CHAPTER 5

SURVIVAL OF *B. licheniformis* IN NISIN ADDED PASTEURIZED IMITATED MILK SYSTEM AS AFFECTED BY CARBOHYDRATE TYPES, LEVELS AND STORAGE TEMPERATURES

5.1 Introduction

A heat treatment is the most common method to reduce, eliminate or destroy pathogenic and spoilage microorganisms. Pasteurization is a processing that heats milk at a specific temperature in a certain length of time to destroy pathogenic organisms and weaken the others (Kon, 1975). The shelf life of pasteurized milks depends on the number and type of microorganisms present in raw milk, the time and temperature of pasteurization, a Post Pasteurization Contamination (PPC) and upon the storage temperature. If the PPC is controlled, organisms survived after pasteurization and growth at refrigeration temperatures become the limiting factor in the shelf life of pasteurized milks. These microorganisms will be dominated by Gram positive spore forming bacteria, especially *Bacillus* genus (Harding, 1999). In fact, reports from researches have found *Bacillus* genus as a common spore forming bacteria that present in raw milk and the genus could cause a serious problem in milk industry. The spores of the bacilli can survive pasteurization processes. The vegetative cells of the organism can produce extra-cellular enzymes, which cause deterioration in milk and milk products (Christiansson *et al.,* 1999). Among the *Bacillus* genus, *B. licheniformis* was the greatest heat resistance bacteria as compared to other species. The spore of this species can germinate after a heat treatment (Lin *et al*., 1998).

Nisin mainly has an activity against Gram positive bacteria. Several reviews have been published recording its antimicrobial potential (Henning *et al.*, 1986; Breukink and de Kruijff, 1999; Thomas *et al.*, 2000; Cleveland *et al.*, 2001; Wirjantoro *et al.*, 2001). In this section, the effectiveness of nisin to inhibit *B. licheniformis* in pasteurized IMS solutions was investigated. Two carbohydrate types at different levels and storage temperatures were included in the experimental

plan to have a better understanding about the effect of carbohydrate on the activity of nisin against the target microorganism.

5.2 Material and methods

5.2.1 Preparation of imitated milks system (IMS)

The IMS solutions was prepared according to the proceduced in the section 4.2.1.

5.2.2 The effect of lactose on the effectiveness of nisin against *B. licheniformis* in IMS solutions

The basic IMS solutions containing fat, casein and WPI were formulated according to the procedure in the section 5.2.1. The IMS solutions were then divided into 5 batches, in which different levels of filter sterilized lactose solutions were incorporated. The studied lactose concentrations included 0, 2.5, 5.0, 10.0 and 20.0% (w/v). The IMS solution without any lactose addition was used as a control treatment in this subsection. After thoroughly mixed the lactose in the IMS solutions, the IMS solutions were aseptically added with 3.76±0.01 log cfu/ml *B. licheniformis* suspension and 100 IU/ml nisin. The whole IMS solutions were subjected into a pasteurization treatment at 72±1ºC for 15 s followed by immediate cooling using a running cold water. The heat treated IMS solutions were stored at 4 and 10ºC for 21 days. During the storage period, samples of each IMS solutions were taken every 3 or 4 days and examined for the chemical and microbiological characteristics.

5.2.3 The effect of sucrose on the effectiveness of nisin against *B. lichiniformis*

In this sub-section, the carbohydrate source of sucrose was used instead of lactose. The experimental method was similar to the section 5.2.2. The concentrations of sucrose investigated in this sub-section were 0, 2.5, 5.0, 7.5, 10.0, 15.0 and 20% (w/v). The IMS solution without any sucrose addition was used as a control treatment in this subsection. The initial *B. licheniformis* population was 3.75±0.01 log cfu/ml.

5.2.4 Chemical analysis

The main chemical composition, total acidity and pH of the IMS solutions were measured according to the method in the section 4.2.4.

5.2.5 Microbiological analysis

Microbiological properties, including Total Viable Microorganisms (TVM), thermoduric bacteria and spore counts were determined following the procedures in the section 4.2.5.

5.2.6 Nisin assay

The method was described in the section 3.2.6**.**

5.2.7 The experimental design

The experiment result of the section 5.2.2 was analyzed statistically using a 5x2 Factorial Experiment in Completely Randomized Design. The first factor was lactose concentrations, including 0, 2.5, 5, 10 and 20% (w/v). The second factor was storage temperatures of 4 and 10°C. If F value was significant, DMRT was used to determine differences between treatment means by using a SPSS program (SPSS 10.0) (SPSS Inc., Chicago, USA).

Data from the section 5.2.3 was analyzed statistically using a 7x2 Factorial Experiment in Completely Randomized Design. The first factor was sucrose levels, including 0, 2.5, 5.0, 7.5, 10.0, 15.0 and 20.0% (w/v). The second factor was storage temperature, which were at 4 and 10°C. If F value was significant, DMRT was used to determine differences between treatment means by using a SPSS program (SPSS version 10.0) (SPSS Inc., Chicago, USA).

5.3 Results and discussion

5.3.1 The effect of lactose on nisin against *B. licheniformis* in the IMS solutions

5.3.1.1 Chemical composition of IMS solution

The basic IMS solutions had 4.17±0.01% (w/v) fat and 4.05±0.00% (w/v) protein. Supplementation of 0, 2.5, 5, 10 and 20% concentrated lactose liquid into the IMS solutions produced final mixtures with 0.25±0.01, 2.66±0.01, 5.27±0.01, 10.56±0.03 and 19.86±0.01% (w/v) carbohydrate contents, respectively (Figure 5.1). As expected, higher lactose levels significantly increased the total solid and solid not fat of the IMS solutions.

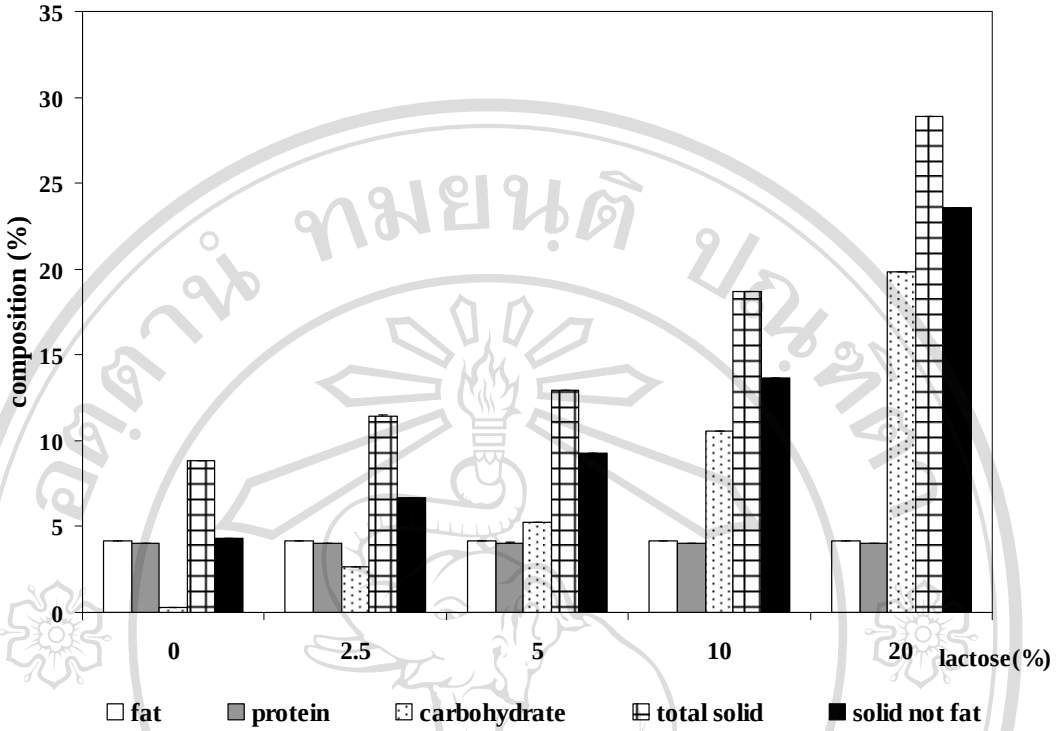


Figure 5.1 Chemical composition of IMS solutions with different lactose levels.

5.3.1.2 TVM count

The initial *B. licheniformis* suspension of 3.76±0.01 log cfu/ml in the IMS solutions added with 100 IU/ml nisin was significantly reduced after the pasteurization treatment at 72°C for 15 s. A reduction between 2.35 and 2.39 log cfu/ml of the TVM in the control treatment (no lactose) was recorded directly after the heat treatment. In the presence of different lactose levels, the reduction in the TVM counts was generally lower than that of the control treatment. The reduction in the TVM number was significantly lower in the IMS solutions supplemented with 20% lactose. This result suggested that at high lactose concentrations, the compound could negatively influence the hurdle effect of nisin and pasteurization. This finding was also in an agreement with the report of Grant *et al*. (1999) that studied about the effect of a pasteurization temperature of 72°C on *Mycobacterium paratuberculosis.* They discovered that after a heat treatment at 72ºC for 15 s, the number of *M. paratuberculosis* (B_2, NCTC 8578 and DVL 943) noticeably decreased.

During the storage period, the effect of storage temperature on the effectiveness of nisin against *B. licheniformis* was clearly displayed (Figure 5.2).

