CHAPTER 3
THE EFFECT OF NISIN ON THE QUALITY OF PASTEURIZED MILKS PRODUCED FROM RAW MILK

3.1 Introduction

Dairy farmers in Thailand are assembled in cooperatives and raw milk is collected collectively from many farms and transferred to the dairy industries. During the collection and transportation, the quality of raw milk will be easily reduced if the temperature of the milk is not strictly controlled under a refrigeration temperature. The quality of raw milk will be worse if the location of the dairy farms is far from the factory or the milk collection centre and the collection of the milk is not done in a hygienic situation. All of these factors will increase the number of microorganisms in the raw milk and emphasize the importance of milk processing in factories to reduce, eliminate or destroy these organisms.

Milk is a good source of nutrients for mammals. Therefore, it is not astonished if microorganisms can grow easily in milk. Most of bacteria and some molds and yeast can grow in milk. However, the presence of these microorganisms is generally undesirable because they can be pathogenic or produce enzymes that cause undesirable transformations in milk. There are several factors that affect the growth of bacteria in milk and milk products, including storage temperatures, initial load of microorganisms, acidity and alkalinity (pH), water availability and oxygen (Brock and Madigan, 1988). The storage temperature has a large effect on the growth rate of bacteria. Keeping milk at low temperatures will generally slow down the bacteria growth because the bacteria will need longer time for their lag phase compared to storage at high temperatures. However, the extension of the milk quality at low temperatures will be depended on the type of organism that is present in milk itself. The psychrotrophic bacteria, for example, will still cause a problem for the milk quality even if the milk is stored at a refrigeration temperature.

Another factor is the initial load of microorganisms in milk. Either keeping a batch of milk at low or high temperatures, a lower initial load of microorganisms will delay spoilage in that batch of milk compared with a higher initial load. Therefore, a

good quality of raw milk can be produced if the milk is directly stored at temperature ≤ 4°C after milking and the milk is processed as soon as possible because the low storage temperature can prevent the multiplication of bacteria only for 24 h (Bramley and McKinnon, 1990; Walstra *et al*., 1999).

The predominance spoilage organisms in milk at low storage temperature are Gram negative rods psychrotrophic species, including *Pseudomanas* spp., in which *Ps. fluorescens* is the dominance species and other species will be *Ps. putida*, *Ps. fragi* and *Ps. aeruginosa*; *Flavobacterium*; *Alcaligenes* and coliforms. The Gram positive microorganisms will include genera *Bacillus*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus* and *Microbacterium* (Bramley and McKinnon, 1990; Varnam and Sutherland, 1994; Sorhaug and Stepaniak, 1997; Harding, 1999; Prescott *et al*., 2002).

Psychrotrophic *Bacillus* spp. are aerobic bacteria that can become pathogenic microorganisms to men and animals because they form endospores that are heat resistant. *B. cereus* and *B. licheniformis* were the most commonly isolated species of *Bacillus* in milk. *B. licheniformis* was present in the farm environment and was higher in raw milk than *B. cereus* during the winter months. However, *B. cereus* could come to dominate the *Bacillus* population and reach a level of enterotoxin production, because *B. cereus* grew faster than *B. licheniformis* at ambient temperatures (Crielly *et al.*, 1994). Research showed that a low level of environmental contamination of *B. cereus* spores in raw milk would lead to a major source of psychrotrophic *B. cereus* in the pasteurized milks (Larsen and Jorgensen, 1999).

Since *B. cereus* and *B. licheniformis* can grow in dairy products, non-aseptic packaged refrigerated fluid milk can be spoiled because of the growth of these organisms. *Bacillus* spp. is present in more than 80% of raw milk samples. The spores of microorganism can germinate after pasteurization as a result of heat-shocked mechanism. In addition, the organisms can also present in milk products as a result of PPC from the processing plant. The defect characteristics in milk products as a result of *Bacillus* spp. growth are recognized as sweet curdling, coagulation by a chymosin-like protease and bitter flavor in the milk products (Frank, 1997).

To further control the growth of *Bacillus* spp. in pasteurized milks during their storage period, an application of an antimicrobial protein, such as nisin would be an alternative solution. 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, raw milk was used and supplemented with different concentrations of nisin before passed a pasteurization process. The pasteurized milk was stored at 4 and 10ºC and analyzed for its microbiological and chemical properties during 21 days of storage. The objective of this work was to determine the effect of nisin on the growth of microorganisms from raw milk during the shelf life of pasteurized milk. In addition, spore colonies that were growth on the agar media were isolated, purified and identified to find out the species of the survival spore forming bacteria in the pasteurized milk without nisin addition.

3.2 Material and methods

3.2.1 Raw milk

Raw milk from local dairy farmers in Chiang Mai was used. An amount of 5 l of raw milk during an experimental period of February to July 2004 was bought from a local dairy cooperative (Huay Kaew, Chiang Mai) and transported to the laboratory at low temperature (not over than 4°C).

3.2.2 Study the effect of nisin on the keeping quality of pasteurized milk produced from raw milk

In this section, the experimental plan was divided into 2 sections. In the first sub-section, nisin concentrations of 0, 100, 250, 500 and 1,000 IU/ml were used, whereas in the second sub-section, lower nisin levels of 0, 25, 50, 75 and 100 IU/ml were investigated. Cold raw milk from the section 3.2.1 was aseptically added with nisin at different levels 30 min before a pasteurization process. The pasteurization process at 72ºC for 15 s was carried out in a water bath (Memmert®, Germany). To do the heating process, the water bath temperature was firstly set into a temperature of 75ºC. When the temperature was reached, different treatments of raw milk in screw

glass bottles were immersed into the water bath with one screw glass bottle containing raw milk and a probe of a digital thermometer (Tecpel 307, Taiwan) to monitor the internal temperature of milk treatments. As the digital thermometer showed an internal temperature of milk of 72ºC, the holding time of the pasteurization process of 15 s was initiated and at the same time, the temperature of the water bath was slightly reduced to maintain the milk temperature to be at 72±0.5ºC. After 15 s of the holding time, the pasteurized milk treatments were quickly cooled down using a running cold water and stored at 4 and 10ºC. Directly after the pasteurization and during 14 to 21 days of storage, samples of pasteurized milk were determined for their chemical and microbiological properties. The microbiological standard for pasteurized milk in Thailand was $5x10^4$ cfu/ml (Ministry of Public Health, 2002a; Ministry of Public Health, 2002b). Each of the pasteurized milk treatments was done in triplicate.

3.2.3 Nisin

Nisin used in this research was bought from Danisco Co., England under a brand name of 'Nisaplin'. The antimicrobial compound was in the form of white powder and had a concentration of nisin of $1x10^6$ IU/g. The addition of nisin into raw milk was carried out aseptically in a laminar flow cabinet (Heal Force 1200/C, China).

3.2.4 Microbiological analysis

3.2.4.1 Total viable microorganisms

The number of total bacteria in raw milk samples was determined by a Standard Plate Count (SPC) method (Marshall, 1992).

3.2.4.2 Spore count

The number of spores in milk samples was measured by heating the samples in a water bath at 80°C for 10 min. The samples were then cooled and plated on Plate Count Agar (PCA). The plates were incubated at 30°C for 3 days for mesophiles spore (Harrigan, 1998).

3.2.4.3 Thermoduric bacteria

The analysis of thermoduric bacteria was carried out by heating the milk samples in a water bath at 63.5±0.5°C for 35 min. After the time finished, the samples were removed from the water bath and cooled rapidly in ice water. The milk samples were diluted as appropriately and plated on PCA (Merck, Germany). The plates were incubated at 30°C for 2 days (Harrigan, 1998).

3.2.5 Chemical analysis

3.2.5.1 pH measurement

pH values of the milk samples were measured by a pH meter (Consort C830, Belgium).

3.2.5.2 Total titrable acidity measurement

Milk samples of 9 g were measured and transferred into a 100 ml beaker. An amount of 0.5 ml phenopthalein (Merck, Germany) was added into the milk samples. The samples were then titrated against 0.1 N sodium hydroxide (Merck, Germany) until a pink color appeared for 30 s (Marshall, 1992).

3.2.6 Nisin assay

Nisin concentration in the milk samples was determined by an agar plate diffusion method using *Micrococcus luteus* as a test microorganism (Fowler *et al*., 1975; Pongtharangkul and Demirci, 2004).

3.2.7 Microorganisms identification

During storage of pasteurized milk samples without nisin addition, spore colonies that grown on the PCA medium were further isolated by transfering selected colonies on fresh solid PCA medium. The medium was incubated at 30ºC for 48 hr. Single colony from each of the PCA medium was streaked for another 2 more times on fresh solid PCA medium to produce pure colonies of microorganism. The final isolated colonies was checked with Gram straining (Harrigan, 1998), identified the cell structure using microscope (Olympus, USA) and subjected to some biochemical reactions, including Hugh and Liaison's, starch hydrolysis, acid from mannitol, citrate

utilization and Voges-Praskauer test. The biochemical results were compared with bacteria characteristic on the Bergey's manual of determinative bacteriology (Priest, 1989; Holt *et al*., 1994; Harrigan, 1998). The bacterium species was determined using an API 50 CHB test kit (Bio Merieux, France) that was incubated at 30ºC for 24 and 48 h. The positive reaction results from the test kit were compared with the database for the microorganism profile supplied by Bio Merieux, (France).

3.2.8 Experimental design

The experiment results of the section 3.2.2 were analyzed statistically using a Factorial in Completely Randomized Design with 2 factors. The first factor was nisin concentrations, which were 0, 100, 250, 500 and 1,000 IU/ml for the first subsection or were 0, 25, 50, 75 and 100IU/ml in the second subsection. The second factor was storage temperatures, which were at 4 and 10°C. Duncan's New Multiple Range Test (DMRT) was then used to determine differences between treatment means. The analyses were carried out using a SPSS program (SPSS version10.0) (SPSS Inc., Chicago, USA).

