003
5	52.16	5.22	30	–0.425 ± 0.002
6	52.16	8.78	30	–1.050 ± 0.000
7	87.84	5.22	30	–0.744 ± 0.001
8	87.84	8.78	30	–1.196 ± 0.005
9	70	4	25	–0.402 ± 0.006
10	70	10	25	–1.286 ± 0.000
11	40	7	25	–0.597 ± 0.007
12	100	7	25	–0.916 ± 0.009
13	70	7	16.6	–0.322 ± 0.000
14	70	7	33.4	–0.792 ± 0.010
15	70	7	25	–0.746 ± 0.004
16	70	7	25	–0.746 ± 0.004
17	70	7	25	–0.759 ± 0.001
18	70	7	25	–0.800 ± 0.022
19	70	7	25	–0.804 ± 0.009
20	70	7	25	–0.816 ± 0.001

*Mean and SD of the three analytical replicates

Table 3: Analysis of variance values of the response effects.

Source	Df	SS	MS	*F*-Value	*P*-Value
Model	9	1.2945	0.1438	102.32	<0.0001
NaCl	1	0.8425	0.8425	599.37	<0.0001
NaOCl	1	0.1279	0.1279	91.01	<0.0001
time	1	0.2132	0.2132	151.66	<0.0001
NaCl*NaCl	1	0.0114	0.0114	8.13	0.017
NaOCl*NaOCl	1	0.0001	0.0001	0.08	0.779
time*time	1	0.0776	0.0776	55.21	<0.0001
NaCl*NaOCl	1	0.0046	0.0046	3.27	0.100
NaCl*time	1	0.0077	0.0077	5.50	0.041
NaOCl*time	1	0.0026	0.0026	1.85	0.204
Error	10	0.0141	0.0014		
Lack-of-Fit	5	0.0091	0.0018	1.82	0.263
Pure Error	5	0.0050	0.0010		
R^2		98.93			
Adjusted R^2		97.96			

$P < 0.05$ is considered as significant.
Df = degrees of freedom; SS=sum of squares; MS=mean square.

The addition of NaCl to the CO_2 washing process represented two positive effects, namely, no aerobic bacteria in the solution and the inhibition of gas bubble coalescence. The numbers of aerobic bacteria were determined at the beginning and the end of the washing process for all the experiments when NaCl was added, and the results were undetectable. This implied that an inactivation process occurred in the free bacteria removed from the fish fillet surfaces during the washing process. For the inhibition of gas bubble coalescence with the addition of NaCl, which was mentioned as indicated above by Sanchis *et al*., who reported that bubble coalescence decreased with increasing NaCl concentration in the solution and no bubble coalescence after adding 0.68% NaCl in the solution [19]. This result agreed well with this study by representing the number of bubbles observed with an increase in NaCl concentration from 4% to 10%. In the CO_2 MNB solution, the mechanism of inactivation by using the CO_2 MNB treatment with NaCl solution is yet unknown, but it could be brought about by two factors. First, synergistic effects of low pH in NaCl solutions were obtained. Since CO_2 MNB treatment created a solution pH of about 4.0, the effective inactivation might occur with two factors rather than with NaCl alone. This result is supported by Shabala *et al*., who studied the effects of acid and NaCl on *Listeria monocytogenes*, wherein the treatment with hydrochloric acid (pH 3.5) or 14% NaCl alone was less effective than combined treatment [27]. Van der Waal *et al*., also reported that *Staphylococcus aureus* and *Enterococcus faecalis* were reduced more rapidly when treated with malic acid, citric acid, or potassium sorbate combined with NaCl than when treated with acid alone. Second, NaCl helped to increase the number of MNBs [28]. These significant capabilities, which include shock waves generated by the collapse of MNBs, cavitation and shear force [29], caused the bacteria to detach from the fish fillet surfaces and fall into the washing solution. When increasing NaCl in an acidic solution, the bubble surface tension increases, which then decreases the size of the bubbles. Additionally, the NaCl addition in the acidic solution caused the coalescence inhibition of the bubbles and had an outstanding effect on the number of bubbles in the CO_2 MNB treatment [30].