At 4ºC storage temperature, the TVM of the IMS solutions was slowly reduced throughout 21 days of storage. A reduction in the TVM population for up to 1.15 log cfu/ml was recorded in different IMS solutions at the end of the storage period. Although the presence of lactose in the IMS solutions produced higher reduction in the TVM count than the control, the effect was found not to be significantly different. Continue reduction in the TVM count could be due to a combination effect of nisin that was available throughout the storage period (Figure 5.7a) and the low storage temperature.

At higher storage temperature of 10ºC, the presence of 100 IU/ml nisin was not able to inhibit the growth of *B. licheniformis* in the IMS solutions added with different levels of lactose (Figure 5.2b). A significant increase in the TVM population was shown within the first week of storage. During the first 7 days of storage, the IMS solutions supplemented with lactose had significantly higher TVM counts than that of the control treatment. This result indicated that the presence of lactose might support the growth of bacilli and/or reduced the nisin activity at higher storage temperature. After the TVM population reached its maximum population on the 7^{th} or 10^{th} day of storage, the TVM count of different IMS solutions was slightly declined and was not significantly different between different IMS treatments.

5.3.1.3 Spore count

After the pasteurization treatment, different IMS solutions had a spore count between 1.26 and 1.45 log cfu/ml. This spore number was stepwise reduced throughout 21 days at both storage temperatures (Figure 5.3). The presence of lactose did not give a significant effect on the effectiveness of nisin against the bacilli spore form, but higher spore numbers were noticed in the lactose added IMS solutions compared to that of the control treatment. The reduction rate of the spore population was also lower at higher storage temperature. This might correlate with higher storage temperature that gave a better support for the microorganisms growth as was seen for the TVM result (Figure 5.2). A good control of nisin towards the bacilli spore during 21 days of storage might be affected by the continuous presence of the antimicrobial compound throughout the studied storage period (Figure 5.7). A similar result had been reported by Mansour *et al*. (1999) that studied about the presence and absence of nisin to inhibit spores outgrowth.

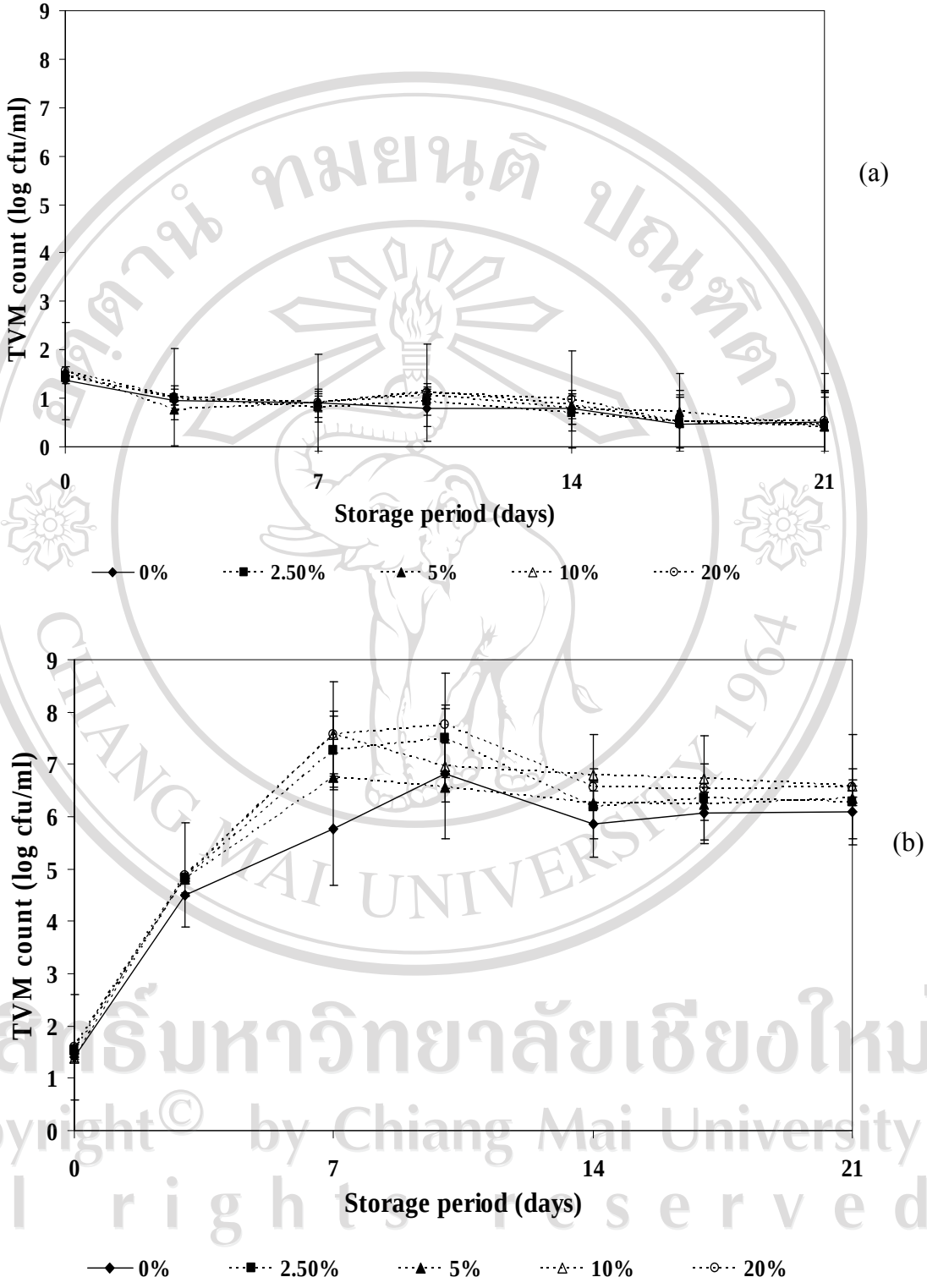


Figure 5.2 Total Viable Microorganisms of IMS solutions with different lactose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

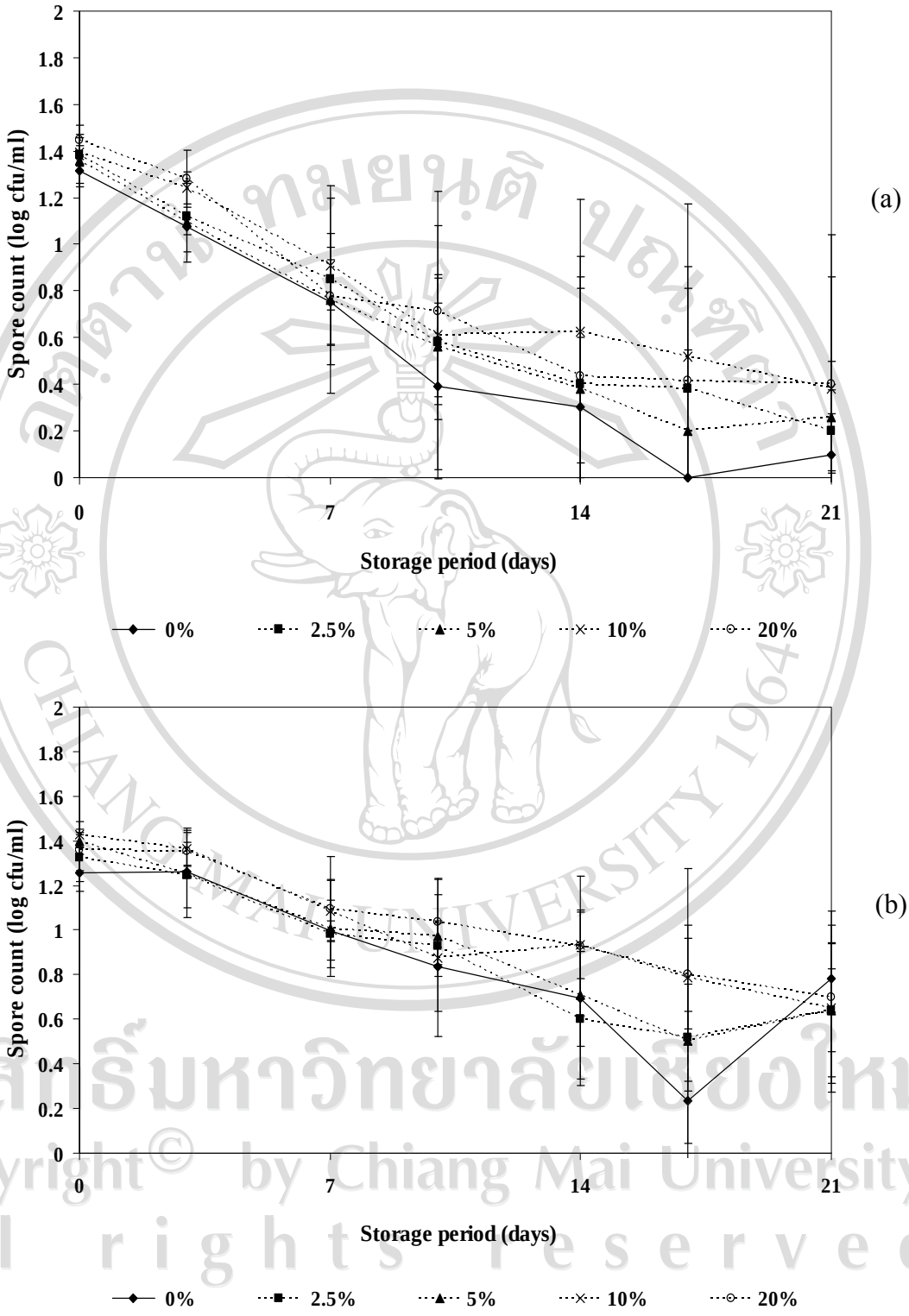


Figure 5.3 Spore count of IMS solutions with different lactose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