3.3 Results and discussion

3.3.1 The effect of 0 to 1,000 IU/ml nisin on the keeping quality of pasteurized milks produced from raw milk

3.3.1.1 TVM count

The effectiveness of nisin to inhibit microorganisms in raw milk was displayed directly after the pasteurization process at 72ºC for 15 s and during 21 days of storage at 4 and 10ºC. The initial TVM population of the control pasteurized milk was significantly ($P \leq 0.05$) higher than those of the pasteurized milk supplemented with nisin at the beginning of the storage period (Figure 3.1). Higher nisin concentrations also significantly ($P \leq 0.05$) produced lower initial TVM enumeration. This finding suggested that nisin worked synergistically with pasteurization in reducing the microbial load in pasteurized milk. At the same time, some losses of nisin during the heating process should be expected (Figure 3.6; Thomas *et al*., 2000).

During 21 days of storage, the control pasteurized milk had a significant ($P \leq 0.05$) increase in its TVM population and was significantly ($P \leq 0.05$) affected by the storage temperatures. On the other hand, the pasteurized milk supplemented with nisin experienced further reduction within the first 3 days of storage. After this time period, the concentrations of nisin had a significant effect on the growth of the TVM population in the milk samples. In the presence of 100 IU/ml nisin, the TVM population in the milk treatments was increased to 2.56±0.03 and 2.79±0.01 log cfu/ml at the end of the storage period when the milk was stored at 4 and 10ºC, respectively, and was significantly ($P \leq 0.05$) affected by the storage temperature. However, the supplementation of 250 to 1,000 IU/ml nisin continued to inhibit the growth of the vegetative bacilli to be below 10 cfu/ml throughout the rest of the storage period. This result suggested that the residual nisin activity in the pasteurized milk (section 3.3.1.6) played an important role in controlling the growth of the TVM population during the storage period. When the nisin activity was between 62.59 and 66.30 IU/ml (the pasteurized milk supplemented with 100 IU/ml nisin), the vegetative bacilli was able to start to grow in the milk samples, whereas the residual nisin activity of 103.33 or higher (the pasteurized milk supplemented with 250 IU/ml or higher nisin) could continue to inhibit the growth of the TVM population even though the milk was kept at 10ºC.

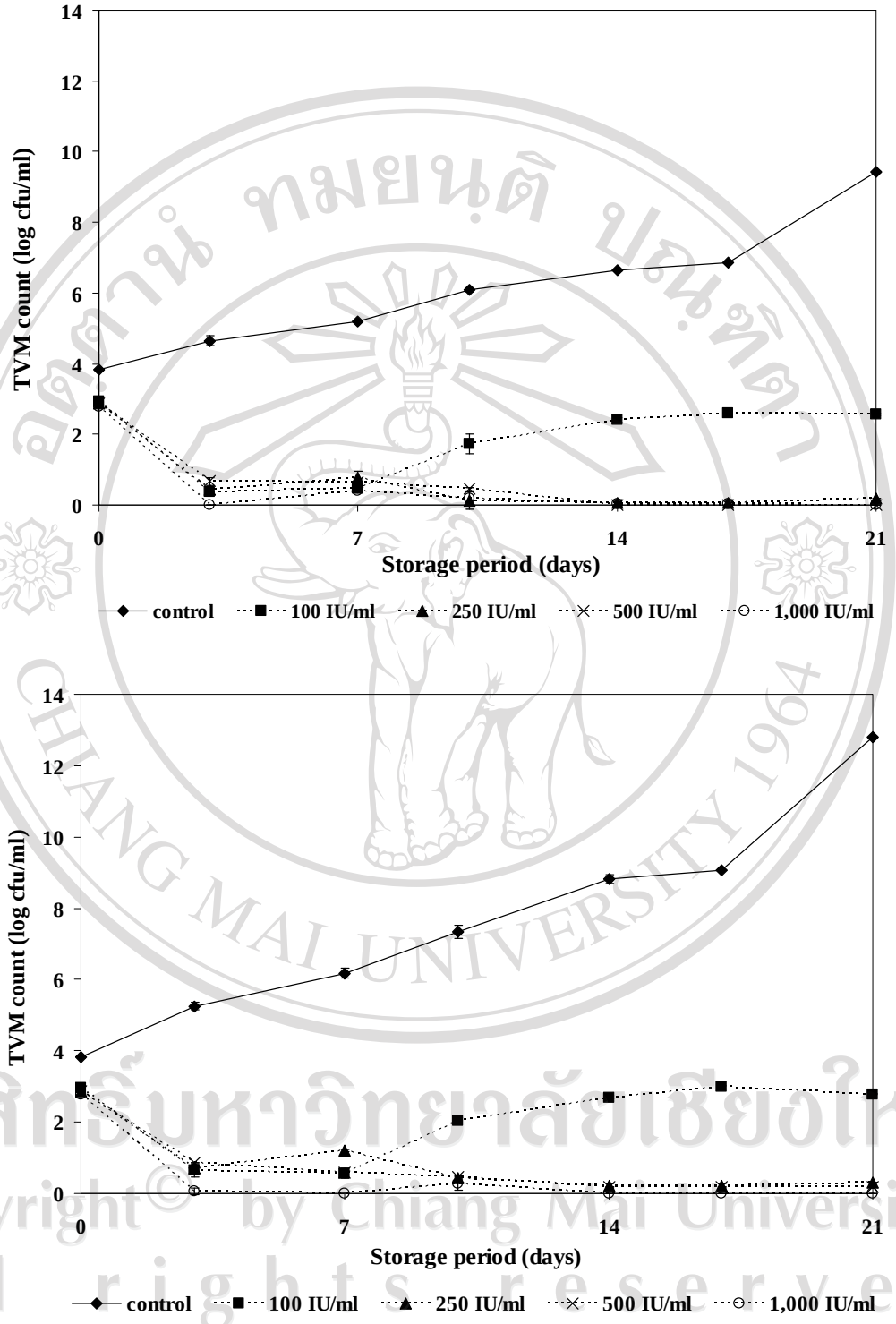


Figure 3.1 Total Viable Microorganisms of pasteurized milk supplemented with 0 to 1,000 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.1.2 Spore count

Nisin was also found to be significantly effective in reducing the spore number in the pasteurized milk treatments after the pasteurization process (Figure 3.2). However during the storage period, the spore in the control pasteurized milk samples experienced a further reduction for up to 0.92 log cycle within the first 3 days of storage and was significantly ($P \leq 0.05$) affected by different storage temperatures (lower spore reduction at higher storage temperature). After the 3rd day of storage, the spore of the control pasteurized milk increased to reach spore populations of 4.16±0.09 and 5.77±0.09 log cfu/ml in the milk treatments kept at 4 and 10ºC, respectively, on the 21st day of storage. In contrast, a higher reduction of the population between 2.55 and 2.82 log cycle was found in the nisin added pasteurized milk at the beginning of the storage period. Following this high reduction, the spore of the pasteurized milk supplemented with 100 IU/ml nisin had an increase in its spore population to become 1.54±0.01 and 1.74±0.01 log cfu/ml in the milk samples stored at 4 and 10ºC, respectively, at the end of the storage period, and was significantly ($P \leq 0.05$) lower than those of the control pasteurized milk treatments. The pasteurized milk supplemented with 100 IU/ml nisin was also significantly affected by the storage temperatures after 10 days of storage. For the pasteurized milk supplemented with 500 and 1,000 IU/ml nisin, the spore population in the milk samples continued to be lower than 5 cfu/ml at both storage temperatures from the 3rd day of storage onward. This result of the spore enumeration supported the previous finding of the TVM enumeration that the residual nisin activity in the pasteurized milk played an important role in controlling the spore outgrowth.

