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Research Article

Effect of nisin on the survival of *Staphylococcus aureus* inoculated in fish balls

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Abstract

The inhibitory effect of nisin at concentrations of 0-20 µg/ml was determined against *Staphylococcus aureus* (approximately 7 log cfu/ml) and the suitable inhibitory concentration was incorporated with fish balls for shelf-life study. The results showed that inhibition of *S. aureus* increased as nisin concentrations and incubating time increased ($p \leq 0.05$). The number of *S. aureus* decreased from 4.94±0.08 log cfu/ml to 2.73±0.18 log cfu/ml at 4±2°C and 5.37±0.04 log cfu/ml to 3.49±0.12 log cfu/ml at 10±2°C after incubating for 42 h when the concentration of nisin increased from 5 to 20 µg/ml. D-values for *S. aureus* were between 17.92-10.74 h at 4±2°C and 18.58-12.10 h at 10±2°C. According to the D-value results, the suitable nisin concentration of 15 µg/ml was incorporated with fish balls inoculated with 7 log cfu of *S. aureus*. The samples were packed in polyethylene bags before being kept at 4±2°C and 10±2°C. Microbiological results indicated that *S. aureus* of both control groups increased from 7.27 ± 0.33 log cfu/g to 9.53±0.34 log cfu/g and 10.28±0.39 log cfu/g when the samples were kept at 4±2°C and 10±2°C, respectively while that of nisin treated samples reduced to 5.70±0.53 log cfu/g on day 12 and 6.43±0.27 log cfu/g on day 6 when the samples were kept at 4±2°C and 10±2°C respectively. In addition, the total plate counts of control samples increased to 10.2±0.36 log cfu/g and 11.25±0.31 log cfu/g at 4±2°C and 10±2°C, respectively whereas those of nisin treated samples reduced to 6.86±0.20 log cfu/g on day 12 and 7.27±0.44 log cfu/g on day 6 at 4±2°C and 10±2°C, respectively. For chemical results, total acid of fish ball was ranged from 0.0046% to 0.0049% and their pH values were between 6.77 and 6.92 during keeping for 30 days at both storage temperatures. In conclusion, the use of nisin at 15 µg/ml would be an alternative method to decrease the contamination level and it could be used in combination with other preservation means.

Keywords: food microbiology, contamination, Thailand, total plate count, D-value

Introduction

Fish ball is a commonly cooked food in southern China and overseas Chinese communities. It is also very popular in Thailand. Fish ball is made of fish meat that has been finely pulverized. It is cooked in noodle soup, fried or grilled to be eaten as a snack. The product is generally for local consumption as its shelf-life is not very long. Since fish ball requires considerable handling during preparation and is often kept at slightly elevated temperatures after preparation, it is frequently involved in microbial contamination. Meechai [1] reported that the average total bacteria count in fish ball ranged from $3.4x10^4$ to $2.11x10^8$ colonies/g and almost of them were *Stapylococcus aureus*. Staphylococci not only exist in air, water and food, but also on food equipment, environmental surfaces, humans and animals as well. It grows and reproduces at temperatures from 10^oC (50^oF) to 48.89^oC (120^oF), with the most rapid growth occurring near body temperature (about 37^oC). The toxin produced by *S. aureus* is very heat-stable. Symptoms of staphylococcal food poisoning are usually rapid and serious in many cases. The most common symptoms are nausea, vomiting, abdominal cramping and prostration. In more severe cases, headache, muscle cramping and changes in blood pressure and pulse rate may occur [2].