Contact time is a critical factor that significantly impacts both product quality and operating costs in washing processes. The number of aerobic bacteria was reduced with the increase in contact time until it reached 25 min. For example, the number of aerobic bacteria on the tilapia fillets at concentrations of a 7% NaCl and 70 mg/L NaOCl solution decreased with increased contact time from 16.6 (trial 13) to 25 min (trials 15–20, center point) but there was no significant change in those on the tilapia fillets when the contact time increased from 25 min to 33.4 min (trail 14). Trial 1 and trial 5 also showed the inactivation to be 0.333 and 0.425 log CFU/g reduction with increasing contact time from 20 to 30 min, respectively. This can be explained by the fact that most of the aerobic bacteria on the fish surfaces are washed out by MNBs into the solution during the first 25 min.

3.2 *Statistical analysis for aerobic bacteria inactivation*

The analysis of variance (ANOVA) results obtained from the response surface (regression) model in Equation (2) were analyzed and are shown in Table 3. The goodness of fit for the model was also evaluated using coefficients of determination (R^2) and adjusted coefficients of determination (R^2 adj) at 98.93 and 97.96, respectively. Moreover, the lack of fit was non-significant (p-value = 0.263 > 0.05) and "pure error" was rather low. The F-value of the model was 102.32 and the p-value was lower than 0.005 (p-value < 0.0001), indicating that the model was significant. These criteria indicated that this model satisfactorily described the experimental data and that the model was appropriate for predicting the observed data in the present study. The regression model was:

$$\log(N/N_0) = 2.052 - 0.00690\text{NaOCl} + 0.0191\text{NaCl} - 0.1332\text{T} + 0.000009\text{NaOCl}*\text{NaOCl} - 0.00885\text{NaCl}*\text{NaCl} + 0.002935\text{T}*\text{T} + 0.000754\text{NaOCl}*\text{NaCl} - 0.000202\text{NaOCl}*\text{T} - 0.00349\text{NaCl}*\text{T} \quad (2)$$

where
$\log(N/N_0)$ is the log reduction
NaOCl is sodium hypochlorite concentration (mg/L),
NaCl is sodium chloride concentration (%w/v) and
T is time (min).

The linear terms NaOCl, NaCl and contact time were highly significant (p-value < 0.0001) for aerobic bacteria inactivation on tilapia fillets using CO_2 MNB treatments at 4–7 °C, but the quadratic terms showed only both NaCl and contact time to be significant (p-value < 0.05) and NaOCl was not significant (p-value > 0.05). The F-value for each linear term shows the degree to which it affects the experimental model from largest to smallest [31], indicating that NaCl has the most impact, followed by contact time and NaOCl.

The interaction term between the concentration of NaCl and contact time was significant for the inactivation. On the other hand, the interaction between contact time and the concentration of NaOCl, and the interaction between the concentration of NaCl and NaOCl were not significant for the inactivation (*p*-value > 0.05), meaning that the individual parameters were independent of each other. This result agreed with the experiment that fish fillet was sequentially treated by soaking in a NaOCl solution before being washed in NaCl solution with the CO_2 MNB treatment. However, adding higher concentrations of NaOCl in the first step followed by NaCl in the CO_2 MNB treatment can individually help reduce the number of aerobic bacteria.

The response surface plots and the contour plots of the aerobic bacteria inactivation on tilapia fillets using CO_2 MNB treatments for each condition at the center point of the CCD are displayed in Figure 2. Figure 2(a) shows the number of aerobic bacteria on tilapia fillets using CO_2 MNB treatments with a constant contact time of 25 min decreased with increasing NaOCl concentration and NaCl percentage. Aerobic bacteria inactivation was the highest at high concentrations of NaOCl (100 mg/L) and an NaCl percentage of 10% resulting in less than a 1.2 log CFU/g reduction. Figure 2(b) shows that the inactivation of aerobic bacteria on tilapia fillets was highly improved during the contact time in the range of 25–34 min and the NaCl percentage was higher—up to 10%. Finally, Figure 2(c) shows that NaOCl has the least effect on the aerobic bacteria inactivation on tilapia fillets. According to the response surface plots, the addition of NaCl to the washing process using CO_2 MNB treatments was significant for the reduction of aerobic bacteria on tilapia fillets.