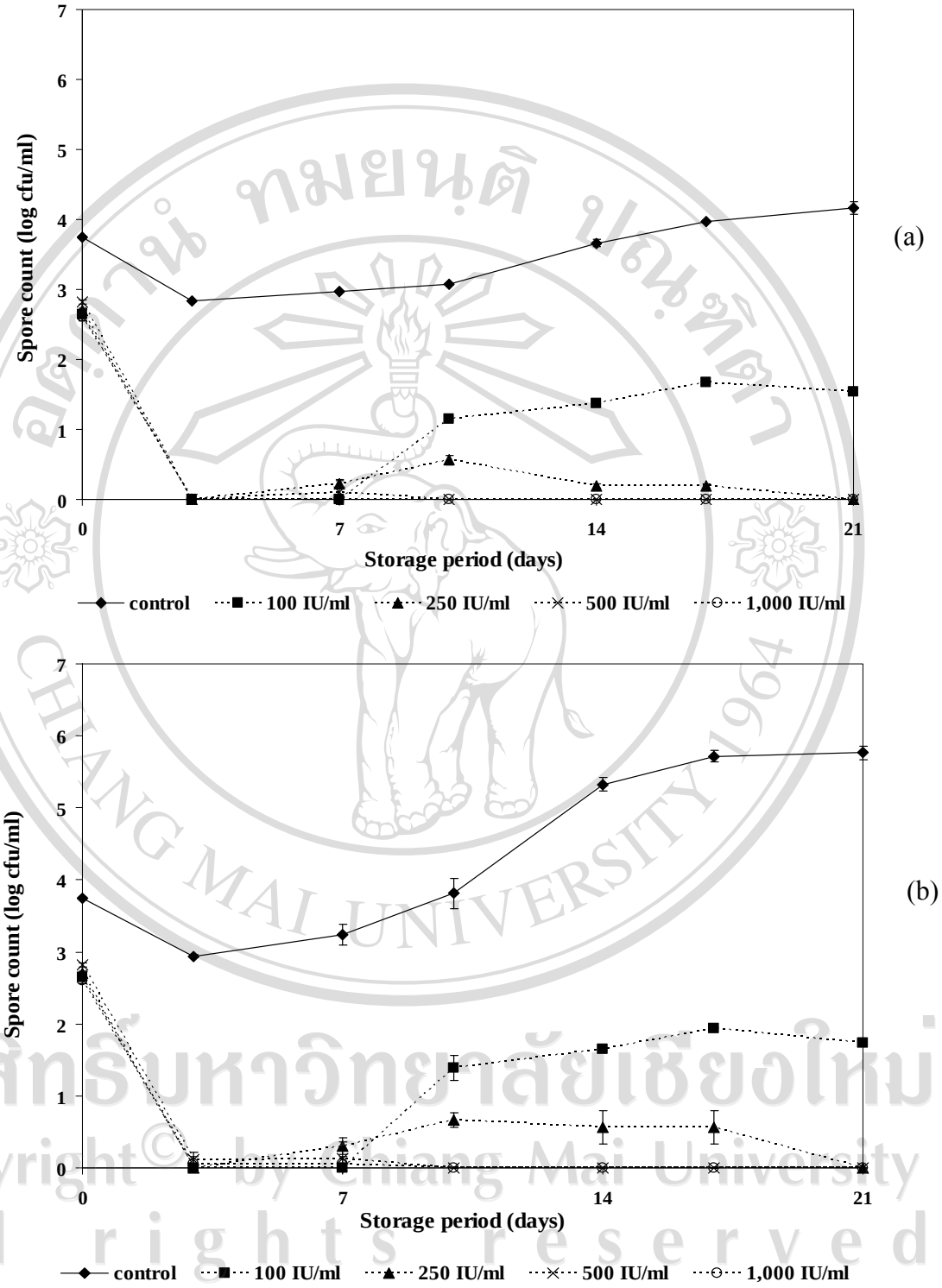


Figure 3.2 Spore count of pasteurized milk supplemented with 0 to 1,000 IU/ml nisin during storage at 4ºC (a) and 10ºC (b)

3.3.1.3 Thermoduric count

In general, the development of the thermoduric microorganisms in different pasteurized milk treatments (Figure 3.3) reflected the result of the TVM enumeration (Figure 3.1) at lower microbial numbers. This finding indicated that the thermoduric bacteria would be the main spoilage microorganisms in the pasteurized milk in the absence of PPC (Frank, 1997). Although different concentrations of nisin were effective in reducing the thermoduric population when combined with a pasteurization treatment, during the storage period the residual nisin activity significantly ($P \leq 0.05$) affected the growth of the thermoduric bacilli. The storage temperature also significantly ($P \leq 0.05$) affected the increase in the thermoduric population in the pasteurized milk without nisin addition and with 100 IU/ml nisin.

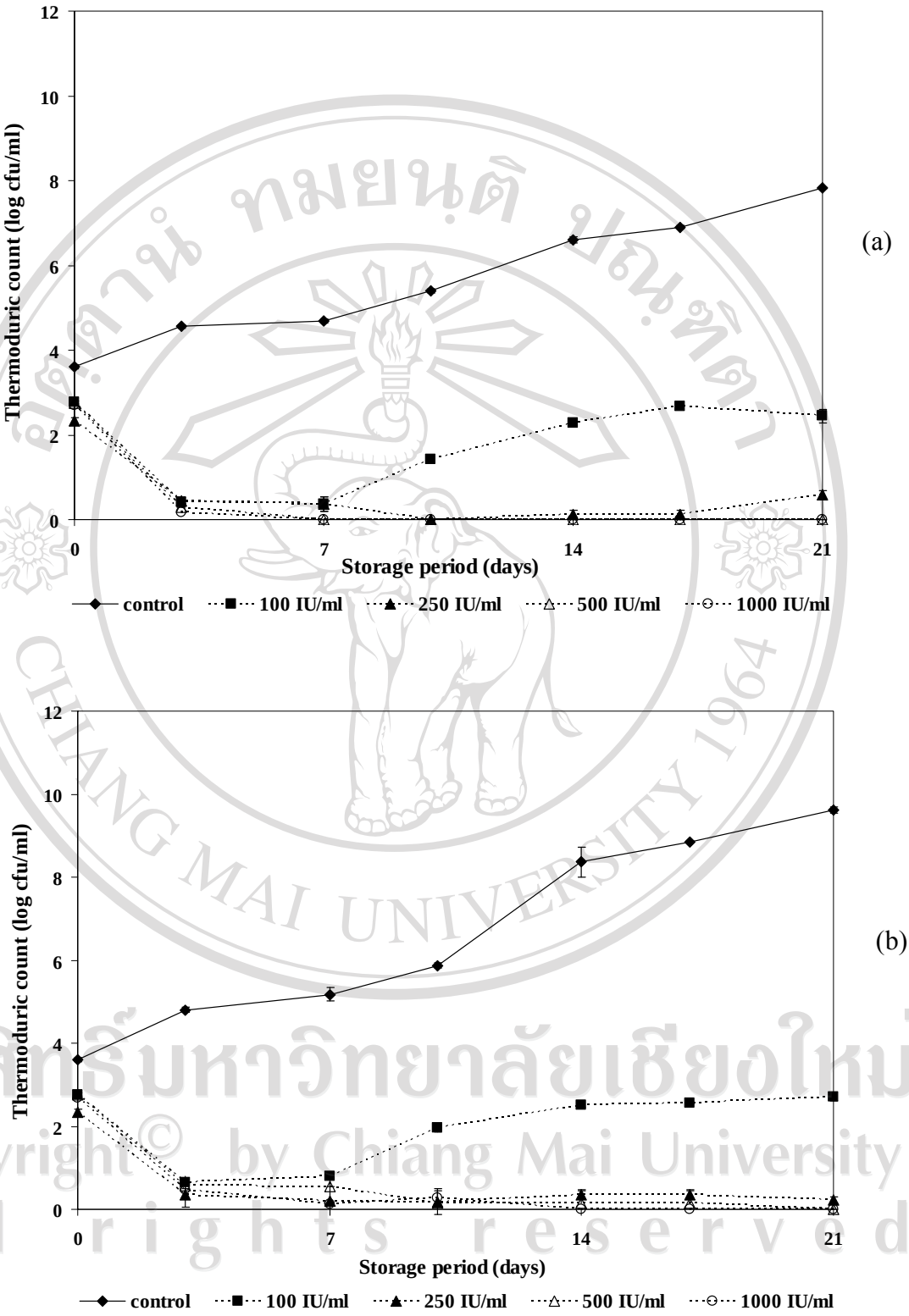


Figure 3.3 Thermoduric count of pasteurized milk supplemented with 0 to 1,000 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.1.4 pH value

Decrease in the pH of pasteurized milk samples (Figure 3.4) confirmed the finding for the growth of microorganisms in the milk during the storage period (Figures 3.1-3.3). The control pasteurized milk had a significant ($P \leq 0.05$) decrease in its pH value and was significantly ($P \leq 0.05$) lower than those of the nisin added pasteurized milk after 3 days of storage. Higher storage temperature also caused the pH of the control pasteurized milk to be significantly ($P \leq 0.05$) lower. Although reduction in the pH of the pasteurized milk supplemented with 100 IU/ml nisin supported the result of the microbial growth in the milk samples, pH reduction between 0.46 and 0.68 unit in the pasteurized milk supplemented with 250 to 1,000 IU/ml nisin stored at 4 and 10ºC storage temperature indicated that there was microbial activities in the milk samples even though no significant viable microorganism population was detected in the milk samples. This finding suggested that the work of nisin against the microbial population in pasteurized milk might only act as an inhibitor for the growth of the microbial population rather than to directly kill the microorganisms.

3.3.1.5 Total acidity

Responding to the decrease in the pH value, the acidity of different pasteurized milk treatments increased during the storage period (Figure 3.5). The control pasteurized milk had a significant ($P \leq 0.05$) higher increasing rate of acidity compared to those of the nisin added pasteurized milk treatments. Storage temperatures were found to significantly affect the acidity of the control pasteurized milk and the pasteurized milk supplemented with 100 IU/ml nisin. The presence of higher levels of nisin of 250 to 1,000 IU/ml reduced the effect of the storage temperature on the milk acidity, which could be due to a better control of the antimicrobial peptide towards the microbial growth in the milk samples.