5.3.1.4 Thermoduric count

The initial thermoduric count of the IMS solutions that was between 1.24 and 1.35 log cfu/ml at the beginning of the storage period, was within the range of spore count in the milk samples. Changing in the thermoduric bacilli population during storage (Figure 5.4) was also shown to be similar to the result of the spore count (Figure 5.3). Both lactose concentrations and storage temperatures were not significantly affected the effectiveness of nisin against thermoduric *B. licheniformis*. Compared to the results of the spore count, a higher rate of thermoduric reduction was recorded in the first 7 days of storage. At the same time, after reaching the minimum thermoduric count in the middle of the storage period, the number of the thermoduric population was slightly increased after 14 days of storage. This finding suggested that the heated thermoduric bacilli might be more susceptible towards nisin compared to the spore form at the beginning of storage period. However, at the end of the storage period when the nisin activities were reduced (Figure 5.7), the thermoduric bacteria could start to grow. This result showed that the availability of nisin was important throughout the storage period, particularly for thermoduric bacilli.

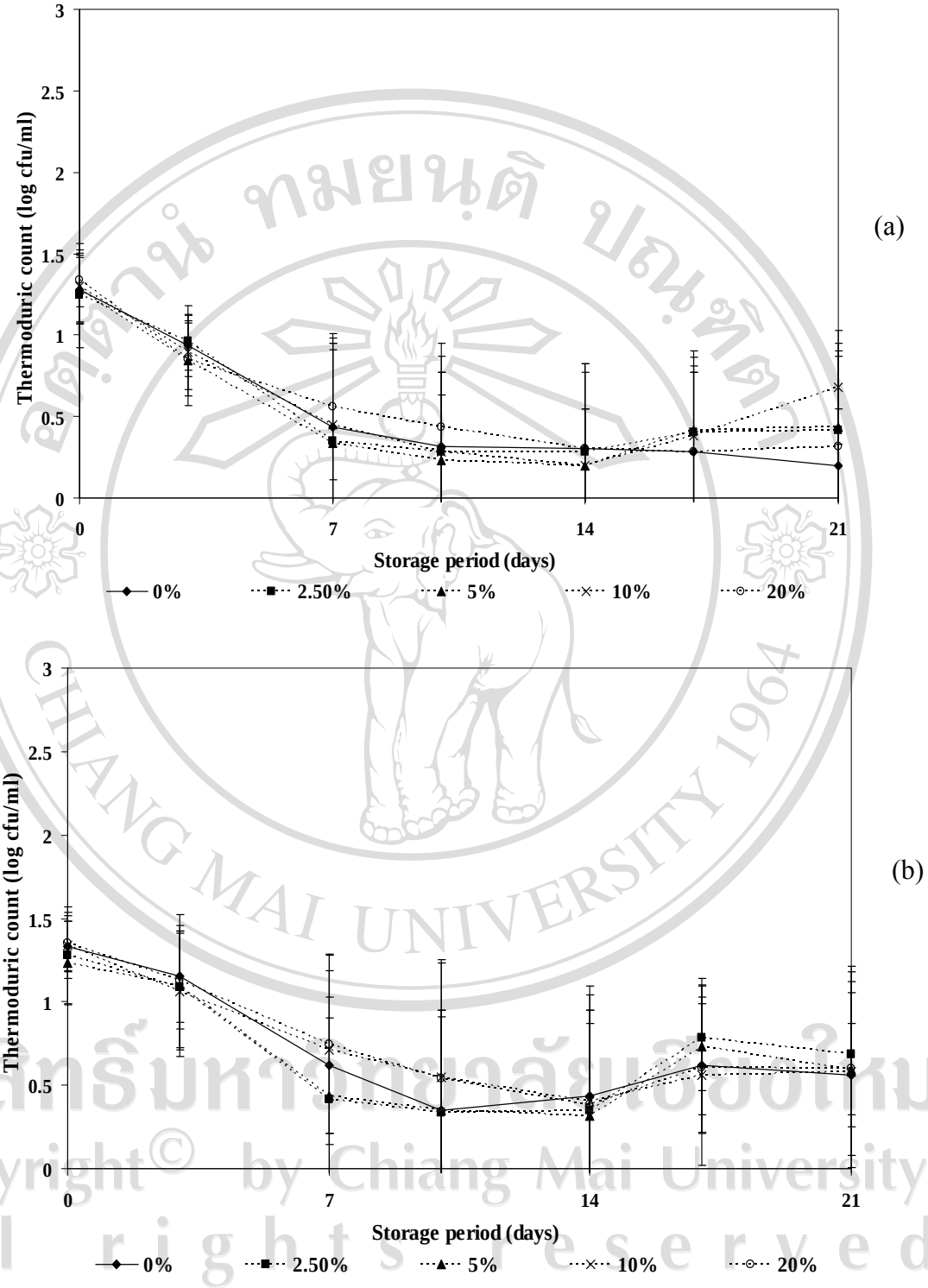


Figure 5.4 Thermoduric count of IMS solutions with different lactose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

5.3.1.5 Chemical properties of the IMS solutions

pH value

Different IMS solutions experienced reduction in their pH values during storage at 4 and 10ºC (Figure 5.5). The presence of lactose in the IMS solutions did not produce a significant effect on the pH of the IMS samples, but the pH of these treatments was lower than that of the control treatment. This result might correlate with higher numbers of the TVM count in the lactose added IMS solutions stored at 10ºC (Figure 5.2). Storage temperatures were significantly affected the pH of the IMS solutions after 21 days of storage. The pH of the IMS samples kept at higher storage temperature was significantly lower than that of the samples stored at 4ºC, particularly for the lactose added IMS solutions.

Acidity value

The initial acidity value of 0.008% lactic acid of different IMS solutions was increased to 0.011-0.013% lactic acid at the end of the storage period (Figure 5.6). Different storage temperatures and lactose levels did not significantly affect the acidities of the IMS solutions. However, the control treatment stored at 4ºC had a significant lower acidity value than that of the IMS solution supplemented with 20% lactose and kept at higher storage temperature. The increase in the acidity value might mainly attribute to the growth of microorganisms in the IMS solutions.

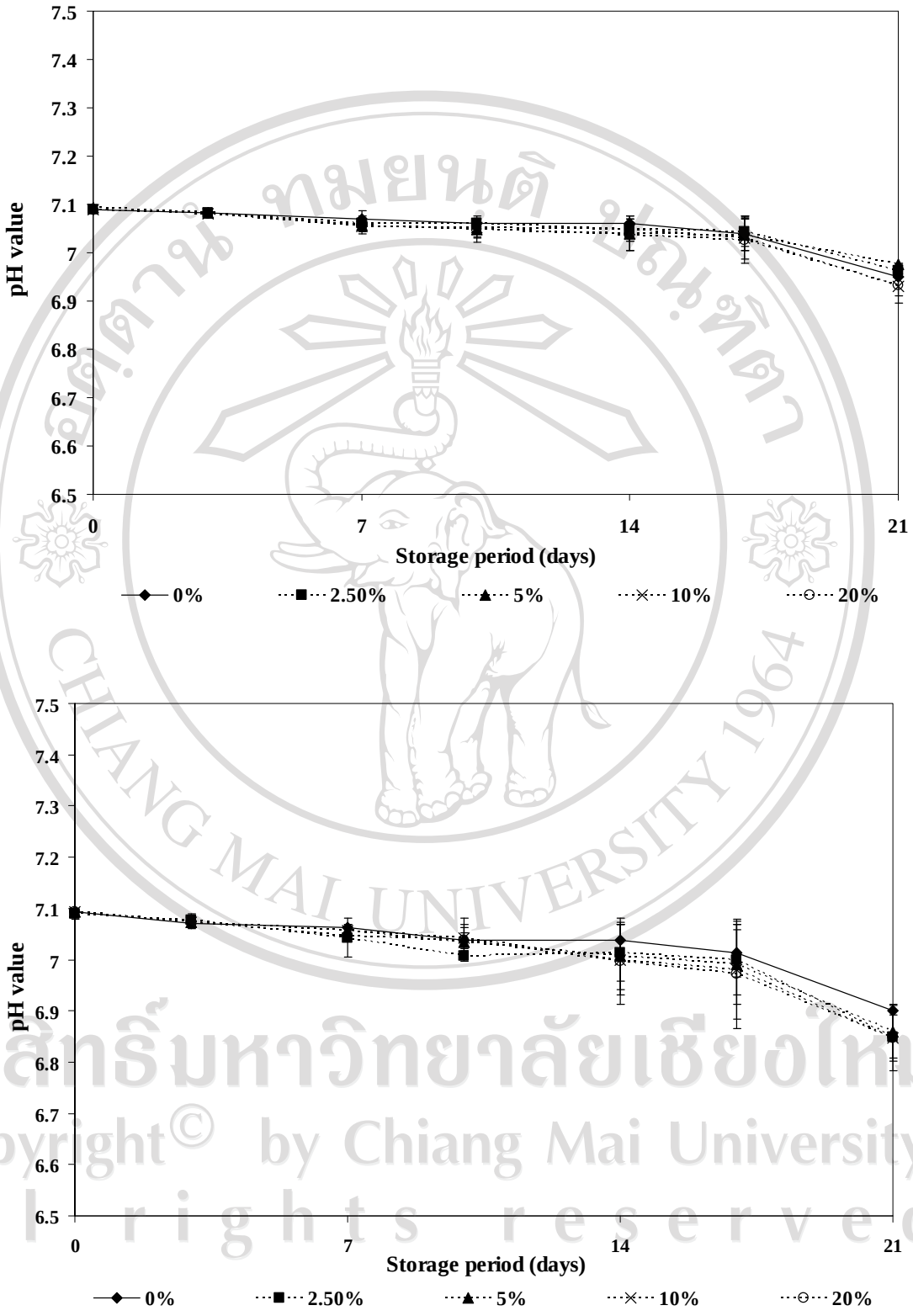


Figure 5.5 pH values of IMS solutions with different lactose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