Nisin is a polycyclic peptide antibacterial produced by fermentation using the bacterium *Lactococcus lactis*. It is used as a food preservative in many food items to extend shelf life by suppressing Gram-positive spoilage and pathogenic bacteria [3]. As reported by Gallo, Pilosof and Jagus [4], the action of nisin against bacteria is that it binds electrostatically to the negatively charged phospholipids then inserts itself into the cytoplasmic membrane resulting in pore formation. The efflux of essential intracellular constituents through those forming pores causes a complete collapse of the proton motive force and subsequently results in cell death. Nisin is soluble in water and can be effective at levels nearing the parts per billion range. It has been approved as a food additive in Europe and achieved GRAS (Generally Recognized As Safe) status in the USA [5]. In food products, it is common to use nisin at levels ranging from 0.25-37.7 mg/l, depending on the food type and regulatory approval [6].

Much research has been undertaken on the efficacy of nisin against pathogenic and spoilage microorganisms such as *Listeria monocytogenes* [7, 8, 9], *Clostridium sporogenes* [10, 11], *Bacillus* sp. [6, 8], and *Staphylococcus aureus* [8, 10]. However, inactivation kinetics of nisin on *S. aureus* and the application of nisin in fish ball have not been studied so far. The present study deals with the evaluation of this potential to preserve and/or extend the shelf life of this meat product. The objective of this investigation was to determine the effective inhibitory effect of nisin on the growth *S. aureus* in liquid medium and in fish ball. This work was not only to limit contamination of the end product with *S. aureus* as much as practically possible, but to provide a control strategy that suppresses the pathogen growth [12].

Materials and Methods

Preparation of nisin stock solution

The stock solution was prepared as described by Paik et al. (2006). Briefly, 0.0012g of commercial nisin (Sigma-Aldrich, Germany) was dissolved in 1 ml of 0.02 N HCl. The solution volume was adjusted to 10 ml with distilled water before subjecting to heating for 10 min and kept at -20^oC until used.

Bacterial strain and culture media

Staphylococcus aureus TISTR 29 was purchased from the culture collection at Thailand Institute of Scientific and Technological Research (TISTR). The bacteria was cultured on the nutrient agar slant and kept at 4 ± 2^oC. In the preparation of seeding culture for antimicrobial test, the

bacteria from agar slant was inoculated in nutrient broth (Britania, Argentina) and incubated at 37±2°C for 24 h. A serial dilution was taken to meet required bacterial population by sterile peptone water (Fluka, Germany).

Inhibitory effect of nisin against Staphylococcus aureus

The nisin stock solution was diluted with distilled water to obtain nisin concentrations of 0, 5, 10, 15 and 20 µg/ml. To establish sensitivity test, 2 ml of each nisin concentration was added to bacterial suspension in nutrient broth to obtain the microbial concentration of 7 log CFU/ml. Samples were incubated at 4±2°C and 10±2°C. For each incubation group, aliquots were taken every 6 h interval and estimated for *S. aureus* count using direct plating on nutrient agar (Britania, Argentina).

Kinetics determination

For each temperature, the logarithm of survivors was plotted against incubation times. D-values were calculated as the inverse negative slope of the regression line. Z-values were determined from the regression as the inverse negative slope when plotting the logarithm of D-values against the treatment concentrations.

Effect of nisin on the survival of Staphylococcus aureus inoculated in fish balls

Fish ball treatment

The fish balls were made in the Department of Microbiology at King Mongkut's University of Technology Thonburi. The ingredients of the fish balls are given in Table 1. Nisin at appropriate concentration obtained from the previous section was added to the fish mass prior to cooking. The fish balls were set in warm water (40±1°C) for 20 min and then cooked until the core temperature reached 90±2°C. Cooked samples were inoculated by dipping in calibrated bacterial suspension at approximately 7 log cfu of bacterial cells/ml for 10 seconds. The treated samples were packed in polyethylene bags and then incubated separately at 4±2°C (refrigerated temperature) and 10±2°C (abused storage temperature). Fish balls without nisin treatment were used as the control. All samples were taken for analysis every 3 days.

Table 1. Ingredients of the fish balls.