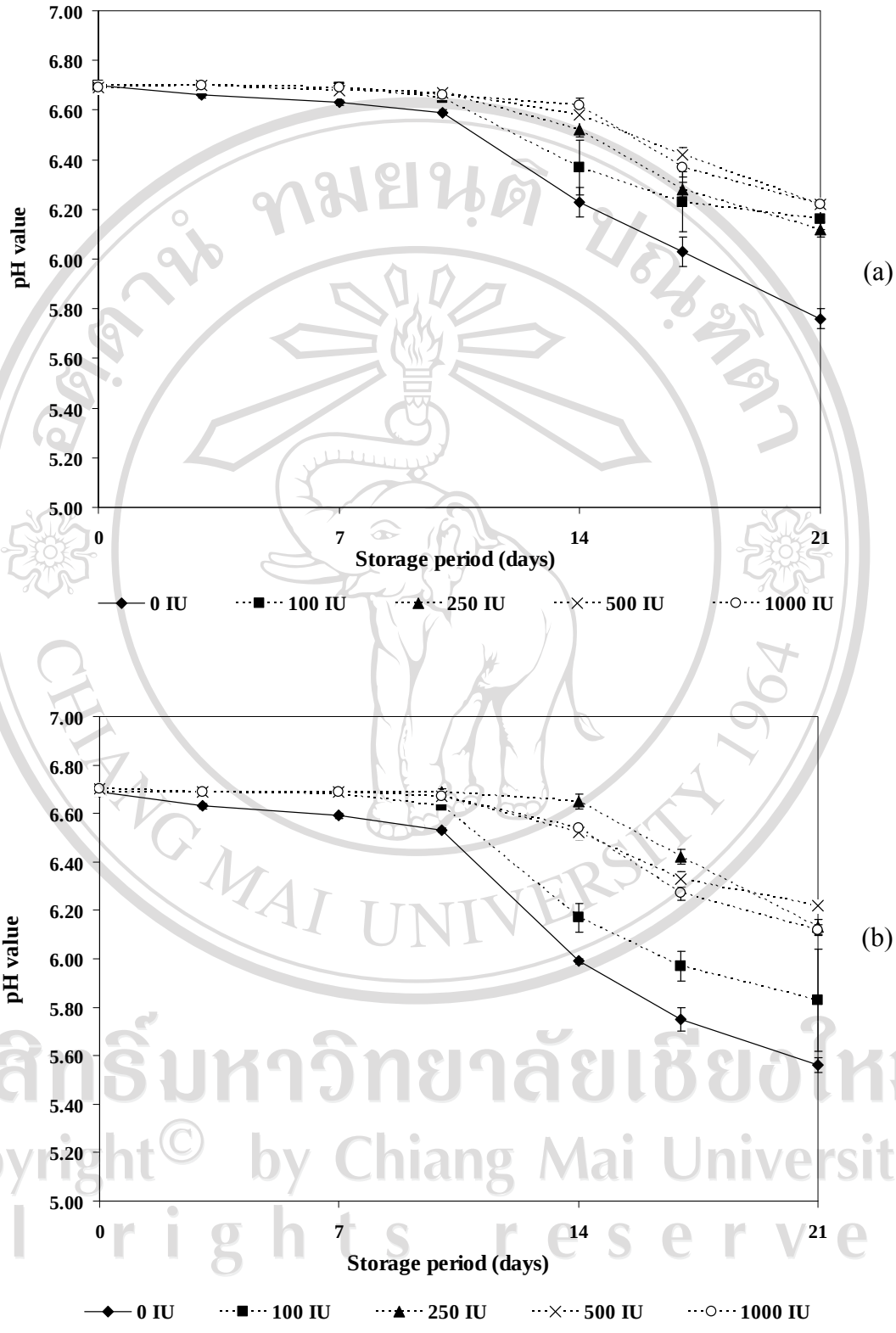


Figure 3.4 pH value of pasteurized milk supplemented with 0 to 1,000 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

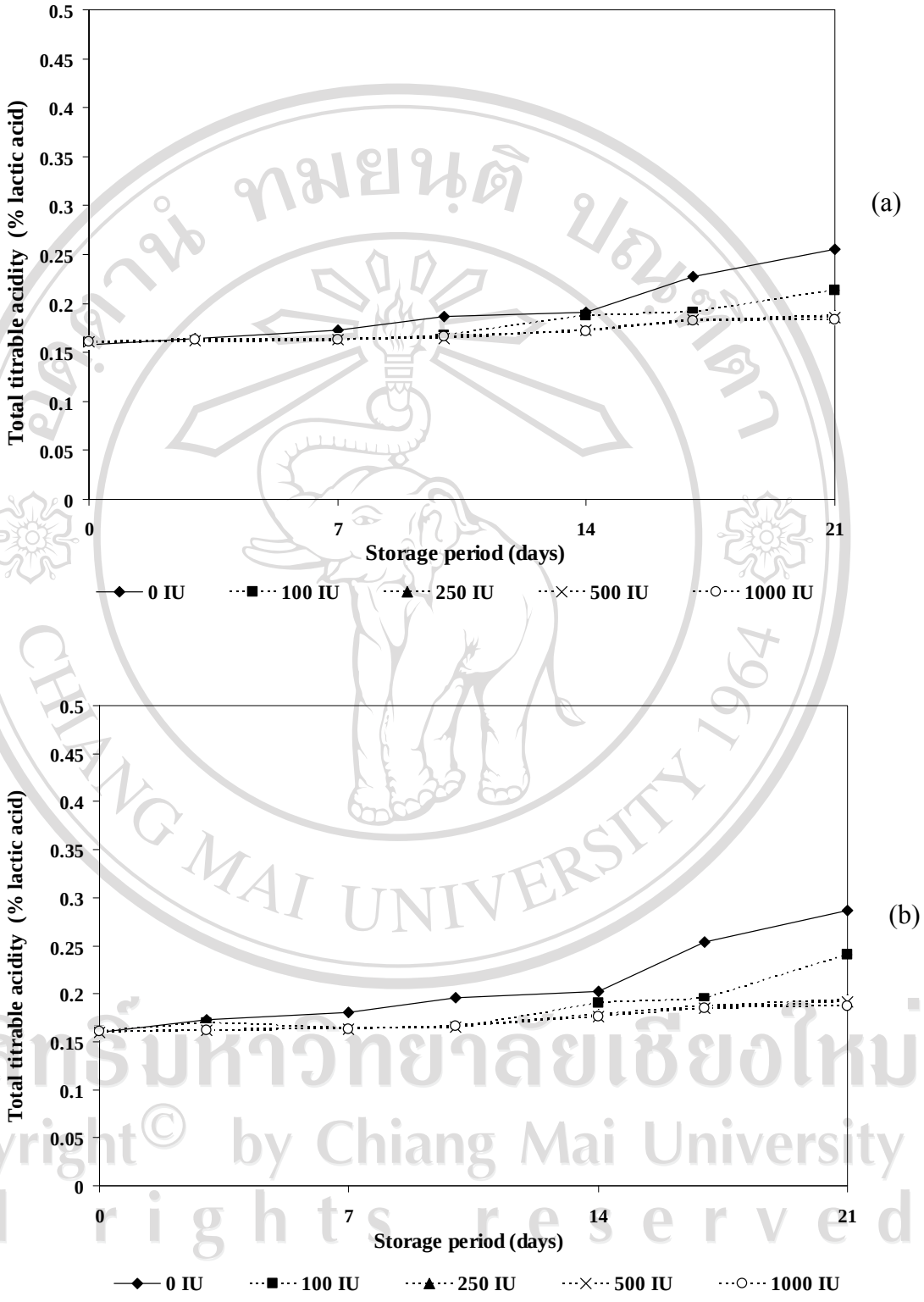


Figure 3.5 Total titrable acidity of pasteurized milk supplemented with 0 to 1,000 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.1.6 Nisin assay

Reductions of the nisin activity between 10.04 and 30.00% were found directly after the pasteurization process (Figure 3.6). Higher supplementation levels of nisin reduced the loss of the nisin activity during the heat treatment. During 21 days of storage, the reduction of the nisin activity was occurred at a higher rate compared to the reduction during pasteurization. When the pasteurized milk was kept at 4ºC, the supplementation of nisin at 100, 250, 500 and 1,000 IU/ml in the milk samples experienced reduction for 37.04, 43.18, 22.14 and 16.47%, respectively, at the end of the storage period. Applying a higher storage temperature caused a higher reduction in the nisin activity. Reductions for 47.61, 51.81, 35.43 and 24.70% were recorded in the pasteurized milk supplemented with 100, 250 500 and 1,000 IU/ml nisin, respectively. This finding suggested that the nisin activity in the pasteurized milk was influenced by the initial nisin levels, the pasteurization condition, the storage temperatures and storage time.

Although the presence of 250 to 1,000 IU/ml nisin demonstrated a good control for the microbial growth in the pasteurized milk, the supplementation of 100 IU/ml nisin had already produced a significant improvement in the microbial quality of the milk compared to the control pasteurized milk. In order to have a batter understanding about the effectiveness of nisin against the microbial population in the pasteurized milk and to further reduce the additional cost to add nisin in the pasteurized milk, lower nisin concentrations of 0 to 100 IU/ml were investigated in the next section.

