CHAPTER IV

RESULTS AND DISCUSSION

4.1 Properties of Nisin

4.1.1 Minimum Inhibitory Concentration of Nisin

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that could inhibit visible growth of microorganism after overnight incubation (Gill et al., 2002; Anonymous, 2007). Table 4.1 shows the absorbance at 600 nm of MRS broth and *Lactobacillus plantarum* without and with nisin at different concentrations both before and after overnight incubation. Figure 4.1 shows tubes containing the solution. It can be seen that absorbance at 600 nm of the solution did not increase at nisin concentration from 0.05 mg/mL to 5 mg/mL which means that the MIC of nisin is 0.05 mg/mL. The result in Table 4.1 also shows that without nisin addition the absorbance of the solution increased from 0.01 to 1.960 and 0 to 1.858, respectively, after incubating overnight at 37 $^{\circ}$C in anaerobic condition.

Table 4.1 Absorbance at 600 nm of MRS broth containing nisin at different concentrations and *Lactobacillus plantarum* both before and after incubating overnight at 37 °C in anaerobic condition.

Concentration of nisin (mg/mL)	Absorbance (600 nm)					
	Replication 1			Replication 2		
	before	after	difference	before	after	difference
0.00	0.010	1.960	1.950	0.000	1.858	1.858
0.05	0.023	0.029	0.006	0.009	0.009	0.000
0.10	0.040	0.038	-0.002	0.020	0.020	0.000
0.15	0.047	0.033	-0.014	0.032	0.031	-0.001
0.20	0.063	0.046	-0.017	0.046	0.043	-0.003
0.25	0.072	0.052	-0.002	0.054	0.053	-0.001
0.30	0.094	0.068	-0.026	0.063	0.063	0.000
0.50	0.143	0.130	-0.013	0.073	0.067	-0.006
1.00	0.231	0.223	-0.008	0.108	0.105	-0.003
2.00	0.429	0.420	-0.009	0.354	0.350	-0.004
5.00	1.500	1.433	-0.067	1.258	1.256	-0.002

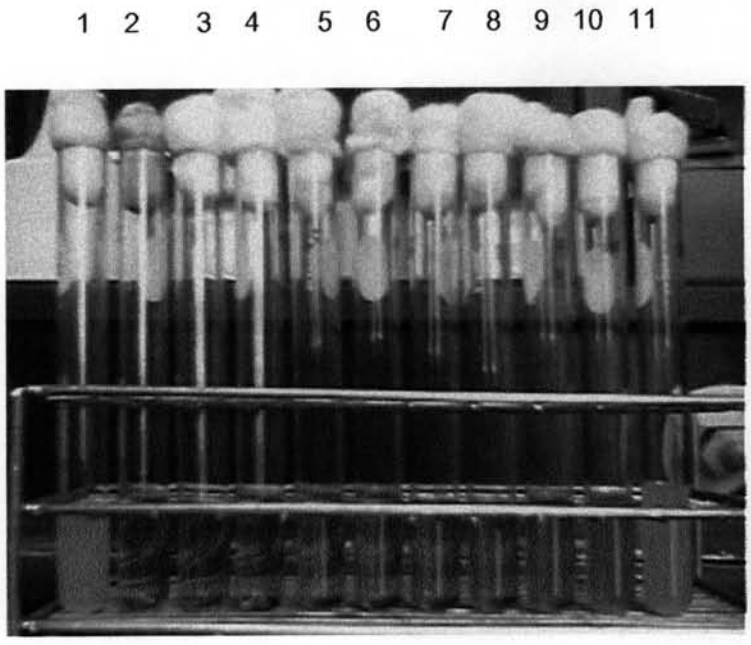


Figure 4.1 Tubes containing solution of MRS broth and *Lactobacillus plantarum* with/without nisin at different concentrations after overnight incubation.
1) without nisin, 2) 0.05 mg/mL nisin, 3) 0.10 mg/mL nisin, 4) 0.15 mg/mL nisin, 5) 0.20 mg/mL nisin, 6) 0.25 mg/mL nisin, 7) 0.30 mg/mL nisin, 8) 0.50 mg/mL nisin, 9) 1.00 mg/mL nisin, 10) 2.00 mg/mL nisin, and 11) 5.00 mg/mL nisin.