Based on the RSM results, the maximum aerobic bacteria inactivation on tilapia fillets using CO_2 MNB treatments at 4–7 °C was determined at 100 mg/L NaOCl, 10% NaCl, and a contact time of 32 min (optimum CO_2 MNB process). The predicted value of aerobic bacteria reduction was 1.509 log CFU/g. In order to check the validity of the quadratic model in Equation (2), a test sample was treated under the abovementioned conditions. An aerobic bacteria reduction of 1.503 ± 0.009 log CFU/g (n = 3) was found, which was not a significant deviation from the predicted value of 1.509 log CFU/g. This indicated that this model is suitable for predicting the reduction of aerobic bacteria on tilapia fillets using CO_2 MNB treatments.

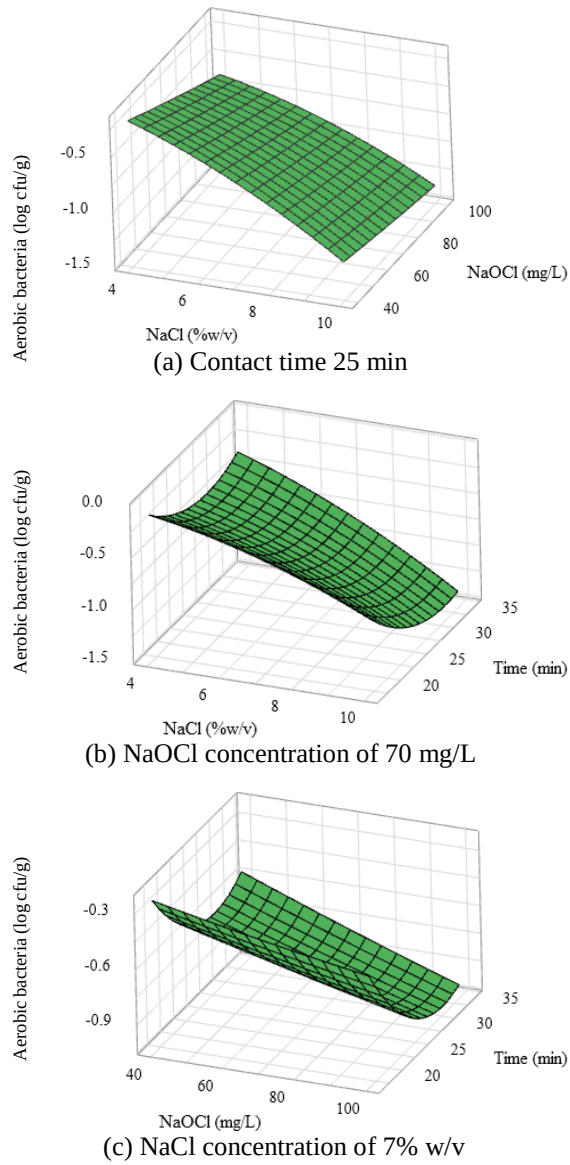

Figure 2: Response surface plots and contour plots of aerobic bacteria inactivation on tilapia fillets. (A): effect of NaCl (%w/v) and NaOCl (mg/L). (B): effect of NaCl (%w/v) and contact time (min). (C): effect of NaOCl (mg/L) and contact time (min).

Table 4: Changes in aerobic bacteria counts (log CFU/ g) in tilapia fillets stored at 4 °C in different treatment conditions.