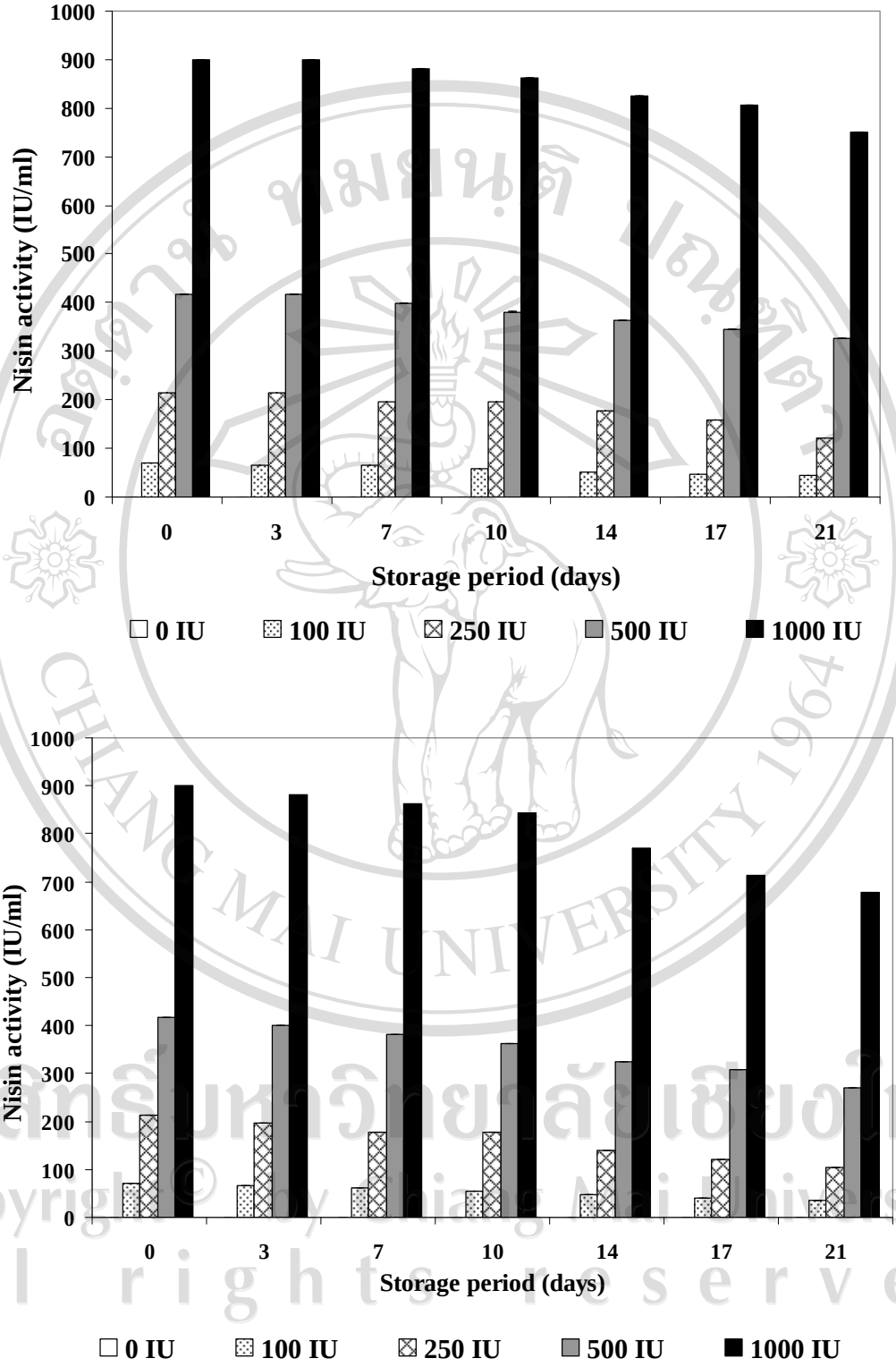


Figure 3.6 Nisin activity of pasteurized milk supplemented with 0 to 1,000 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.2 The effect of 0 to 100 IU/ml nisin on the keeping quality of pasteurized milks produced from raw milk

3.3.2.1 TVM count

In this section, concentrations of nisin between 0 and 100 IU/ml were supplemented into raw milk before a pasteurization process at 72ºC for 15 s to further understand whether lower nisin concentrations could produce a similar effectiveness as 100 IU/ml nisin in controlling the microbial population in pasteurized milk. Data from TVM enumeration showed that directly after the pasteurization process, the TVM population in the pasteurized milk was significantly ($P \leq 0.05$) affected by the nisin concentrations supplemented in the milk. Reductions for 1.23, 1.48, 1.88, 1.93 and 1.97 log cycle of the TVM populations were found in the pasteurized milk supplemented with 0, 25, 50, 75 and 100 IU/ml nisin, respectively (Figure 3.7). After this initial reduction, the control pasteurized milk had a significant ($P \leq 0.05$) increase in its TVM population during 2 weeks storage and was significantly ($P \leq 0.05$) affected by the storage temperature. In contrast, the pasteurized milk supplemented with nisin had a further reduction in its TVM population at the beginning of the storage period. This second reduction was also influenced by the levels of nisin in the pasteurized milk. A reduction for up to 0.26 log cycle was recorded in the pasteurized milk supplemented with 25 IU/ml nisin, whereas the presence of 100 IU/ml could produce a second reduction for up to 2.52 log cycle. Following this second reduction, all of the nisin added pasteurized milk had an increase in their TVM population at a similar rate until 14 days of storage. A higher storage temperature also caused higher TVM enumeration in the nisin added pasteurized milk.

The result of the TVM enumeration demonstrated that lower nisin concentrations of 25 to 100 IU/ml still gave a significant effect on the microbial population of the pasteurized milk compared to that of the control pasteurized milk. However the microbial control produced by these nisin concentrations was not able to maintain low numbers of the TVM population after 7 days of storage, except in the presence of 75 and 100 IU/ml nisin. The supplementation of 25 IU/ml nisin in the pasteurized milk, for example, caused the milk to have 6.20±0.15 log cfu/ml TVM population after 2 weeks of storage at 10ºC, which was followed by a significant change in the milk chemical property (Figures 3.10–3.11). Due to this reason, the

supplementation of 100 IU/ml nisin was noted to be the best concentration, since the nisin level significantly (P≤0.05) produced the lowest TVM population throughout the studied storage period (Figure 3.7).

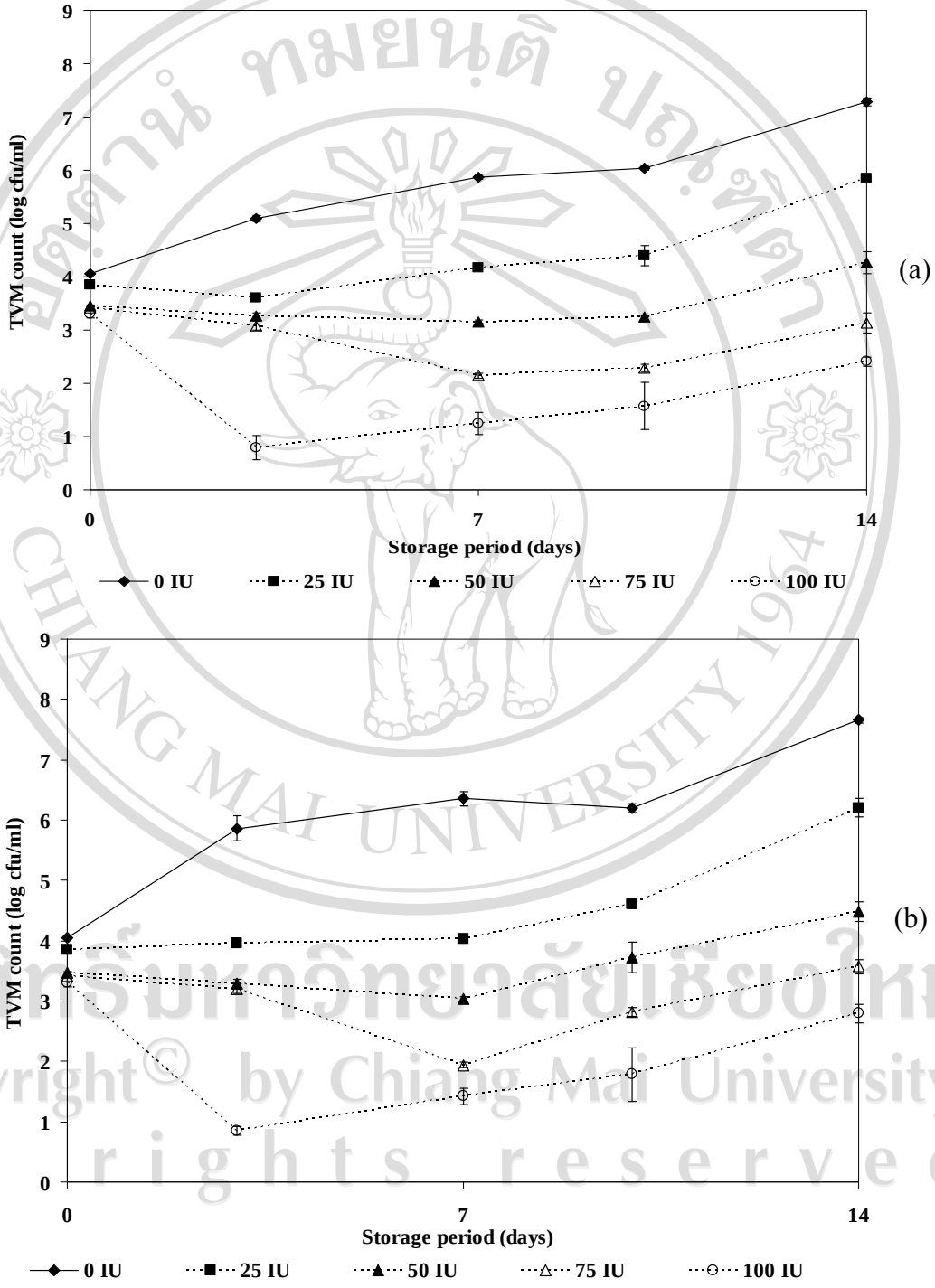


Figure 3.7 Total Viable Microorganisms of pasteurized milk supplemented with 0 to 100 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.2.2 Spore count

The initial spore count of different pasteurized milk treatments was significantly ($P \leq 0.05$) affected by the presence of nisin (Figure 3.8). Lower initial spore count was also noted at higher concentrations of nisin. From the initial spore count between 2.97 and 3.31 log cfu/ml, the spore in the pasteurized milk experienced reductions within the first week of storage followed by an increase in its number in the last 7 days of storage. The control pasteurized milk had a reduction between 0.19 and 0.25 log cycle for its spore number at the beginning of the storage period. Higher reductions between 1.17 and 1.30 log cycle and between 2.17 and 2.32 log cfu/ml were found in the pasteurized milk supplemented with 75 and 100 IU/ml nisin, respectively. After 7 days of storage, the increasing rate of the spore in the pasteurized milk supplemented with 25 IU/ml nisin was similar or even higher than that of the control pasteurized milk causing spore numbers in the both treatments to be not significantly different on the 14th day of storage. The presence of 50 to 100 IU/ml nisin in the pasteurized milk caused the spore in the milk to have a lower increasing rate and produced significantly ($P \leq 0.05$) lower spore population at the end of the storage period. However, it was only the pasteurized milk supplemented with 100 IU/ml that significantly had the lowest spore number throughout the storage period at 4 and 10ºC storage temperatures.

3.3.2.3 Thermoduric count

As in the section 3.3.1.3, the results of the thermoduric enumeration in this section (Figure 3.9) displayed similar microbial growth rates as in the finding for the TVM population (Figure 3.7). However, the thermoduric count of different pasteurized milk was not significantly ($P > 0.05$) different directly after the pasteurization process. During the storage period, the thermoduric organisms in the control pasteurized milk continued to have an increase in the population number after the pasteurization process. On the other hand, the thermoduric population in the nisin added pasteurized milk experienced reductions in the first 3 days of storage followed by an increase in its population number for the rest of the storage period. Higher reductions in the thermoduric count were found at higher levels of nisin. The lowest number at thermoduric population was found in the pasteurized milk supplemented

with 100 IU/ml nisin, especially when the milk was stored at 4ºC, throughout the studied storage period.