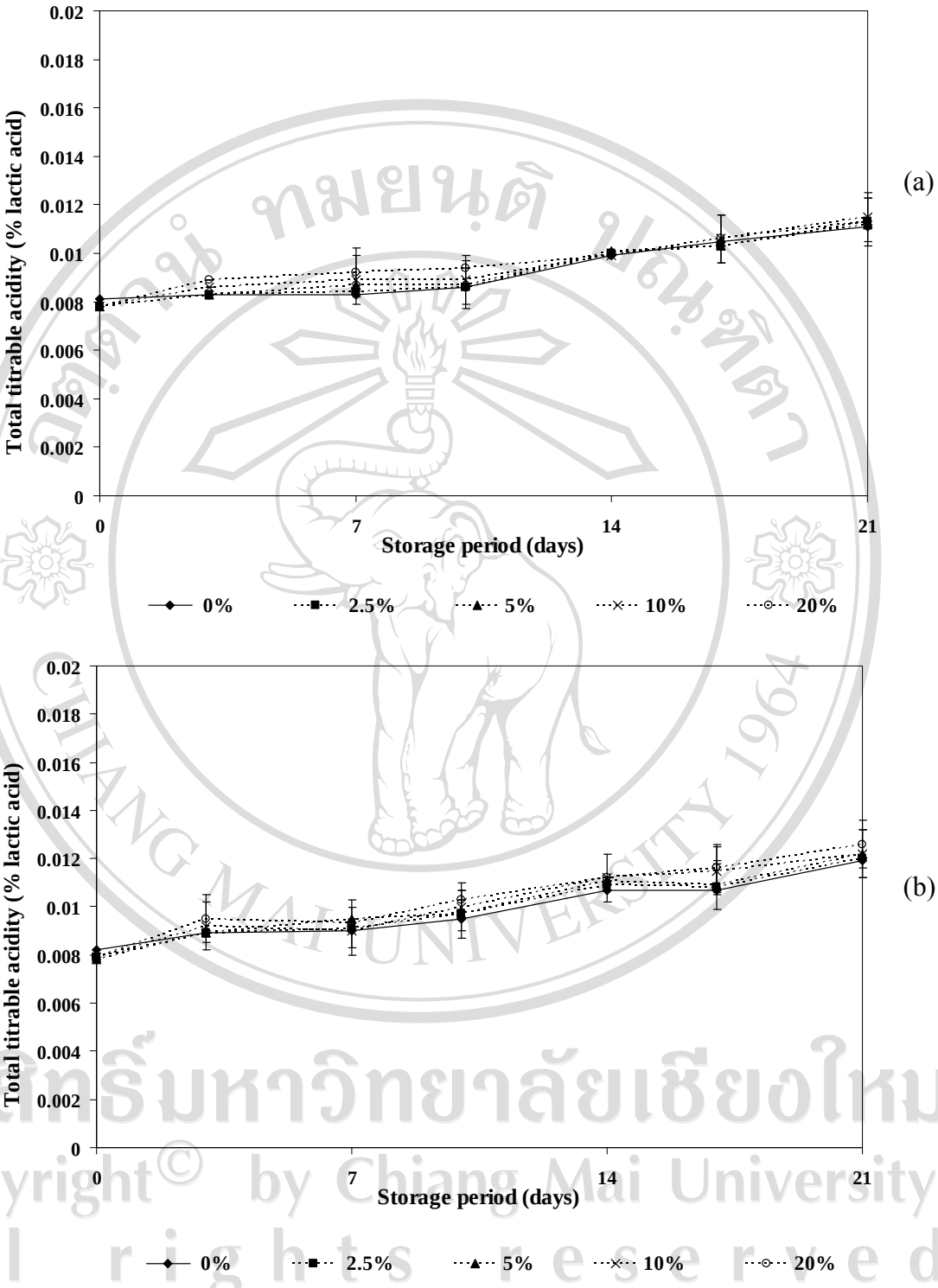


Figure 5.6 Acidity values of IMS solutions with different lactose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b)**.**

5.3.1.6 Nisin assay

The residual nisin activity in the IMS solutions stored at 4 and 10ºC was reduced during 21 days of storage (Figure 5.7). High reduction rates were occurred during the pasteurization process and within the first 3 days of storage. Higher reduction rates were also noted at higher storage temperature. At the same time, higher lactose levels produced lower nisin activities at longer storage period (after 10 days of storage). Higher nisin activities at lower storage temperature might contribute to lower TVM counts in the IMS solutions (Figure 5.2).

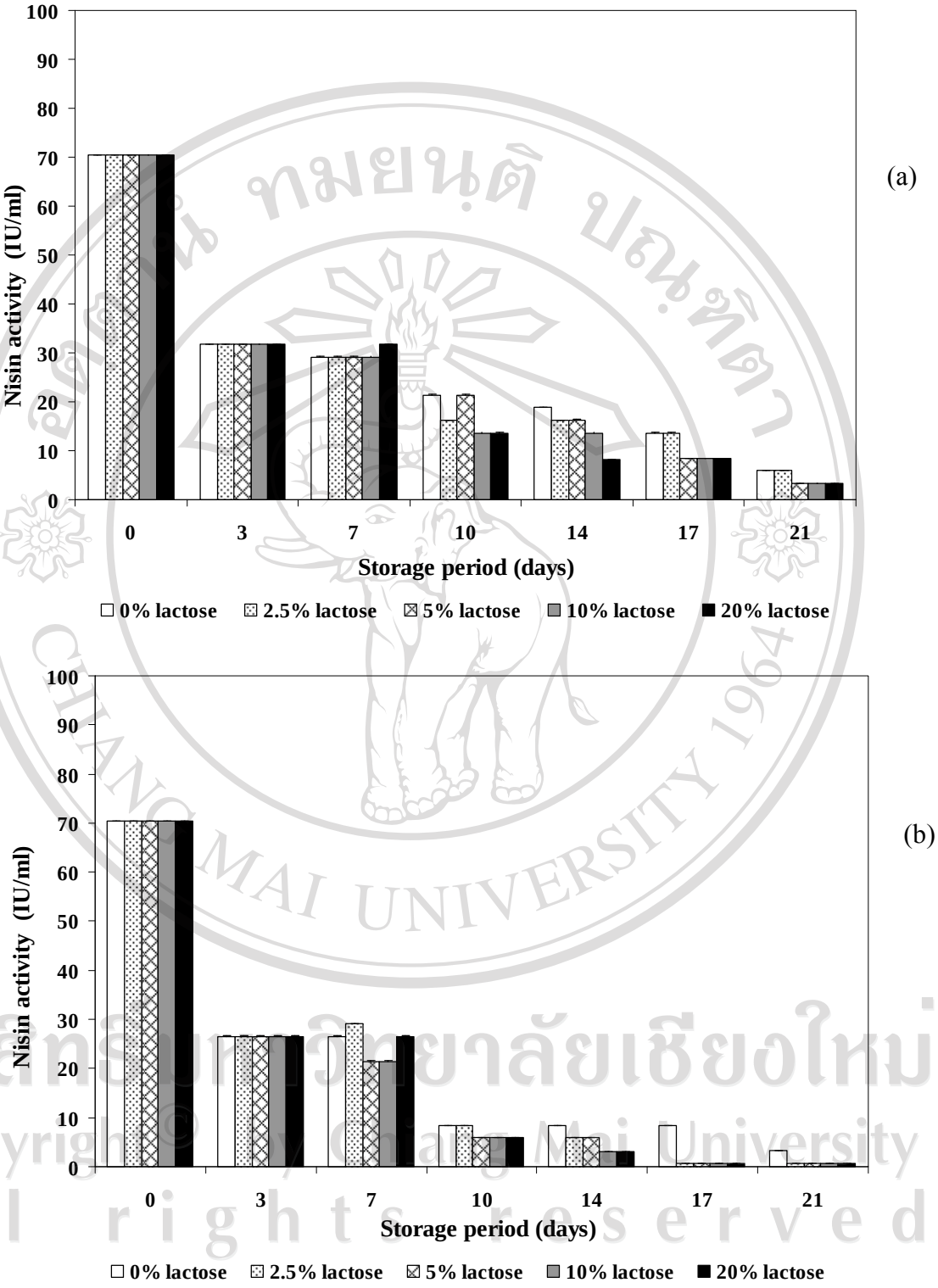


Figure 5.7 Nisin activity of IMS solutions with different lactose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

5.3.2 The effect of sucrose on the effectiveness of nisin against *B. licheniformis* in the IMS solutions

5.3.2.1 Chemical composition of IMS solution

In this subsection, the addition of sucrose in the IMS solutions was carried out to simulate some dairy products that are supplemented with the carbohydrate, such as sweetened flavored milk and drinking yogurt. The addition of sucrose in sweetened flavored milks can be in the range of 3 to 0.5 %, whereas sucrose can be present up to 8% in yogurt products. Using a basic IMS solution containing 4.29±0.04% fat and 4.01±0.03% protein (no lactose), different sucrose levels of 0, 2.5, 5, 7.5, 10, 15 and 20% (w/v) were supplemented into the IMS solutions. The final IMS solutions had 0.06±0.01, 2.66±0.02, 4.95±0.01, 7.28±0.02, 9.55±0.01, 14.24±0.03 and 18.95±0.01% carbohydrate, respectively (Figure 5.8). Higher sucrose levels significantly increased the total solid and solid not fat of the IMS solutions.