Ingredients	Amount (%)
Fish meat	94.0
Ice	1.0
NaCl	2.8
Sugar	1.9
Other spice assortment	0.3

Analysis

Microbiological analysis

A sample (25g) was removed aseptically and transferred to 225 ml of sterile 0.1% peptone water solution. The sample was homogenized in a stomacher (AES Smasher, Australia) for 1 min. A 10-fold dilution was made of the peptone water as needed for plating. The bacterial counts in the resultant slurry were determined by pour plate technique on manitol salt phenol red agar for *S. aureus* (Fluka, Germany) and on plate count agar for total plate count (Merck, Germany). The samples were incubated at 35±2 °C for 24-48 h.

Chemical analysis

- Total acid determination

Total acid was determined as amount of standardized 0.1 M sodium hydroxide (NaOH) required to neutralize the meat using phenolphthalene as indicator [13]. Titratable acidity was expressed as lactic acid in grams per 100 g sample.

- pH measurement

The pH value was recorded using pH meter (Metrohm, Switzerland). Samples (10g) were thoroughly homogenized with 100 ml of distilled water and the homogenate was used for pH determination.

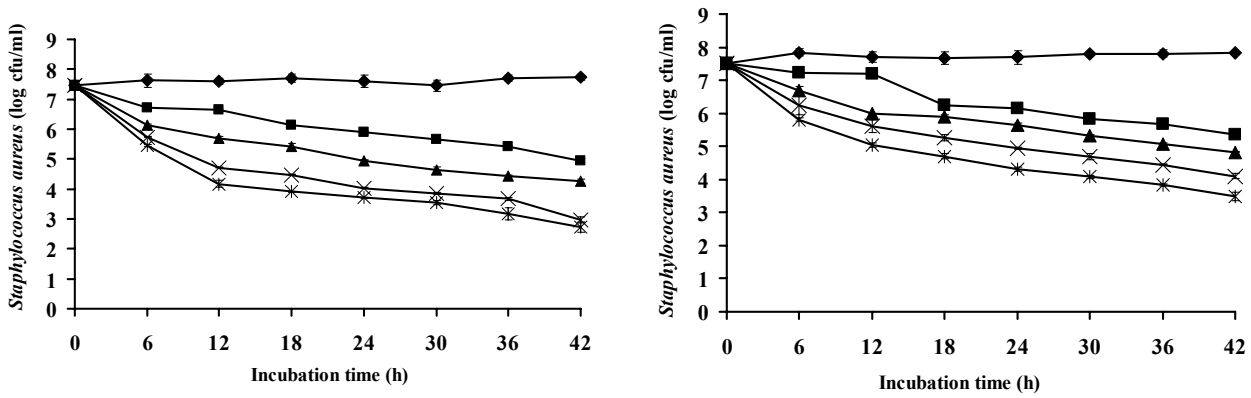
Statistical evaluation

Analysis of variance of the data was performed using the ANOVA procedure by Duncan's multiple range test. Significant differences ($p < 0.05$) between mean values of triplicate samples were determined.