Treatment	Storage time (day)							
	0		2		5		7	
	Aerobic Bacteria	pH	Aerobic Bacteria	pH	Aerobic Bacteria	pH	Aerobic Bacteria	pH
Control	5.623±0.000	6.46±0.06	5.826±0.028	6.37±0.11	6.653±0.057	6.60±0.03	6.957±0.044	6.70±0.13
Tap water	5.364±0.060	6.36±0.01	5.511±0.046	6.21±0.04	6.330±0.044	6.38±0.06	6.677±0.029	6.56±0.04
CO_2 MNB	4.121±0.009	6.27±0.02	4.344±0.146	6.00±0.11	5.089±0.035	5.98±0.01	5.340±0.151	6.15±0.05

*Mean and SD of the three analytical replicates

In comparison with other additives and techniques, the mesophilic aerobic bacteria reduction of tilapia fillets treated under the optimum CO_2 MNB process from this research represents a better result than those treated with 5 mg/l NaOCl combined with 5 mg/L ozone solution [12]. However, Abouel-Yazeed [5] reported psychrophilic bacteria counts of tilapia fillets soaked with a 2% solution of sodium tripolyphosphate to be 2.25 log CFU/g reduction before keeping under modified atmosphere packaging.

3.3 *Shelf-life of refrigerated tilapia fillets*

Aerobic bacteria inactivation in tilapia fillets was found to be dependent on the type of treatment process, the type of additive, the amount of each used, and the treatment conditions. The shelf-life of tilapia fillets treated under the optimum CO_2 MNB process was compared to the untreated sample (control) and tap water washing. The result showed that the initial number of aerobic bacteria counted on the tilapia fillets for the untreated sample, tap water washing, and optimum CO_2 MNB treatment was 5.623, 5.362 and 4.120 log CFU/g, respectively. The CO_2 MNB treatment resulted in the lowest count of aerobic bacteria, but all of them showed values under the upper limits for consumption of fresh fish proposed by the ICMSF (1992) of 6 log CFU/g [32]. To extend the shelf life, the initial bacteria should remain as low as possible. The washing process is an essential process to eliminate aerobic bacteria from the fish surfaces as much as possible. The CO_2 MNB treatment is one of the most effective washing processes for bacteria reduction before storage.

Table 4 shows that the aerobic bacteria count (log N/N_0) of all the samples increased with increasing storage time at 4 ± 2 °C from the initial day to 7 d. Although the increase of the storage time as long as possible is the target for fish producers, the aerobic bacteria count is restricted to below the standard acceptable limits. The number of aerobic bacteria in tilapia fillets during storage at 4 ± 2 °C on day 7 for the untreated sample, tap water washing and the optimum CO_2 MNB treatment were 6.987, 6.699 and 5.342 log CFU/g, respectively. This indicated that only the tilapia fillet treated under the optimum CO_2 MNB process was below the acceptable limits (<6 log CFU/g). The control group and the group treated with tap water had bacteria counts exceeding the upper microbial limit of 6 log CFU/g for fresh fish on day 7 of storage. This increase in aerobic bacteria could be explained by the fact as stated earlier that the entire body's defense mechanisms failed after the fish died, allowing microorganisms from the outer surfaces (skin and gills) to diffuse and react with the flesh [4]. This was similar to the work of Abouel-Yazeed, who studied maintaining the quality and extending the shelf life of tilapia during storage at 4 °C [5]. It appeared that fresh tilapia packaged in an air package had psychrotrophic aerobic bacteria counts that increased with increasing time of storage at 4 °C and exceeded 7 log CFU/g at 6 d.