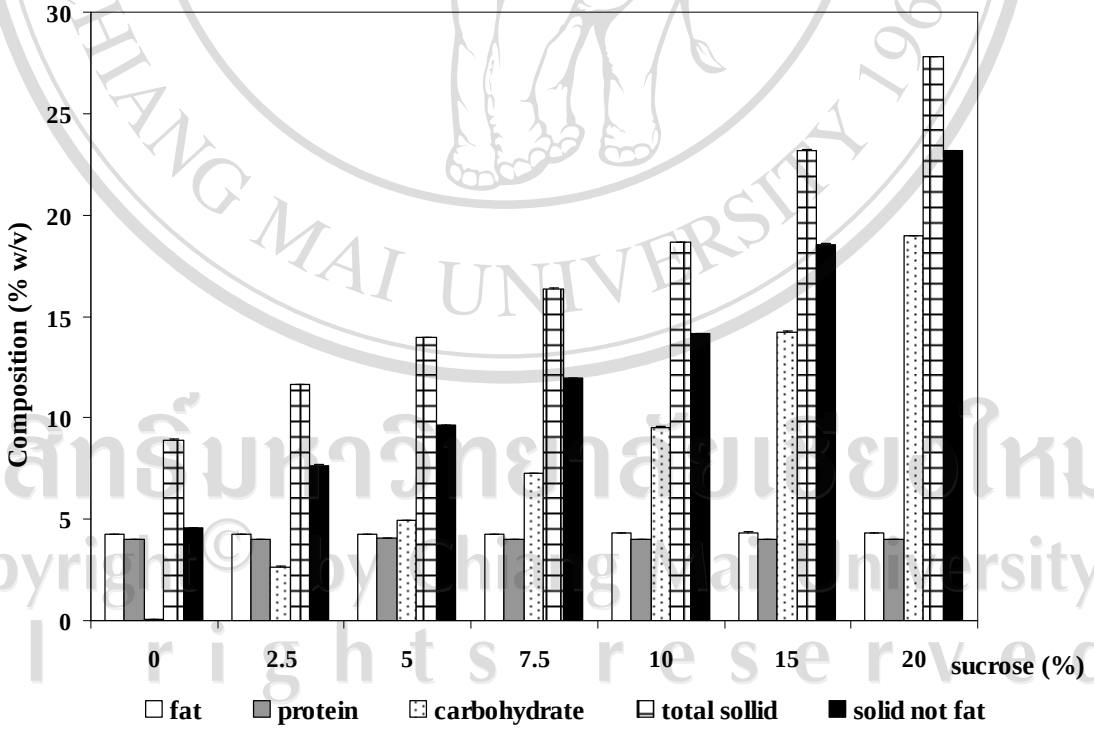
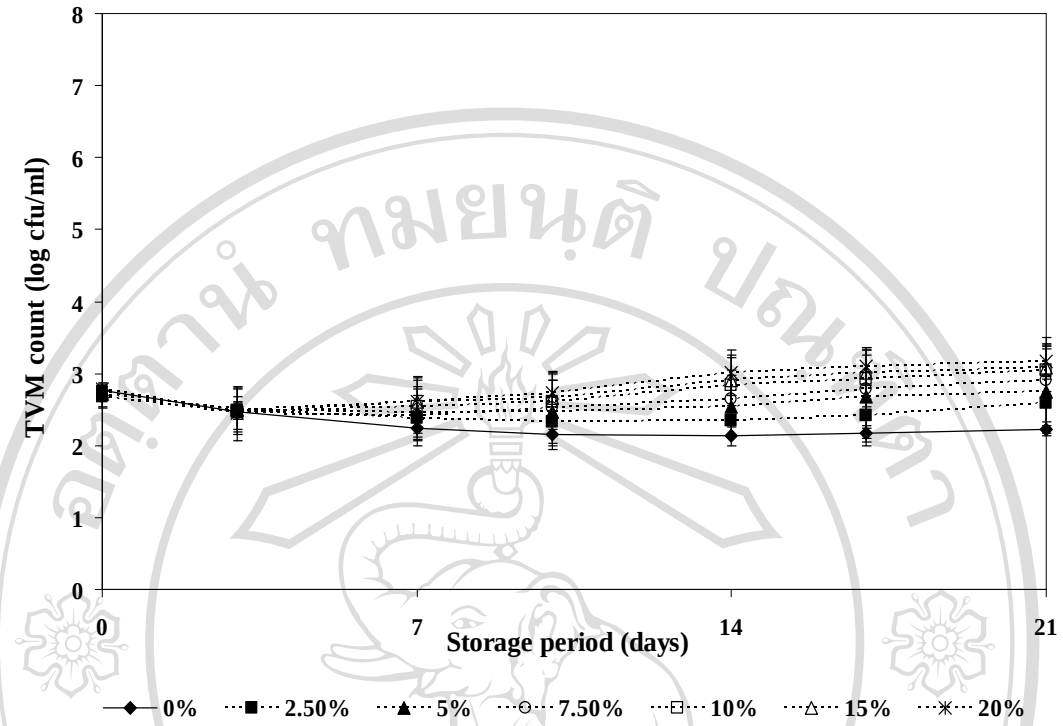


Figure 5.8 Chemical composition of IMS solutions with different sucrose levels.

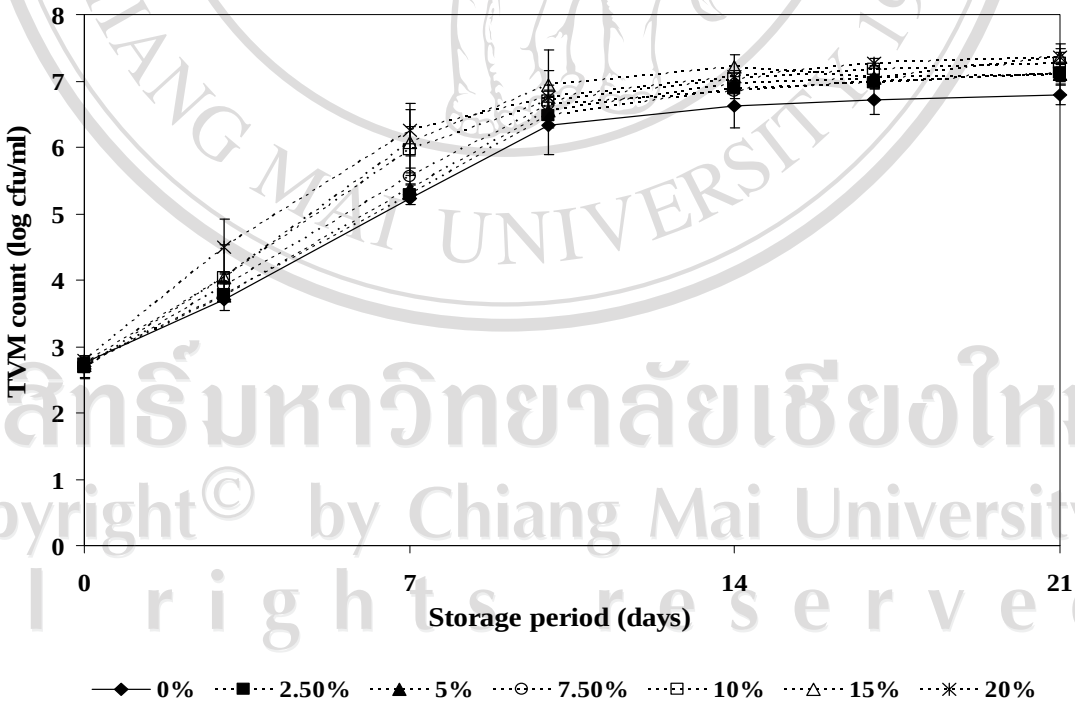
5.3.2.2 TVM count

The combination effect of nisin and pasteurization process could reduce the initial population of *B. licheniformis* in the range of 0.96 to 1.07 log cfu/ml directly after the heat treatment, which was similar to the report of Grant *et al*. (1999) for *M. paratuberculosis*. The presence of different sucrose levels did not significantly affect ($P>0.05$) this hurdle treatment. However, the reduction of bacilli population found in this subsection was lower for more than 1 log cycle compared to the reduction in the previous subsection (the subsection of lactose). Since the experiments of different sections were carried out separately, these differences could not be explained easily. There was a possibility that different batches of *B. licheniformis* population and/or different temperature profiles during the thermal processing (there were 14 treatments in this subsection compared to 10 treatments in the previous subsection) affected the reduction of the bacilli population during the pasteurization treatment.