Results and Discussion

Inhibitory effect of nisin against Staphylococcus aureus

Inhibitory efficacy of nisin (0-20 µg/ml) against *Staphylococcus aureus* in nutrient broth at 4±2°C (Figure 1a) and 10±2°C (Figure 1b) was investigated. In this study, the inhibitory activity was measured based on the reduction of microbial population. Figure 1a demonstrates that *S. aureus* in the control sample (0 µg/ml) slightly increased from 7.51±0.18 log cfu/ml to 7.73±0.08 log cfu/ml as the incubation time increased which meant that no inhibitory effect was detected. The results also revealed that addition of different concentration of nisin (5-20 µg/ml) in bacterial suspension affected *S. aureus* population significantly ($P \leq 0.05$). *S. aureus* counts were reduced by approximately 4.5-4.8 log cfu/ml when nisin at the concentrations of 15-20 µg/ml was applied, whereas there was only a slight decrease of 2.5-3.3 log cfu/ml at nisin concentrations of 5-10 µg/ml at the end of the test period. This finding indicated that the degree of microbial inactivation was directly related to the concentration of nisin and incubation period. This behaviour was consistent with previous findings. Jamuna, Babusha and Jeevaratnam [10] found that bacterial growth decreased from 10.3 to 8.1 log cfu/ml as nisin concentration increased from 40 to 80 AU/ml. This is also in good agreement with Pranoto, Rakshit and Salokhe [8] who observed that inhibitory zone (clear zone) of chitosan films containing nisin markedly increased by the increase of nisin incorporated. A similar result was found when the experiment was conducted at 10±2°C. However, higher incubation temperature tended to slow down the inhibitory effect. As observed in Figure 1b, adding nisin at the concentrations of 5-10 and 15-20 µg/ml could reduce the bacteria counts by 2.2-2.6 log cfu/ml and 3.5-4.1 log cfu/ml, respectively. For each nisin concentration, the number of *S. aureus* incubated at 10±2°C was relatively higher than that incubated at 4±2°C after 42 h. The counts were 7.73±0.08, 4.94±0.08, 4.25±0.07, 2.97±0.1 and 2.77±0.18 log cfu/ml at 4±2°C and 7.83±0.03, 5.37±0.04, 4.83±0.01, 4.09±0.08 and 3.49±0.12 log cfu/ml at 10±2°C for 0 µg/ml, 5µg/ml, 10µg/ml, 15µg/ml and 20µg/ml, respectively. This implied that higher temperature enhanced bacterial growth and thus lowered nisin efficacy.



(a) (b)

(◆)0 µg/ml (Control), (■) 5µg/ml, (▲) 10µg/ml , (×)15µg/ml , (∗) 20µg/ml

Figure 1. Effect of nisin concentration and incubation time on *Staphylococcus aureus* in nutrient broth at 4±2°C (a) and 10±2°C (b).

Kinetics determination

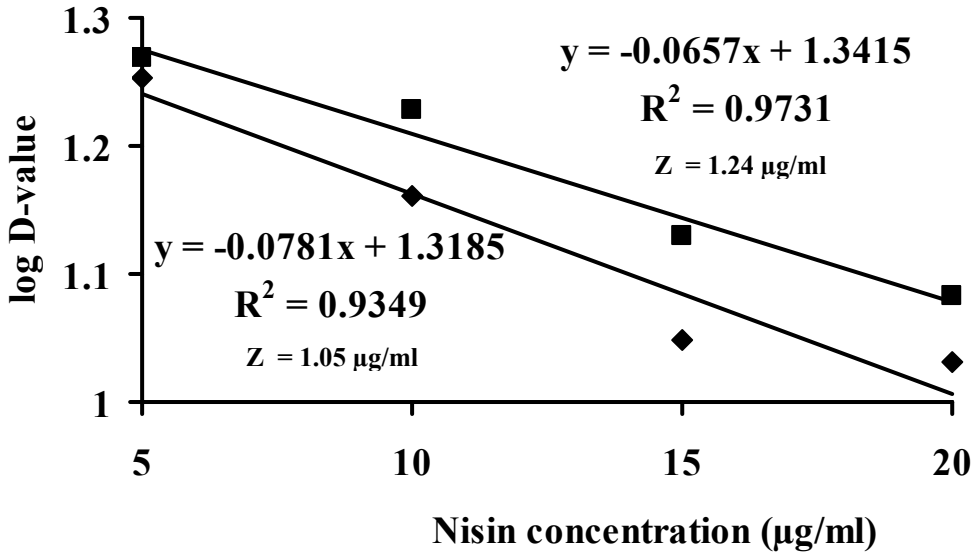
In this study, D-value refers to the time required at a certain nisin concentration to kill 90% of the *S. aureus* [14]. This property is unique for each microorganism hence it measures a disinfectant's efficiency to reduce the number of microbes at a given nisin concentration. Table 2 summarizes D-values for *S. aureus* inoculated in the nutrient broth. The results showed that D-values significantly decreased as nisin concentration increased ($p<0.05$). As compared to the incubation temperature, D-values decreased from 17.92±1.09 to 10.74±0.83 h at 4±2°C and from 18.58±1.12 to 12.10±0.49 h at 10±2°C when the concentration of nisin increased from 5 to 20 µg/ml. The results explicated that a higher efficacy of nisin was observed when the sample was incubated at 4±2°C. This is to be expected as nisin activity depends upon its concentration and incubation temperature [6]. However, the results from statistical analysis revealed that there was no significant difference of D-values between the sample treated with nisin at concentration of 15 µg/ml and 20 µg/ml at both temperatures. Therefore, the concentration of nisin at 15 µg/ml was used for further study.