4.1.2 Stability of Nisin

Table 4.2 shows the average width of inhibition zone which denotes the activity of 0.28 mg/mL nisin solution after 120 °C heating for 30 minutes compared with non-heated nisin solution (0.28 mg/mL). The average width of inhibition zone (Figure 4.2) for the heated nisin was slightly less than that of the non-heated nisin solution. This means that nisin is stable under heating at the above condition. This result is consistent with that of Hoffman et al. (1997) who reported that nisin was stable at 100 °C in the absence of water and of Nicechem (2005) who also reported that nisin was very stable at room temperature or under acid-heating (pH 2.0, 121 °C, 30 minutes).

Table 4.2 Average width of inhibition zone of non-heated and heated nisin (0.28 mg/mL) against *Lactobacillus plantarum* TISTR 850.

Sample	Average width of the inhibition zone (cm)
Nisin [Non-heated]	1.97±0.035
Nisin [Heated at 120 °C for 30 minutes]	1.92±0.024

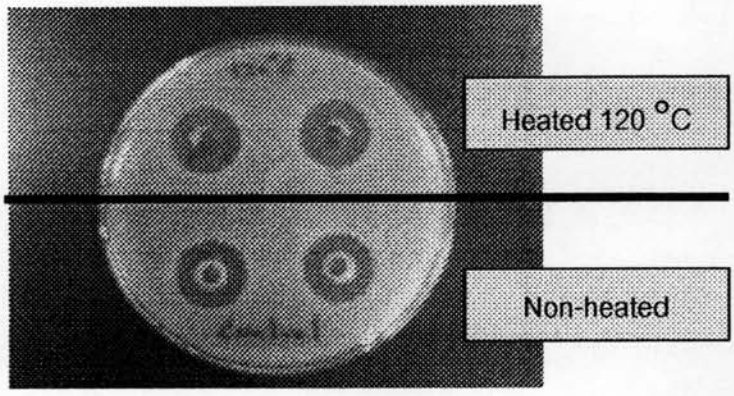


Figure 4.2 The width of inhibition zone of non-heated and heated nisin (0.28 mg/mL) against *Lactobacillus plantarum* TISTR 850 for both heated (upper zone) and non-heated (lower zone).

4.2 Effects of Nisin Concentration and Diameter of Gelatin Nanofiber on Nisin Release

4.2.1 Characteristics of Gelatin-nisin Solution

Gelatin-nisin solutions (22% w/v gelatin concentration) were prepared in 70:30 v/v acetic acid: distilled water with varying initial nisin concentrations in the range of 0% – 3% w/w. The properties of the solutions were measured following the procedure in 3.4.2.1. The average pH of samples was found to be 3. Viscosity and conductivity of gelatin solution (22% w/v), which was blended with nisin at different concentrations (0% – 3% w/w), are shown in Figure 4.3. It could be clearly seen that viscosity and

conductivity of the solutions increased as nisin concentration was increased. The viscosity of the solution slightly increased from 473.33 cP at 0% w/w nisin to 497 cP at 3% w/w nisin. The conductivity of the solution increased from 1.204 ms/cm at 0% w/w nisin to 2.247 ms/cm at 3% w/w nisin due to the dissolution of nisin in weak acid that caused an interaction between free amino group of nisin chain and hydrogen ions (H^+) of weak acid. This affected nisin chain directly since it had positive charges. Because of an increase in nisin concentration in the solution, there were more positive charges, thus a higher conductivity.