Directly after the pasteurization treatment, different IMS treatments were stored at either 4 or 10ºC for 21 days (Figure 5.9). During this storage period, the effectiveness of nisin to inhibit *B. licheniformis* was significantly ($P\leq0.05$) affected by the storage temperature. At lower storage temperature (Figure 5.9a), the control treatment had a continue slow reduction in the bacilli population until 14 days of storage before the organism number was slightly increased in the last 7 days of storage. Although the sucrose-added IMS solutions showed similar patterns as the control treatment, the minimum bacilli population was reached at shorter storage period. Supplementation of 2.5, 5, 7.5, 10, 15 and 20% sucrose produced minimum TVM counts on 10, 7, 7, 3, 3 and 3 days of storage, respectively. In addition, the TVM count of the IMS solutions added with 5% sucrose was significantly ($P\leq0.05$) higher than that of the control treatment on the 21st day of storage, whereas higher sucrose levels in the IMS solutions produced significantly higher TVM counts than the control treatment after 14 days of storage. These results suggested that the presence of sucrose, especially at high concentrations, gave an adverse effect on the activity of nisin against *B. licheniformis* at 4ºC storage temperature.



(a)



(b)

Figure 5.9 Total Viable Microorganisms of IMS solutions with different sucrose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

For the IMS solution storage at 10ºC storage temperature, the TVM number in the samples was significantly increased during 21 days of storage (Figure 5.9b). The finding showed clearly that the high storage temperature supported the growth of bacilli and affected the activity of nisin. At high sucrose levels of 10, 15 and 20%, the TVM of the IMS solutions was significantly ($p \leq 0.05$) higher than the control treatment after 7, 7 and 3 days of storage, respectively. Similar to the result at 4ºC, the high concentrations of sucrose at high storage temperature demonstrated an antagonistic affect on the activity of nisin to inhibit microorganisms.

5.3.2.3 Spore count

The initial spore counts of different IMS solutions were between 2.44 to 2.62 log cfu/ml and were not significantly different ($P \leq 0.05$) between different IMS treatments. During storage at 4 and 10ºC, the spore counts were slightly decreased in the first 10 to 14 days of storage before they were increased at the end of the storage period (Figure 5.10). Although the trend of the spore count was similar at two storage temperatures, the actual population of the spores was significantly ($p \leq 0.05$) higher when the IMS samples stored at 10ºC compared to those kept at 4ºC from 3 days storage onward. The reduction of the spore population was also affected by the storage temperature and levels of sucrose. At 10ºC storage temperature, the IMS samples with 0, 2.5, 5, 7.5, 10, 15 and 20% sucrose had reductions in the spore population of 0.57, 0.43, 0.38, 0.33, 0.25, 0.20 and 0.22 log cycle, respectively. Whereas, using a lower storage temperature, the IMS treatments experienced reductions in the spore counts of 0.85, 0.86, 0.70, 0.75, 0.67, 0.47 and 0.58 log cycle, respectively. This result showed that even though nisin could inhibit the outgrowth of spore during the studied storage period, which was attributed to the availability of the antimicrobial compound throughout the storage period (Figure 5.14), the effectiveness of nisin reduced at higher sucrose levels and/or high storage temperature. The ability of nisin to inhibit spore outgrowth has also been reported by Mansour *et al*. (1999).

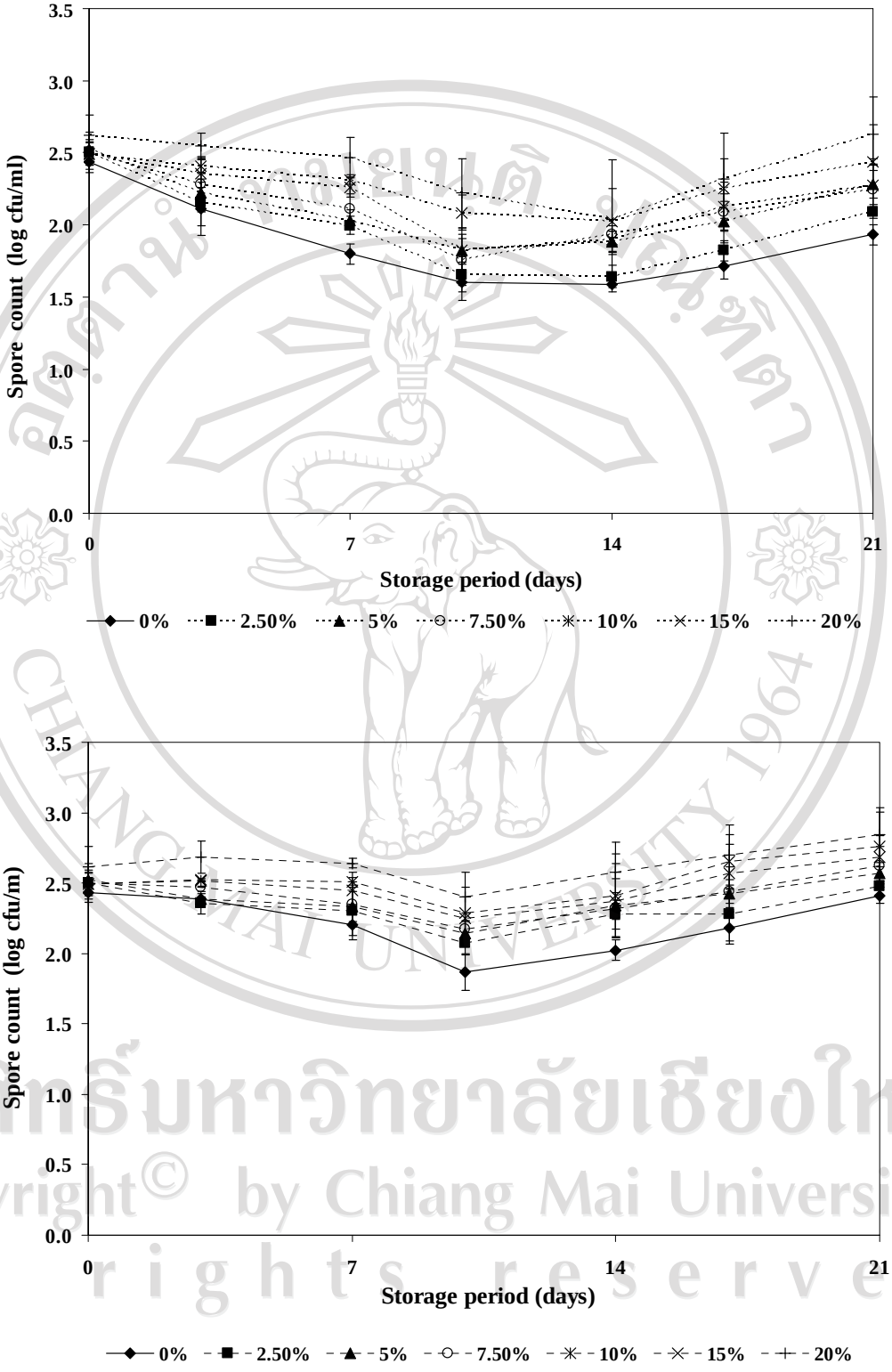


Figure 5.10 Spore count of IMS solutions with different sucrose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

5.3.2.4 Thermoduric count

The effectiveness of nisin against the thermoduric bacilli was more influenced by the storage temperature compared to the sucrose concentration (Figure 5.11). At 4ºC storage temperature, reductions in the number of the thermoduric were occurred in the first 10 to 14 days of storage before the populations of the thermoduric microorganisms were slightly increased in the last 7 days of storage. On the other hand, the thermoduric bacilli in the IMS solutions kept at 10ºC had a continue increase throughout the storage period. The thermoduric counts of the IMS solutions kept at high storage temperature were significantly ($P \leq 0.05$) higher than those of the IMS treatment stored at 4ºC after 3 days of storage.

The presence of sucrose did not produce any positive effect on the activity of nisin to inhibit thermoduric bacteria. At 4ºC, the IMS solutions supplemented with 7.5 to 20% sucrose had significantly ($P \leq 0.05$) higher thermoduric counts than that of the control treatment after 17 days of storage. A similar finding was found for the IMS solutions supplemented with 5% sucrose at the end of the storage period. Using a higher storage temperature of 10ºC, the IMS samples added with 10, 15 and 20% sucrose contained significantly ($P \leq 0.05$) higher thermoduric counts than that of the control treatment since the 3rd day of storage. Whereas the IMS treatment supplemented with 5 and 10% sucrose showed similar results on the 21st day of storage. Findings in this subsection suggested that higher nisin levels were needed to inhibit the growth of thermoduric *B. licheniformis* in the presence of high sucrose concentrations and stored at high storage temperature.