Plotting the log D-values against nisin concentrations provided a straight line (Figure 2) from which the Z-values were estimated. In this study, the Z-value is defined as the concentration of nisin that is required for the inhibition curve to move one log cycle. This value can relate the resistance of *S. aureus* to different nisin concentration. From Figure 2, the Z-values of nisin treated samples were 1.05 µg/ml at 4±2°C and 1.24 µg/ml at 10±2°C.

Table 2. D-values of *Staphylococcus aureus* at nisin concentration of 5-20 µg/ml at different incubation temperature.

Incubation temp. (°C)	Nisin concentration (µg/ml)	D-value (h)*
4±2	5	17.92^{a}±1.09
	10	14.50^{b}±0.71
	15	11.16^{c}±0.59
	20	10.74^{c}±0.83
10±2	5	18.58^{a}±1.12
	10	16.89^{a}±0.04
	15	13.47^{b}±0.97
	20	12.10^{b}±0.49

* means with different letters (a,b,...) in the same column in each temperature are significantly different ($p\leq0.05$)



(◆) 4±2°C, (■) 10±2°C, (—) regression

Figure 2. Z-values of *Staphylococcus aureus* at nisin concentration of 5-20 (µg/ml) at different incubation temperatures.

Effect of nisin on the survival of Staphylococcus aureus inoculated in fish balls

The effect of nisin on the number of *Staphylococcus aureus* in fish ball samples was investigated. All fish ball samples had no detectable *S. aureus* before inoculation. As shown in Figure 3, the control sample (without nisin) allowed *S. aureus* on fish balls to grow rapidly, especially at higher storage temperature. The increase of Staphylococcal population in log cfu from 7.27±0.33 to 9.53±0.34 and from 7.27±0.33 to 10.28±0.39 was detected at 4±2°C and at 10±2°C, respectively. On the other hand, *S. aureus* in treatments incorporating nisin decreased by approximately 1.6 log cfu on day 12 at 4±2°C and 0.84 log cfu on day 6 at 10±2°C after that the inverse effect was observed. There was a slight increase by 1.3 and 2.0 log cfu in fish balls kept at 4±2°C and at 10±2°C, respectively at the end of the test period. The initial decrease in number of *S. aureus* was due to nisin activity while the subsequently increment was due to the lowering of nisin activity and the presence of nisin-tolerant strain [4, 15].

The experimental results also inferred that nisin at the concentration of 15 µg/ml was not effective enough to prevent contamination**.** However, it delayed the growth of *S. aureus* population at both incubation temperatures. In addition, the growth inhibition was more prominent at 4±2°C than at 10±2°C. This also demonstrated that nisin inhibited this microorganism in a temperature dependent fashion.