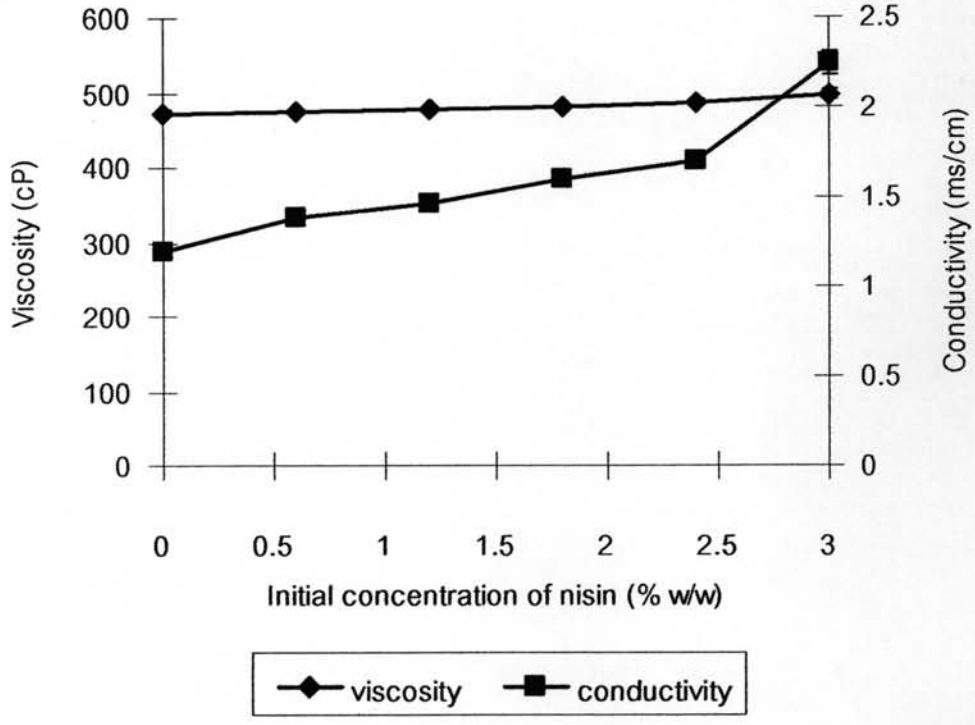


Figure 4.3 Effect of initial nisin concentration on viscosity at 72.5 s^{-1} Shear rate and conductivity of gelatin solution.

Gelatin-nisin solutions (3% w/w nisin concentration) were prepared in 70:30 v/v acetic acid: distilled water with varying gelatin concentrations in the range of 20% - 24% w/v. The properties of the solutions were measured following the procedure

in 3.4.2.1. The average pH of the samples was found to be 3. Viscosity and conductivity of gelatin-nisin solution which was blended with gelatin at different concentrations (20% - 24% w/v) and 3% w/w nisin concentration, are shown in Figure 4.4. From The data obtained, it was found that viscosity and conductivity of the solutions increased as gelatin concentration was increased. The viscosity of the solution increased with increasing gelatin concentration from 355.33 cP at 20% w/v gelatin to 636.67 cP at 24% w/v gelatin. When gelatin dissolves in weak acid solution, the intermolecular bonds between molecules could occur. Moreover, an elongation of gelatin chain occurred by an increase in positive charges causing chain repulsion by electrostatic force resulting in an increase in viscosity of the solution (Huang et al., 2004). The conductivity of the solution was increased from 1.754 ms/cm at 20% w/v gelatin to 2.56 ms/cm at 24% w/v gelatin. This is because the dissolution of gelatin in weak acid that caused an interaction between free amino group of gelatin chain and hydrogen ions (H^+) of weak acid. As gelatin concentration in the solution increased, there were more positive charges, thus a higher conductivity.