The detection of pH is one of the most frequently used physical quality controls for fish and fish products, which is affected by changes in lipid hydrolysis, microorganisms, or enzymes [33]. Moreover, the relationship between pH and fish freshness is a relevant quality parameter. Changes in the pH values of tilapia fillets during storage at 4 ± 2 °C are presented in Table 4. The initial pH value (initial day) of the untreated fish sample was 6.46, indicating that the pH value of live fish muscle is close to 7.00, but post-mortem pH can range from 6.00 to 7.00 depending on the species, season, and other factors [34]. The data indicated that the samples washed with tap water and the optimum CO_2 MNB process showed a slight decrease in pH values after washing on the initial day, which were 6.36 and 6.27, respectively. The decrease in the pH of the fish washed with tap water on the initial day may be due to the washing away of spoilage bacteria on the surface of the fish fillets [35]. In the case of the fish sample washed in the optimum CO_2 MNB process, it could be explained that CO_2 was dissolved in the water and the majority

of dissolved CO_2 was diffused into the fish fillets, causing a decrease in the pH value in the fish sample while a small amount of dissolved CO_2 was hydrolyzed to form carbonic acid [36]. The dissolved CO_2 diffuses into the phospholipid bilayer of the membranes, resulting in a decrease in intracellular pH and bacterial cell death [37]. On day 2, the fish samples showed a decrease in pH value from day 0 for all conditions. This might have occurred from the lactic acid being formed from glycogen [4]. The pH value from day 2 to day 7 then increased slightly. This observation was similar to that of Abouel-Yazeed during storage at 4 °C for tilapia fish fillets [5]. The increasing pH value in fish muscle is associated with the production of alkaline compounds, such as ammonia, resulting from the protein decomposition processes by spoilage bacteria [38]. The increasing pH values from day 2 to day 7 of untreated fillets and tap water washing were 6.70 and 6.56, respectively, but the fish samples pretreated with the optimum CO_2 MNB treatment were slightly lower (6.15). It is currently unknown why the pH of fish samples pretreated with the optimum CO_2 MNB treatment was lower than that of the other samples. However, this result might be explained by the fact that spoilage bacteria were inhibited when dissolved CO_2 in solutions diffused into the fish fillets [13]. Tilapia is an economic fish from Thailand, but it is difficult to export because fresh fish is a highly perishable commodity. As a result, the shelf-life extension of fish is critical to its development. CO_2 MNB combined with additives is one of the effective washing processes for fish, meat and vegetable preservation methods.

4 Conclusions

In the present study, an RSM with a CCD was applied to investigate the operational conditions of NaOCl, NaCl and contact time in relation to the inactivation of aerobic bacteria treated with CO_2 MNB in a washing process of tilapia fillets, compared to untreated samples and soaking with tap water for their shelf-life extensions. In the theoretically optimal results, the maximum aerobic bacteria inactivation on tilapia fillets using CO_2 MNB treatments at 4–7 °C was calculated to be 100 mg/L NaOCl, 10% NaCl, and with a contact time of 32 min. Under these conditions, the experiment of the aerobic bacteria reduction was 1.503 ± 0.009 log CFU/g, compared to the predicted value to be a 1.509 log CFU/g reduction, which was not a significant deviation. The aerobic bacteria were slightly reduced as the concentration of NaOCl increased, while CO_2 MNB with NaCl addition created synergistic effects to reduce the aerobic bacteria counted on the tilapia fillets after washing. The results showed that the initial aerobic bacteria counted on the tilapia fillets for the untreated sample, tap water washing and optimum CO_2 MNB treatment were 5.623, 5.362 and 4.120 log CFU/g, respectively. In the shelf-life study, the samples after treatment with the optimum CO_2 MNB and incubation at 4 ± 2 °C for 7 d showed the number of aerobic bacteria in tilapia fillets lower than the upper limits (<6 log CFU/g). Therefore, the optimum CO_2 MNB conditions for the washing of tilapia fillets are reliable and can be applied to the fresh fish washing process before on-shelf storage in markets and food industry. Additionally, the shelf-life extension of fresh fish will contribute to the reduction of fresh fish waste and increase the potential for fresh fish exports.

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Author Contributions

P.N. and A.P.: conceived and planned the experiments and made final evaluations of the results; P.N.: conducted the experiments and wrote an original draft; A.P.: supervising, reviewing, editing, funding acquisition and project administration. All authors have read and agreed as regards the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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