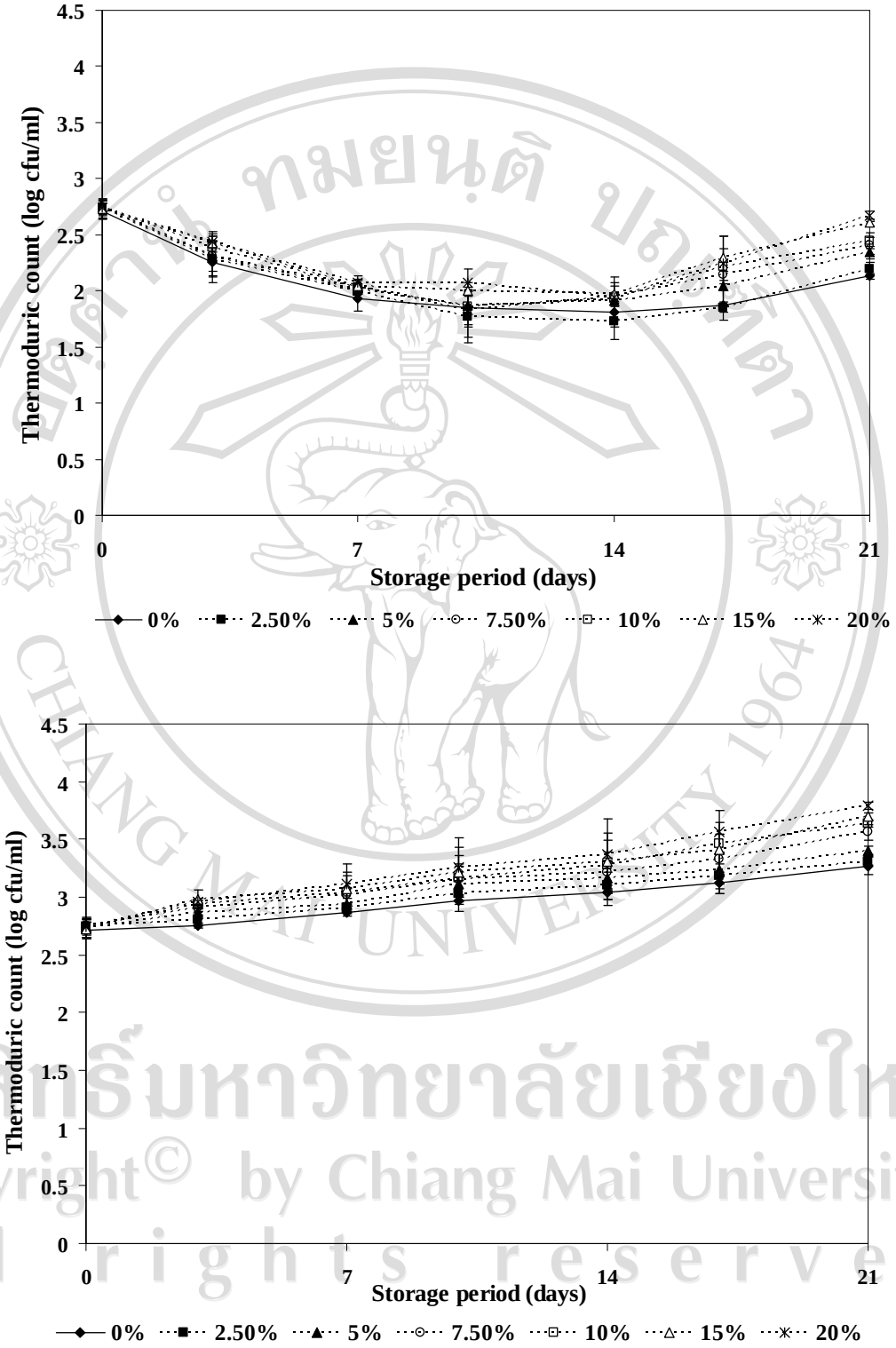


Figure 5.11 Thermoduric count of IMS solutions with different sucrose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

5.3.1.5 Chemical properties of the IMS solutions

pH value

pH of different IMS treatment was slowly decreased during 21 days storage at 4 and 10ºC (Figure 5.12). Reduction in the pH value was higher when the IMS solutions stored at higher storage temperature, but was not significantly different than that of the samples stored at 4ºC, which could be due to a wide variation within a treatment. At the same time, higher sucrose concentrations did not significantly affected ($P>0.05$) the pH of the IMS treatments. However, lower pH values were recorded for the IMS solutions supplemented with higher sucrose levels and stored at 10ºC that corresponded to higher TVM and thermoduric counts in the samples (Figures 5.9 and 5.11).

Acidity value

Responding to the pH decrease in the IMS solutions, the titrable acidity of the samples increased during the storage period (Figure 5.13). Higher values between 0.001 and 0.002% lactic acid were recorded in the IMS treatments stored at 10ºC compared to those of the treatments stored at 4ºC at the end of the storage period. The acidity of the IMS solutions supplemented with sucrose was also higher than that of the control treatment, particularly at higher storage temperature. A similar explanation could be seen in the pH section.

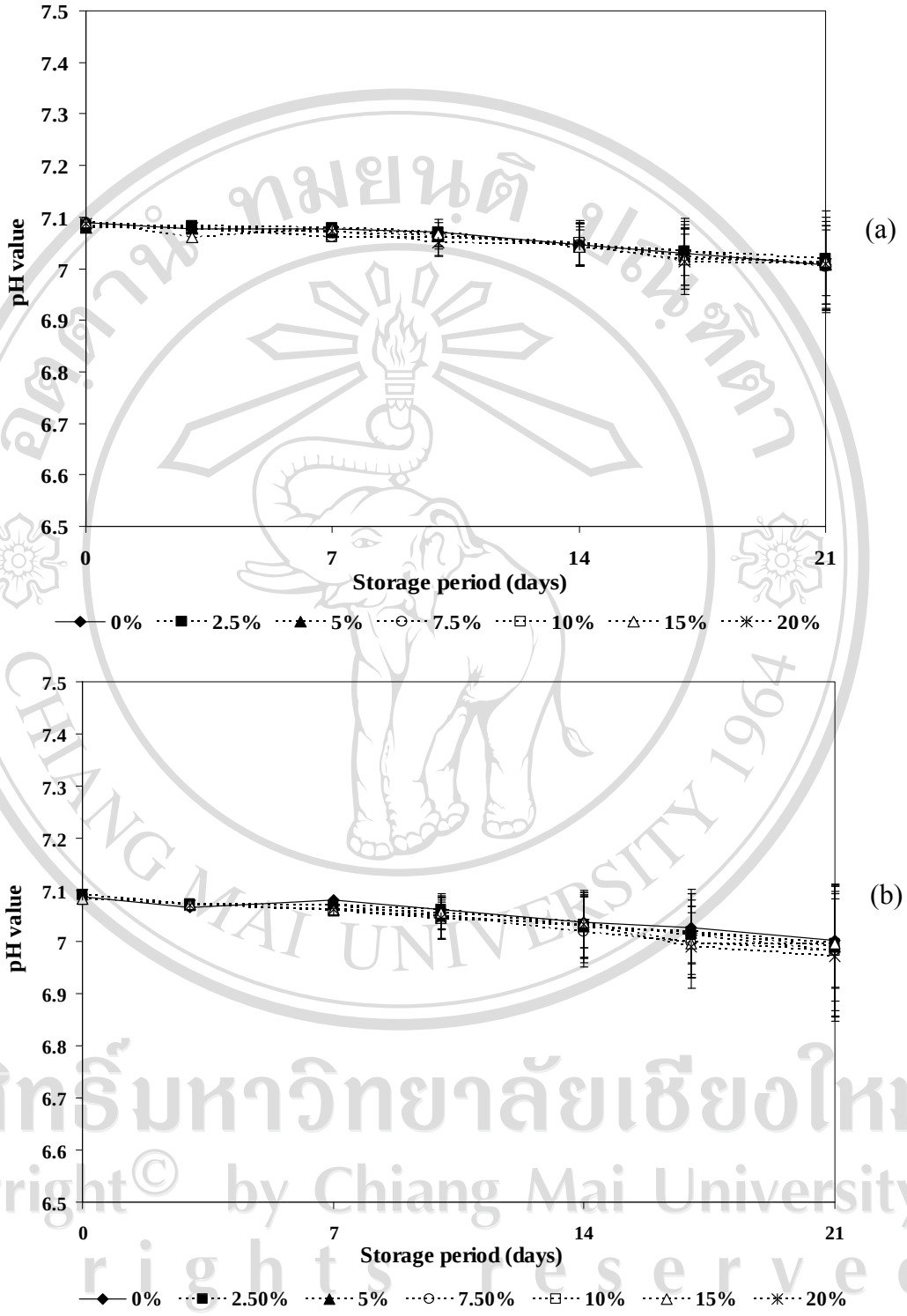


Figure 5.12 pH values of IMS solutions with different sucrose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