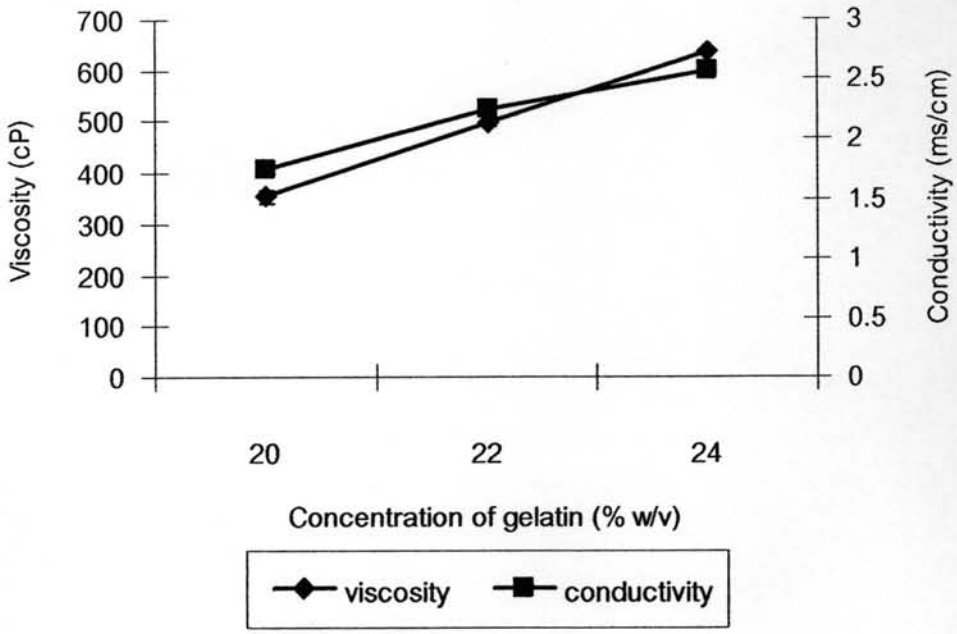
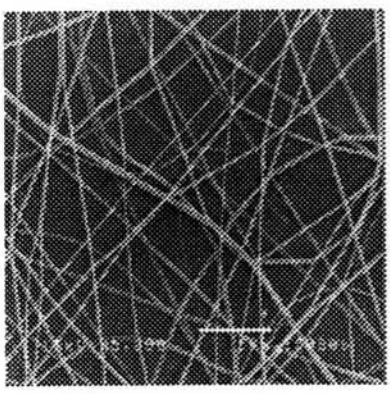


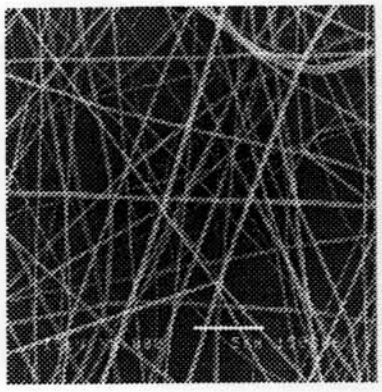
Figure 4.4 Effect of gelatin concentration on viscosity at 72.5 s^{-1} Shear rate and conductivity of gelatin-nisin solution.

4.2.2 Characteristics of Gelatin Nanofibers and Antimicrobial Gelatin Nanofibers

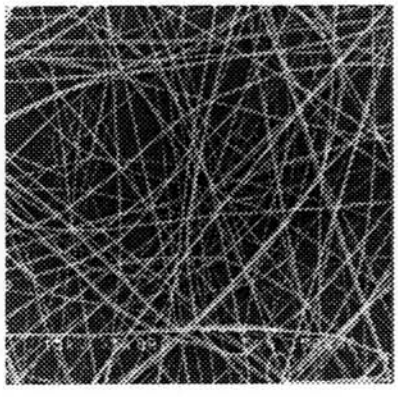
Nanofibers from gelatin solution which was blended with nisin at different concentrations were spun for 5 minutes by electrostatic spinning. Scanning electron micrographs of gelatin nanofibers and antimicrobial gelatin nanofibers are displayed in Figure 4.5. The nanofibers were continuous without beads and were laid to form a non-woven fabric. The random arrangement of nanofiber on collector due to the movement of polymer current which was ejected from the tip to collector was not stable; resulting in a bending instability (Mit-uppatham et al., 2004). Average nanofiber diameter was analyzed by an image analyzer software (Image J, JEOL, USA). Figure 4.6 presents average diameters of gelatin nanofiber which was blended with nisin at different concentrations. The average diameter of gelatin nanofiber was about 220 nm at 0% w/w nisin to 250 nm at 3% w/w nisin. Nanofiber diameter tended to increase when initial nisin concentration was increased harmoniously with increased viscosity. However, when statistically analyzed, there was no significant difference in the average diameter of gelatin nanofiber. In general, increased viscosity of the solution, diameter of nanofiber was increased (Mit-uppatham et al., 2004) and for a given applied potential, the average fiber diameter increased with increasing concentration of the spinning solutions (Varaporn et al., 2005).