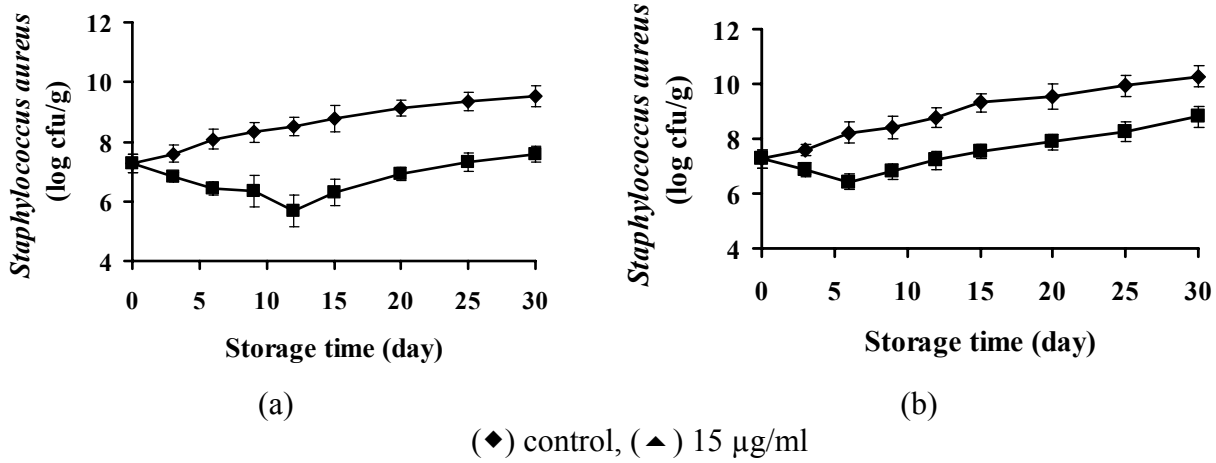


Figure 3. Effect of nisin on survival of *Staphylococcus aureus* inoculated in fish balls kept at 4±2°C (a) and 10±2°C (b).

The effect of nisin on the total aerobic counts is shown in Figure 4. The initial bacterial number was approximately 7.55±0.42 log cfu/g at day 0. This indicated that there were other microflora besides *S. aureus* in the samples. Meechai [1] reported that the contaminated microorganisms on fish balls from local markets were *S. aureus*, *Escherichia coli, coliform*, *Clostridium perfringens* and *Salmonella* sp.. For the control samples (without nisin), the microbial population increased with storage time. The total counts were 10.2±0.36 and 11.25±0.31 log cfu/g after kept at 4±2°C and 10±2°C for 30 days. Unlike the aforementioned samples, nisin in the treated sample was attributed to microbial reduction. The samples incorporating nisin had the minimum counts of 6.86±0.2 and 7.27±0.44 log cfu/g on day 12 at 4±2°C and on day 6 at 10±2°C, respectively. Similar to previous results, the total counts propagated afterwards. At the end of the experiment, total flora increased to 9.18+0.42 log cfu/g for 4±2°C and 9.68±0.37 log cfu/g for 10±2°C**.** Observation on microbial counts, nisin treated samples stored at 4±2°C were consistently lower than those stored at 10±2°C.