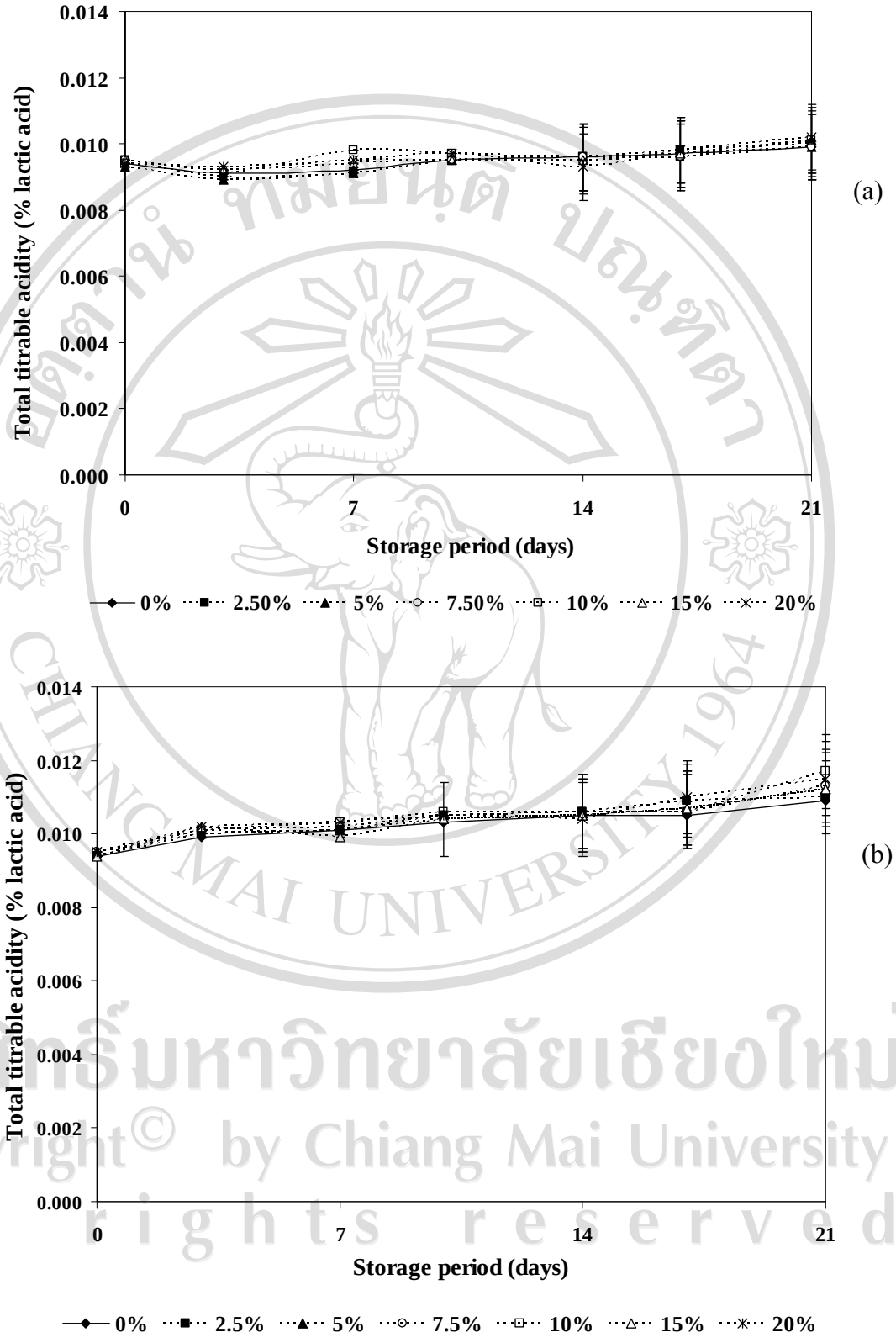


Figure 5.13 Acidity values of IMS solutions with different sucrose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

5.3.2.6 Nisin assay

Residual nisin activity in the IMS solutions was stepwise reduced during 21 days storage at 4 and 10ºC (Figure 5.14). A reduction for 54.27 IU/ml was recorded in the IMS samples stored at 4ºC, whereas a higher reduction of 59.43 IU/ml occurred in the IMS treatment stored at higher storage temperature. It could be concluded that residual nisin activity in the IMS solutions was affected by storage temperature and storage time. The presence of sucrose did not significantly ($P>0.05$) affected the residual nisin activity, even though at higher sucrose concentrations, higher TVM, spore and thermoduric counts (Figures 5.9-5.11) were found. This might indicated that sucrose might give a better support for microorganisms to growth and/or indirectly affected nisin activity in inhibiting microbial growth rather than directly reducing the nisin availability. The result showed that the high storage temperature could reduce the effectiveness of nisin, even though the activity of the antimicrobial compound was 67.93±0.06 IU/ml directly after the pasteurization treatment.(Figure 5.14)

The effectiveness of nisin to inhibit bacilli was highly dependent on storage temperature and the amount of residual nisin activity. The low storage temperature worked synergistically with nisin. At 10ºC, the growth of microorganisms was noted (Figure 5.9) even though nisin was present at 57.59±0.06 IU/ml. At concentrations of lower than 29.17±0.06 IU/ml on the 17th day of storage at 4ºC, increasing numbers of TVM, spore and thermoduric counts (Figures 5.9-5.11) were found suggesting of microorganisms to overcome the presence of nisin.

5.4 Conclusions

The result of this section showed that carbohydrate types, including lactose and sucrose, did not support the activity of nisin to inhibit *B. licheniformis* in the IMS solutions. Nisin was found to work effectively at lower storage temperature of 4ºC. Finding in this section also suggested that nisin could be used to inhibit microorganisms in food products, particularly milk products, after the products passed a mild heat treatment, such as High Temperature Short Time pasteurization and kept at 4ºC. Higher nisin levels would be needed in the presence of carbohydrates in the products.

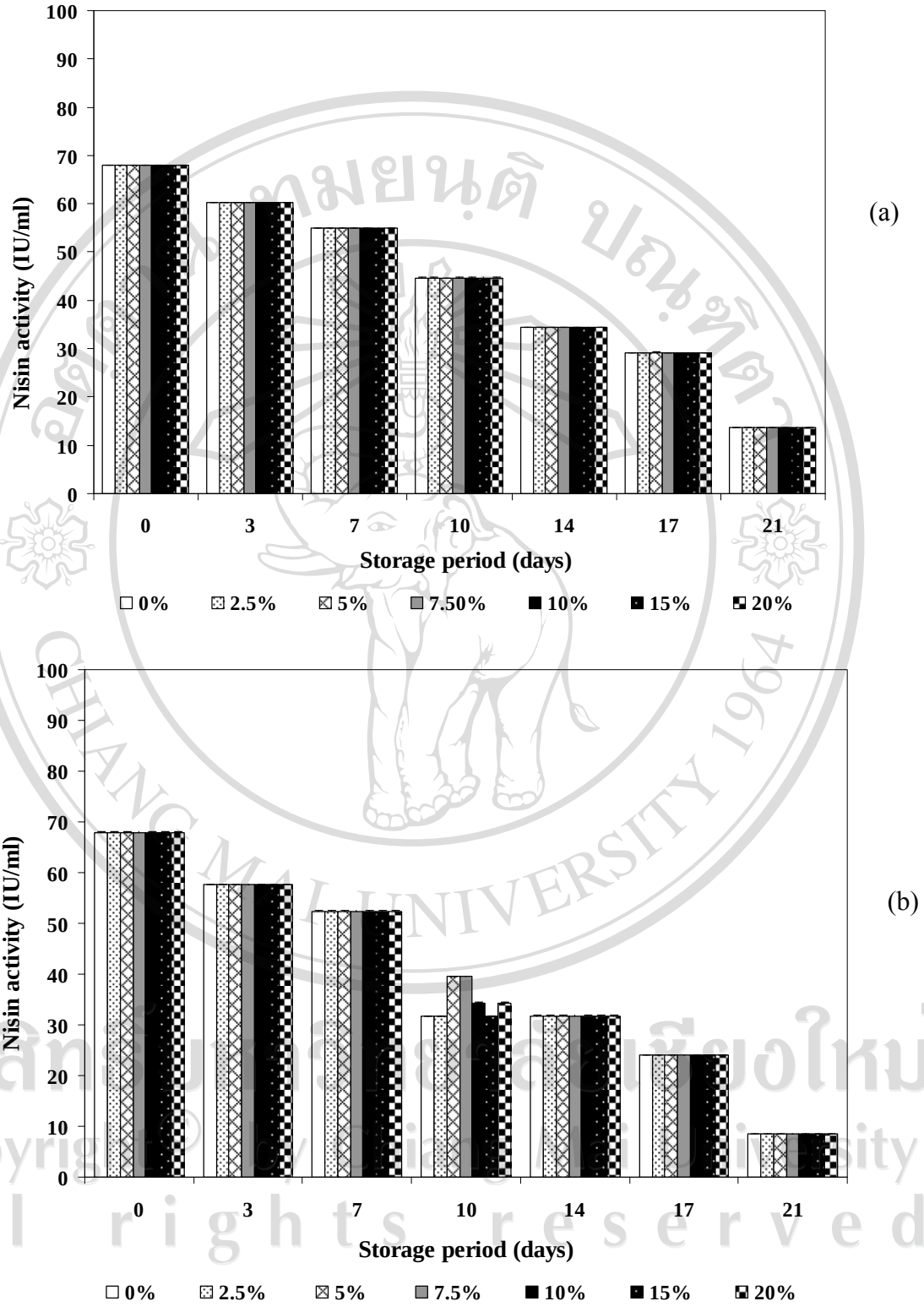


Figure 5.14 Nisin activity of IMS solution with different sucrose levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).