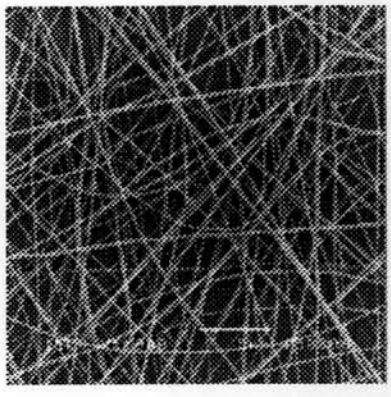
0% w/w Nisin



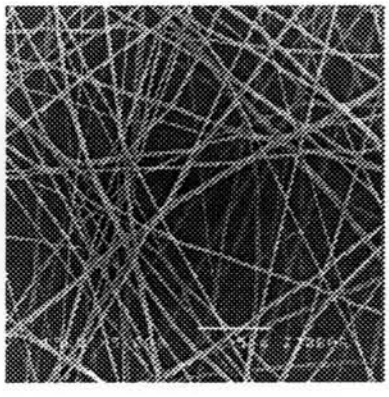
0.6% w/w Nisin



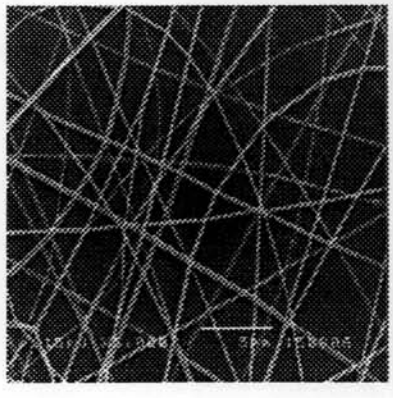
1.2% w/w Nisin



1.8% w/w Nisin



2.4% w/w Nisin



3.0% w/w Nisin

Figure 4.5 Scanning Electron Micrographs of electrospun gelatin-nisin nanofibers (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration).

Note: All SEM photographs are in 5,000 x magnification.

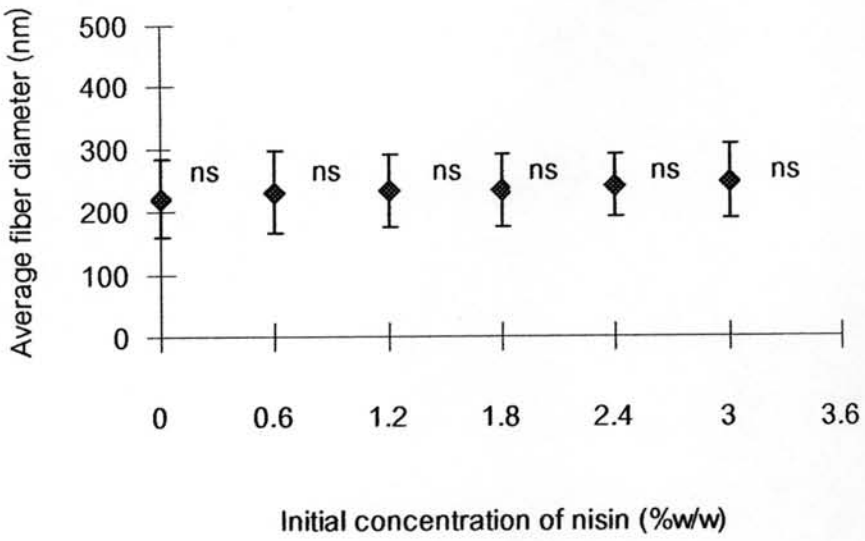
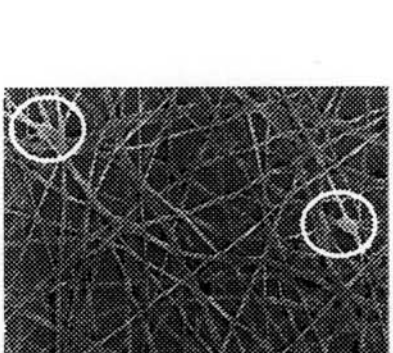