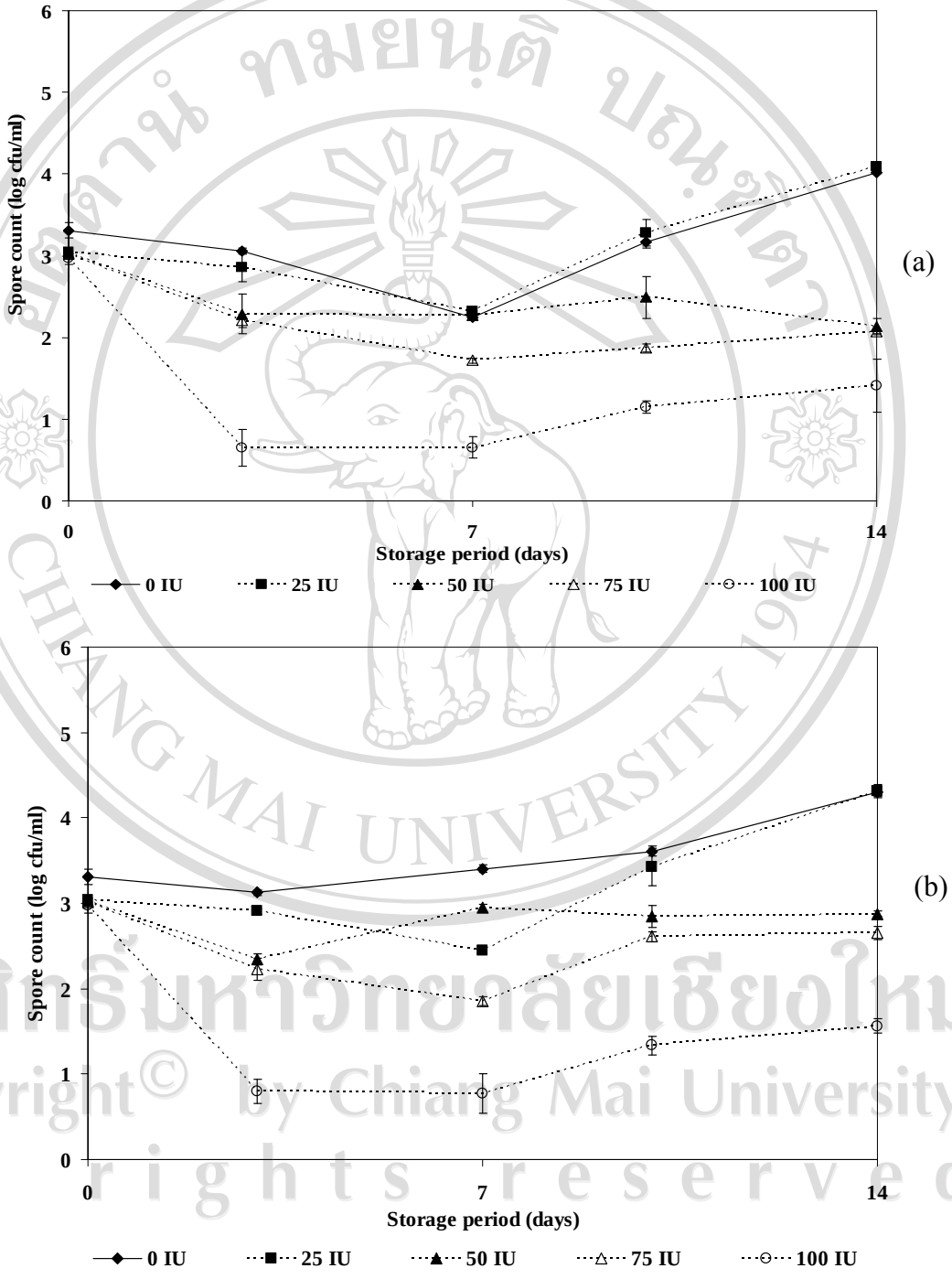


Figure 3.8 Spore count of pasteurized milk supplemented with 0 to 100 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

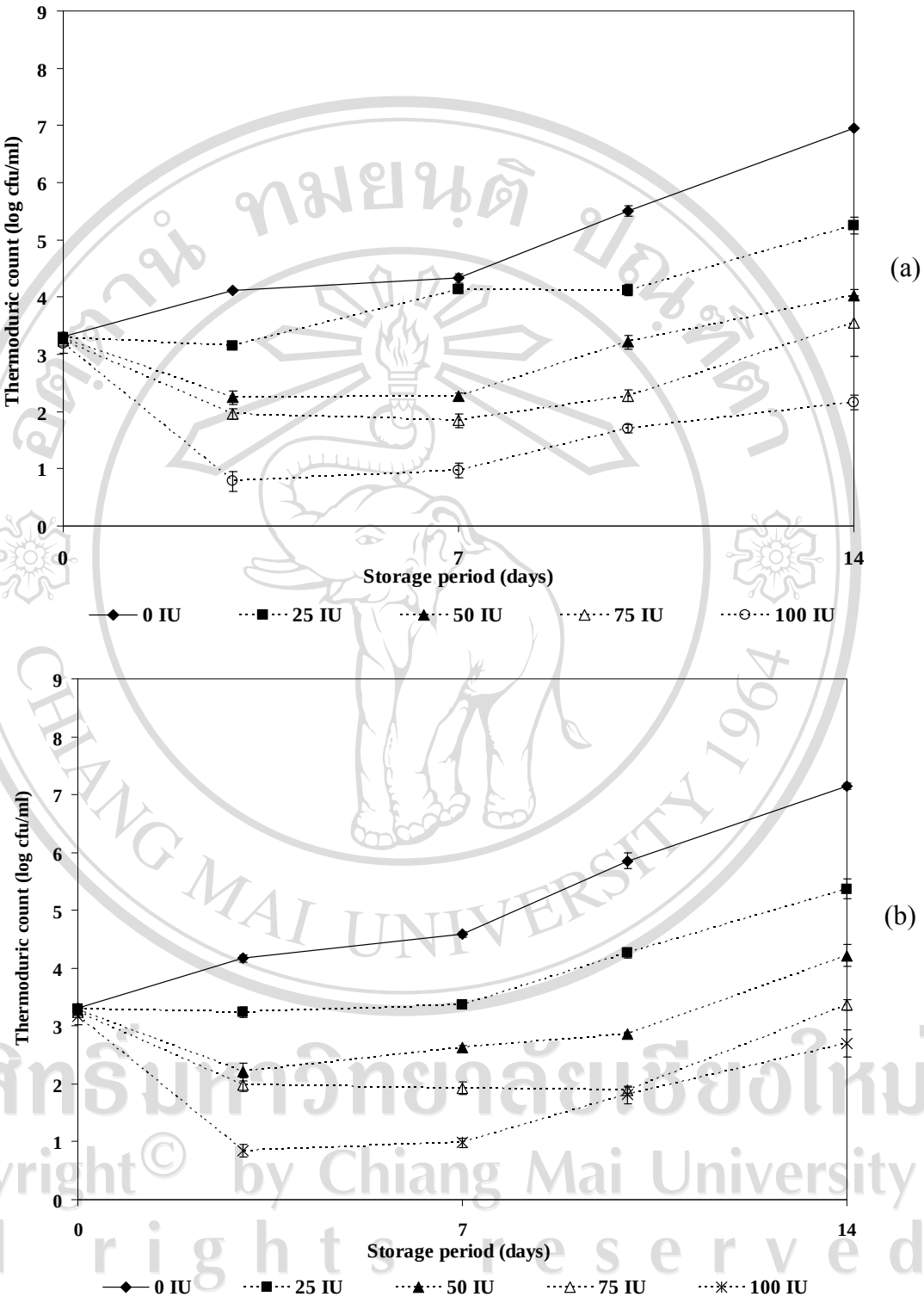


Figure 3.9 Thermoduric count of pasteurized milk supplemented with 0 to 100 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.2.4 pH value

The growth of microorganisms in pasteurized milks (Figures 3.7-3.9) was accompanied by reductions in the pH of the milk samples (Figure 3.10). Different rates of pH reduction were affected by the levels of nisin and storage temperature. The pH of the control pasteurized milk had the highest reduction rate followed by the pasteurized milk supplemented with 25, 50, 75 and 100 IU/ml nisin. The storage temperature was found to significantly affect the pH of the control pasteurized milk after 3 days of storage. The same effect was also noted in the pasteurized milk supplemented with 25 and 50 IU/ml nisin after 1 week of storage, whereas the pH of the pasteurized milk supplemented with 100 IU/ml nisin was not significantly ($P>0.05$) affected by the storage temperature. This last treatment also had the lowest reduction rate of pH, especially when the milk was stored at 4ºC.

3.3.2.5 Total acidity

Responding to the decrease in the pH of pasteurized milk, the acidity of the milk increased during the storage period (Figure 3.11). The presence of nisin significantly ($P\leq0.05$) affected the development of the milk acidity. The environmental factor of the storage temperature was only produced a significant ($P\leq0.05$) effect on the control pasteurized milk. The presence of higher nisin concentrations caused the increasing rate of the acidity of the pasteurized milk to be slower.