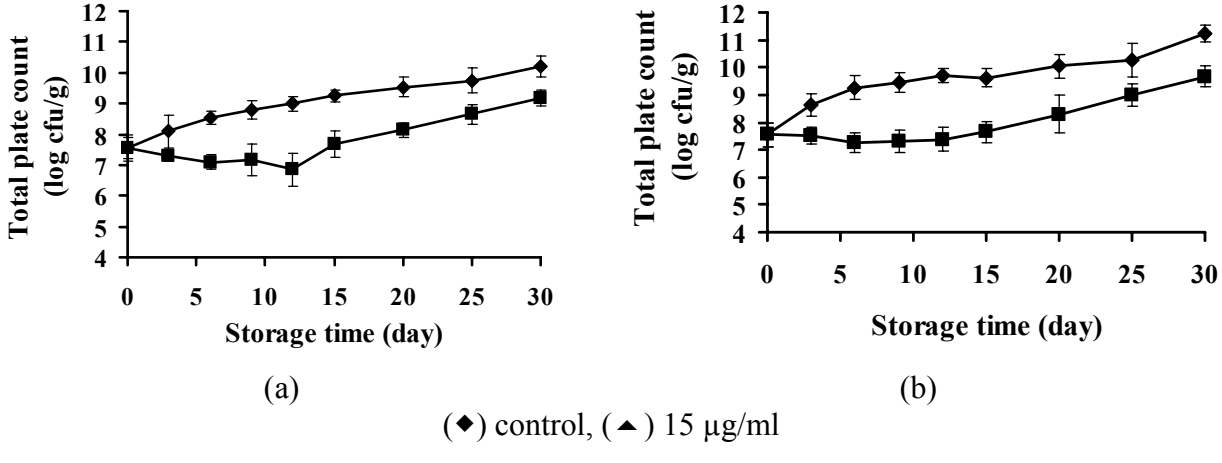


Figure 4. **Effect of nisin on total plate counts of fish balls inoculated with *Staphylococcus aureus* kept at 4±2°C (a) and 10±2°C (b).**

The changes in pH values and acidity of the samples are shown in Figure 5. The initial pH ranged between 6.91 and 6.93 and the pH on the 30th day varied from 6.50 to 6.80, whereas the acidity of the samples was about 0.0046-0.0047% at the beginning and was 0.0048-0.0049% at

the end of the storage period. Statistical analysis indicated that these parameters were not significantly affected by nisin treatment, storage temperatures and time ($p>0.05$).

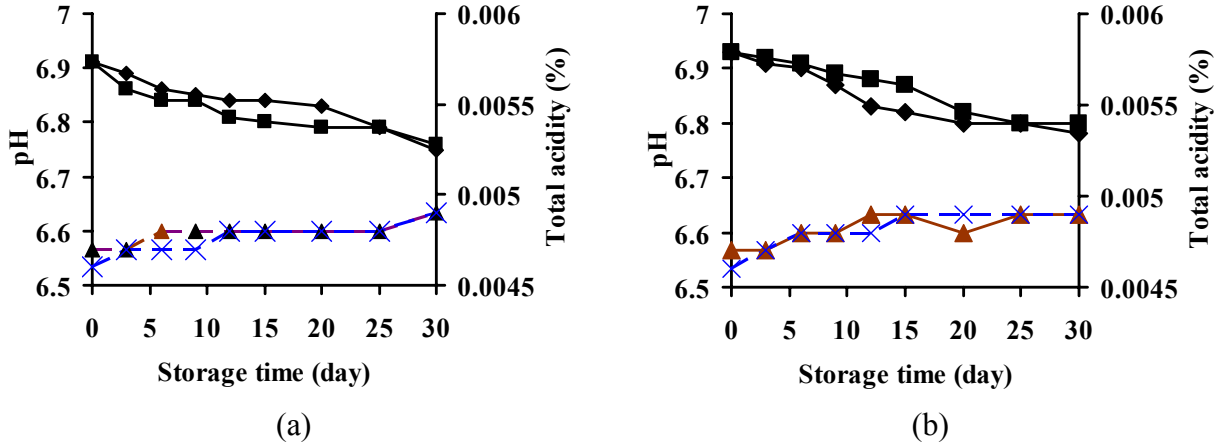


Figure 5. Effect of nisin on total acidity and pH of fish balls inoculated with *Staphylococcus aureus* kept at 4±2°C (a) and 10±2°C (b).

Conclusion

Inhibition of *Staphylococcus aureus* increased as nisin concentration and incubating time increased ($p\leq0.05$). D-values for *S. aureus* were between 17.92-10.74 h at 4±2°C and 18.58-12.10 h at 10±2°C. Nisin at the concentration of 15 µg/ml did not achieve a satisfactory degree of *S. aureus* inhibition in fish balls. The fact that there were initial reduction of microorganisms and the inhibition action of nisin depended upon temperature indicated that nisin might be used together with other preservation methods to control post-processing contamination of *S. aureus* in this product.

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