Figure 4.6 Average diameter of gelatin nanofibers which were electrospun from 22% w/v gelatin concentration and nisin at different concentrations (0% - 3% w/w).

ns indicates no significant difference ($p>0.05$)

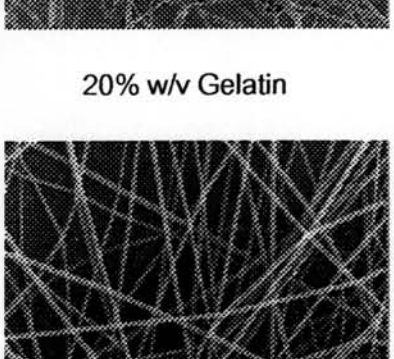
Nanofibers from gelatin-nisin solution (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration) were spun by electrostatic spinning for 5 minutes. Scanning electron micrographs of antimicrobial gelatin nanofibers are illustrated in Figure 4.7. At 20% w/v gelatin concentration, nanofibers were not smooth and had beads on string which was caused by insufficient concentration of the solution. When the solution concentration is insufficient, the surface tensions overcome the electrical force at the surface of polymer solution and viscosity. As the viscosity of the solution was increased, the shape of beads changed from spherical to spindle-link and beads disappeared when concentration which was increased (Mit-uppatham et al., 2004). Fong et al. (1999) stated that beads formation was due to viscosity and surface tension. At a low concentration, the viscosity of the solution was low, while the surface tension was relatively high. Therefore, the solution jet, which would form a nanofiber, could not maintain its own shape at the end of tip due to high surface tension and formed a small drop among the fibers. Besides, the solution was even sprayed because of low viscosity. These effects caused the appearance of the beads instead of the formation of

nanofiber and this reduced the uniformity of electrospun gelatin web. At 22% w/v and 24% w/v gelatin concentrations, nanofibers were continuous without beads. Average nanofiber diameter was analyzed by an image analyzer software (Image J, JEOL, USA). Figure 4.8 shows that an average diameter of nanofibers was increased from 230 nm at 20% w/v gelatin concentration to 320 nm at 24% w/v gelatin concentration. Comparing the average diameters of gelatin nanofiber electrospun from 20% w/v and 22% w/v solutions, there were no significant differences observed. While increasing concentration of gelatin from 22% w/v to 24% w/v, the average diameter of nanofibers was significantly increased ($p \leq 0.05$). Gupta et al. (2005) also reported that the morphology of electrospun materials is influenced by polymer viscosity.



20% w/v Gelatin

22% w/v Gelatin



24% w/v Gelatin

Figure 4.7 Scanning Electron Micrographs of electrospun gelatin-nisin nanofibers. (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration).

Note: All SEM photographs are 5,000 x magnifications.