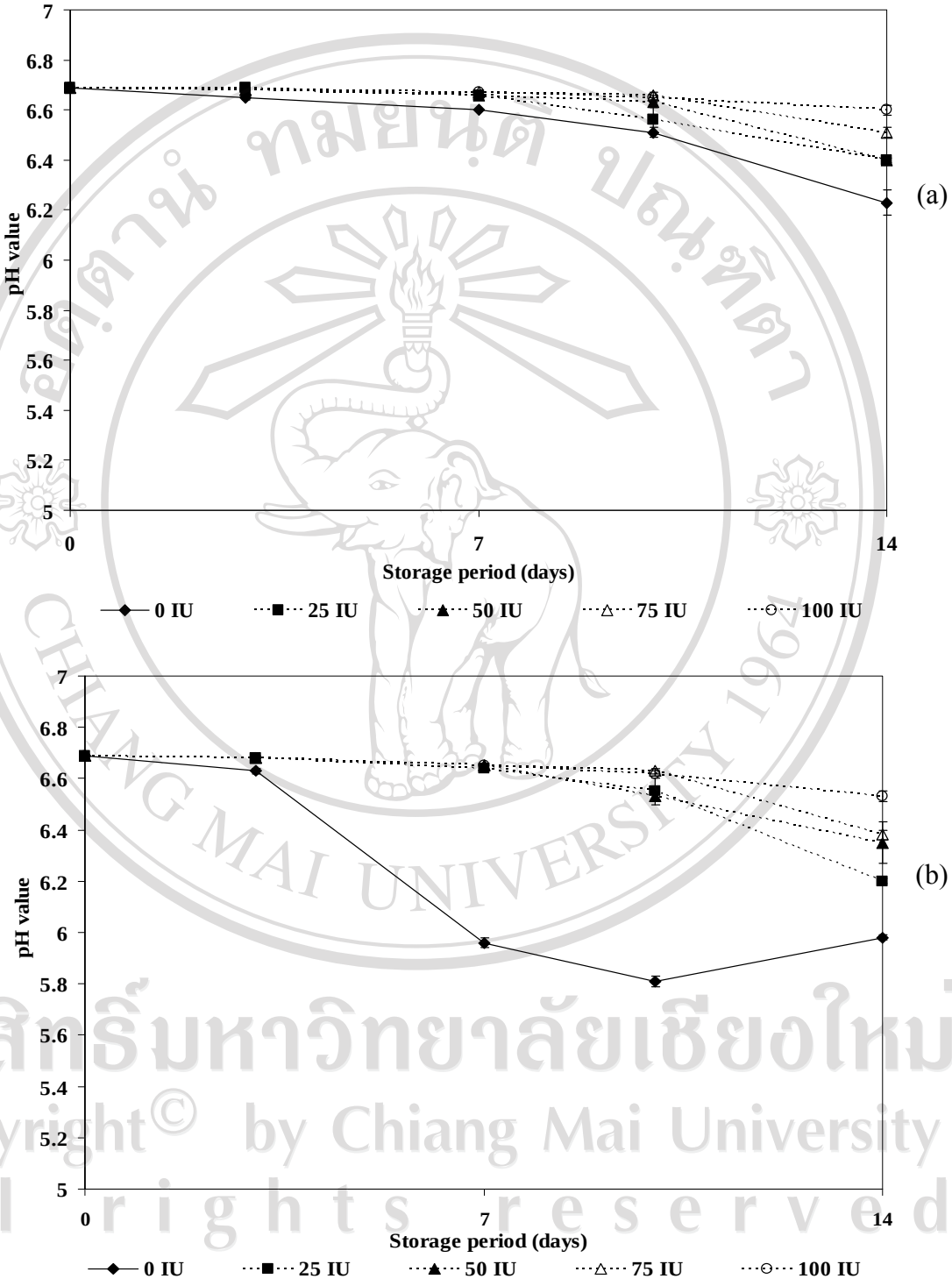


Figure 3.10 pH value of pasteurized milk supplemented with 0 to 100 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

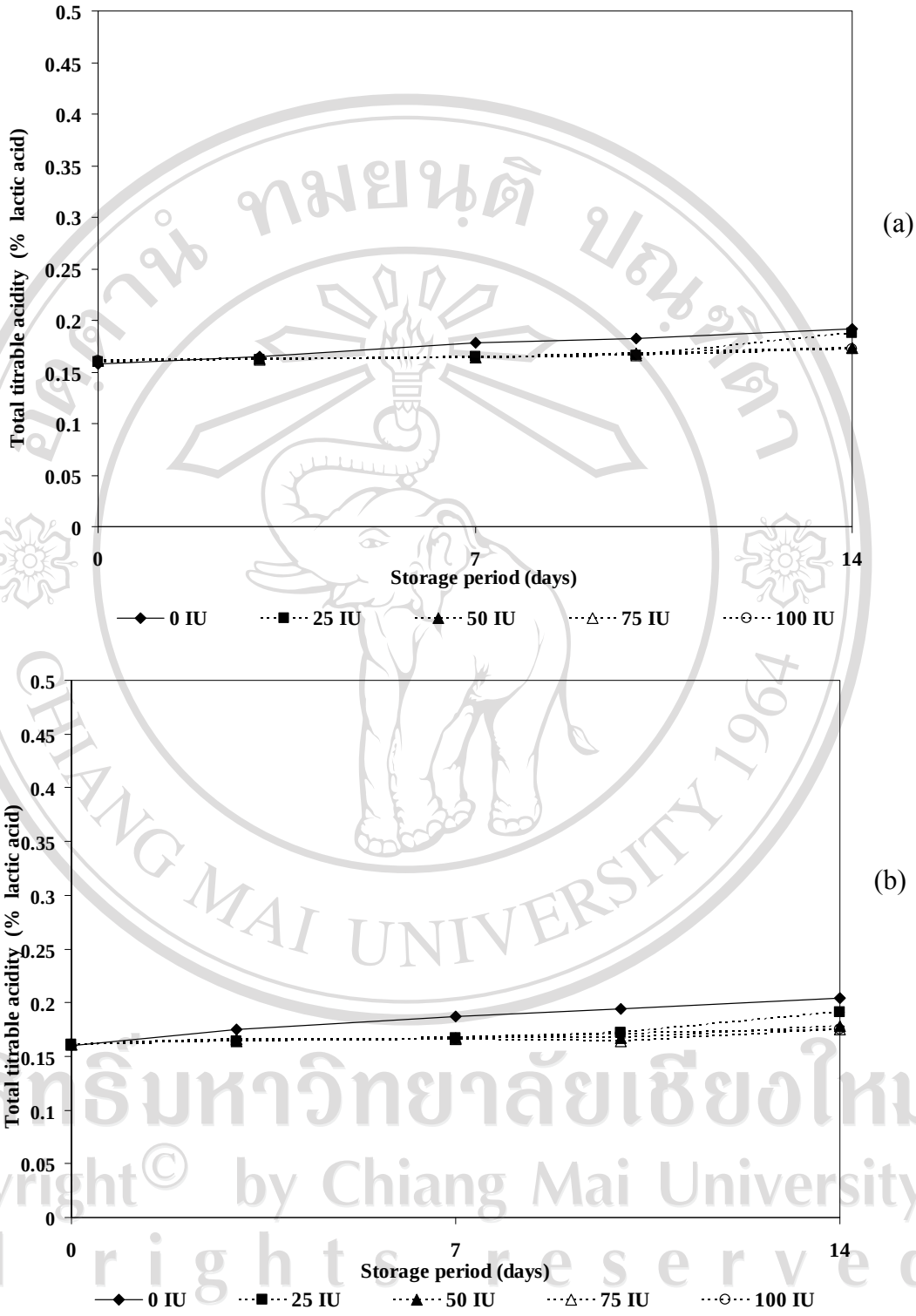


Figure 3.11 Total titrable acidity of pasteurized milk supplemented with 0 to 100 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.2.6 Nisin assay

Residual nisin activity in the pasteurized milk was influenced by the supplementation levels of nisin, pasteurization condition, storage temperature and storage time (Figure 3.12). Applying a pasteurization process, higher storage temperature and longer storage period produced lower nisin activity in the pasteurized milk. Although nisin could be detected in all of the nisin added pasteurized milk treatments throughout the storage period, a high microbial population was found in the pasteurized milk supplemented with 25 IU/ml nisin on the 14^{th} day of storage (Figure 3.7) which indicated that there should be a minimum level of nisin to inhibit the microbial growth in the pasteurized milk. This optimum level of nisin would not only be affected by the processing and storage conditions, but also by the initial microbial load in raw milk and different types of microorganisms (Thomas *et al*., 2000).