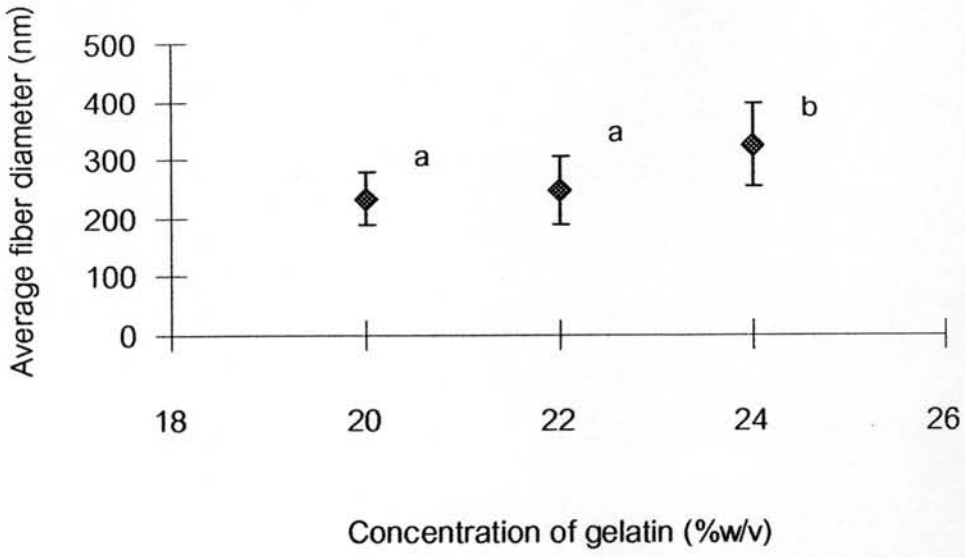


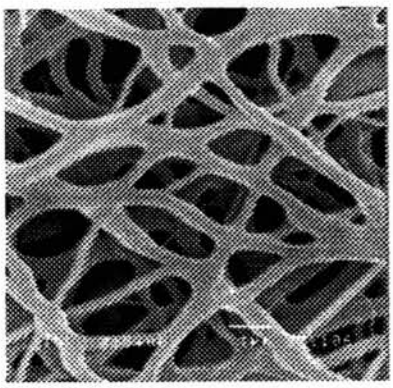
Figure 4.8 Average diameters of antimicrobial gelatin nanofibers which were electrospun from gelatin at different concentrations (20% - 24% w/v) and 3% w/w nisin concentration.

a and b shows significant difference(s) ($p \leq 0.05$)

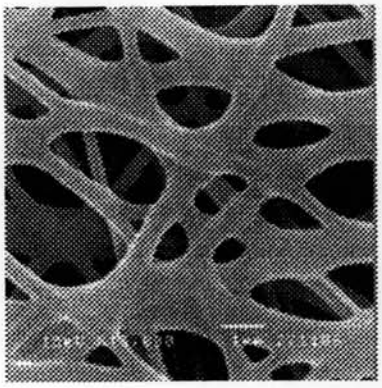
4.2.3 Crosslinking of Gelatin Nanofibers and Antimicrobial Gelatin Nanofibers Mats

Gelatin nanofiber mat has riddled holes property and those holes are very small. Therefore, gelatin nanofiber mat is useful in a variety of applications, such as dressing for wound healing and drug releasing. However, the electrospun nanofibrous structure of gelatin is water soluble and mechanically weak. This could limit its applications. For a long-term biomedical application, an electrospun gelatin nanofibrous membrane must be crosslinked as done on its film counterparts. Crosslinking treatment can improve both water-resistant ability and thermo-mechanical performance of the resulting nanofibrous membranes (Zhang et al., 2006). In this experiment, gelatin solutions blended with different nisin concentrations were spun by electrostatic spinning for 3 days to obtain a nanofiber mat which was later crosslinked by saturated glutaraldehyde vapor at 37°C for 5 minutes. The reasons for using

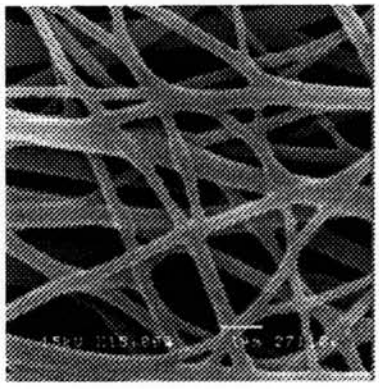
glutaraldehyde were because of its availability, low cost, and capability of accomplishing the crosslinking in a relatively short time period. Besides, it is highly efficient in stabilizing fibrous. Although other crosslinking agents were reported to reduce cytotoxicity, they could not match glutaraldehyde in gelatin stabilization. The risk of cytotoxicity could be reduced by a proper use. The electrospun nanofiber morphology was observed under a Scanning Electron Microscope (SEM). The pictures of which are shown in Figures 4.9 and 4.10. Crosslinked nanofiber was bound between nanofibrous membranes, resulting in an increase in some mechanical properties. Crosslinking of collagenous materials with glutaraldehyde involves the reaction of free amino groups of lysine or hydroxylysine amino acid residues of the polypeptide chains with the aldehyde group of glutaraldehyde (Zhang et al., 2006). This causes an increase in strength of the materials. The result of crosslinking in this study agreed with the study of Songchotikunpan (2006), who reported that tensile strength and Young's modulus of nanofiber mat from fish skin gelatin which was crosslinked by saturated glutaraldehyde vapor at 37^{o}C for 3 hours increased compared to non-crosslinked gelatin nanofiber mat.