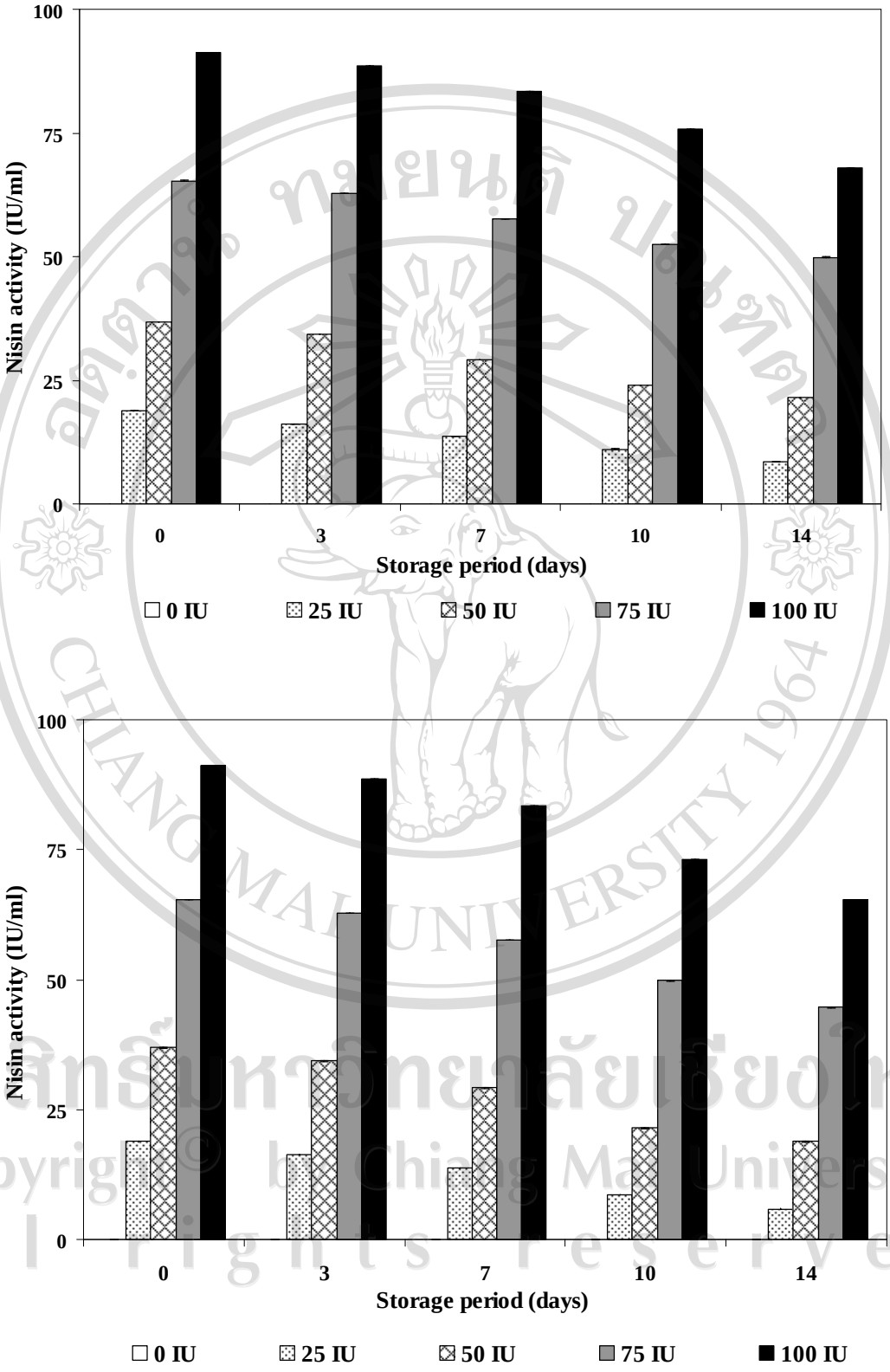


Figure 3.12 Nisin activity of pasteurized milk supplemented with 0 to 100 IU/ml nisin during storage at 4ºC (a) and 10ºC (b).

3.3.3 Identification of microorganisms

Spores from pasteurized milk supplemented with different nisin concentrations that were grown on the agar medium were isolated and purified to be further study. After the pasteurization step, 218 pure colonies were found. These colonies were Gram positive bacteria that had rod shapes and produced spore. Based on Bergey's Harrigan, 1998), the 218 isolated colonies were further divided into 5 groups manual of determinative bacteriology (Holt *et al*., 1994; Priest, 1989; (Figure 3.13). After subjected represented colonies from each group into the API 50 CHB test kit, it was revealed that 4 species of *Bacillus*, including *B. pumilus*, *B. subtilis*, *B. cereus* and *B. licheniformis* could be isolated from pasteurized milk samples. Crielly (1994) reported that *Bacillus* spp. especially *B. licheniformis* and *B. cereus*, were kinds of bacteria normally found in cow milk. The *B. licheniformis* could produce spores which were able to survive at 135ºC (Janstova and Lukasova, 2001).

3.4 Conclusion

Results from this section demonstrated that the presence of nisin significantly affected the microbial load of pasteurized milk during pasteurization and subsequent storage period. Storage temperatures influenced the work of nisin against microorganisms when the antimicrobial compound was supplemented at 0 to 100 IU/ml in the milk. Residual nisin activity in the pasteurized milk played an important role in controlling the microbial growth of the pasteurized milk. Although the nisin levels of 250 to 1,000 IU/ml nisin produced a low microbial population in the pasteurized milk, a nisin concentration of 100 IU/ml was considered to be the optimum concentration in inhibiting the microorganism growth in the pasteurized milk within the shelf life of the product. At the same time, *B. licheniformis* was selected to be further investigated in the next experiment.

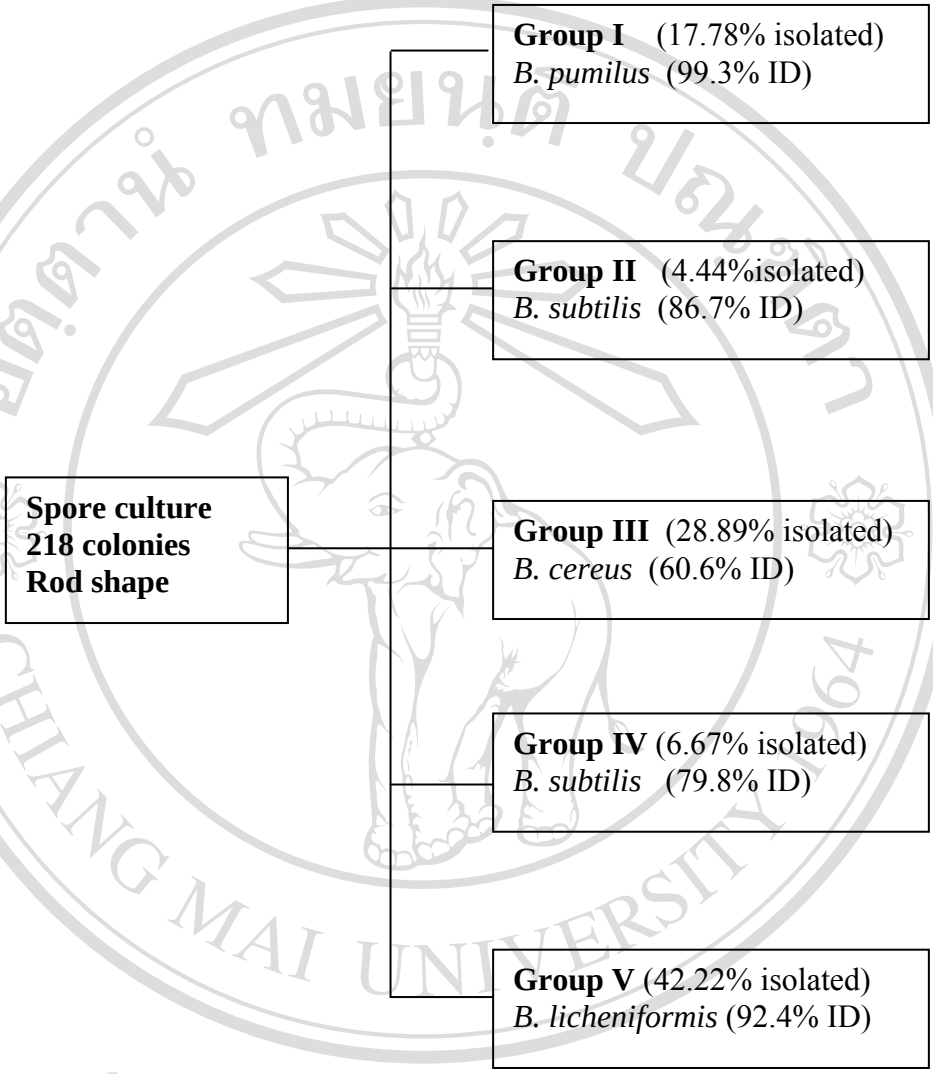
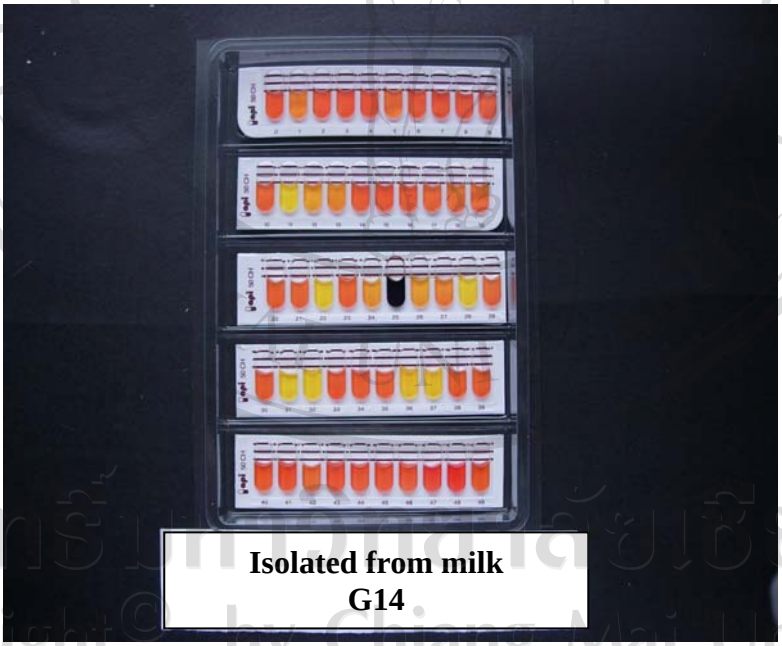


Figure 3.13 A diagram for the *Bacillus* spp. identification isolated from pasteurized milk**.**

(a)



(b)

Figure 3.14 Color changing of the API 50 CHB test kit after 0 h (a) and 24 h (b) incubation at 30ºC by an isolated microorganism from pasteurized milk.

Figure 3.15 Characteristic of the *Bacillus* spp. identification isolated on agar plate from pasteurized milk.

Group		Shape	Size(mm)	Color	Opacity	Elevation	Surface	Edge	Consistency	Catalase
I	→	Circular	0.5 – 5.0	White	Opaque	Flat	Glistening	Entire	-	+
II	→	Circular	3.0 – 6.0	White	Opaque	Flat	Glistening	Entire	Butyrous	+
III	→	Circular	1.0 – 2.0	White	Opaque	Flat	Dull	Entire	Butyrous	+
IV	→	Circular	1.0 – 5.0	White	Opaque	Raised	Dull	Entire	Butyrous	+
V	→	Irregular	0.5 – 4.0	White	Opaque	Flat	Dull	Dentate	-	+