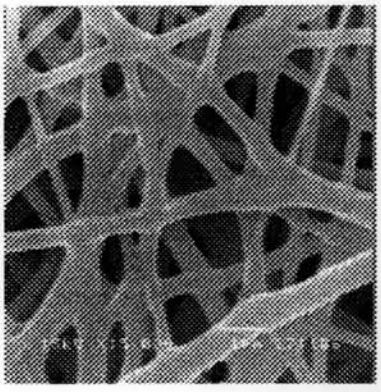
0 % w/w Nisin



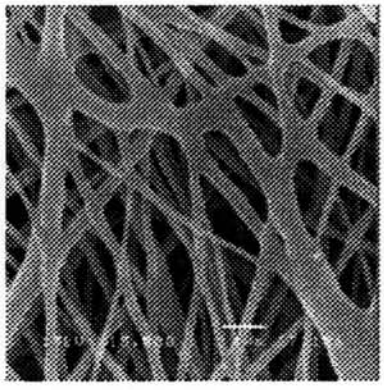
0.6% w/w Nisin



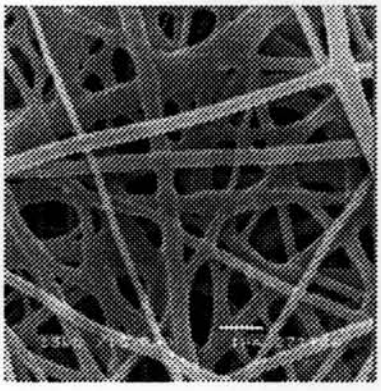
1.2% w/w Nisin



1.8% w/w Nisin



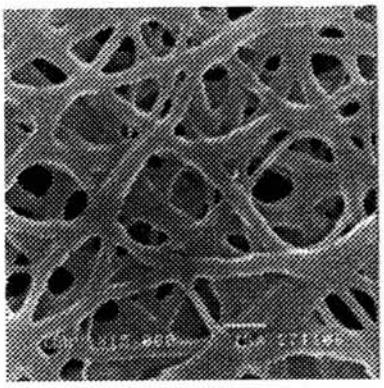
2.4% w/w Nisin



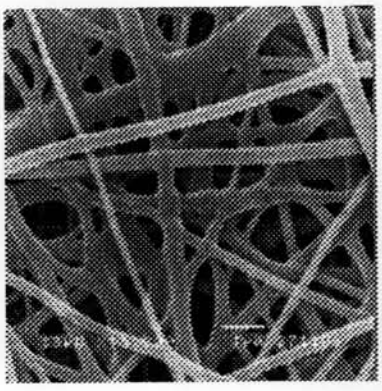
3.0% w/w Nisin

Figure 4.9 Scanning Electron Micrographs of electrospun gelatin-nisin nanofibers which were crosslinked for 5 minutes (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration).

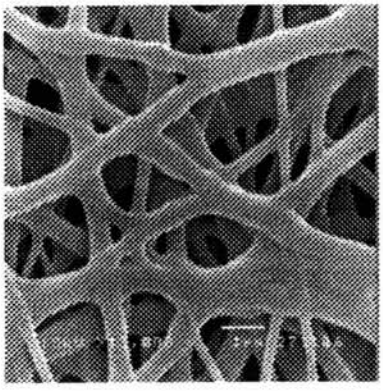
Note: All SEM photographs are in 15,000 x magnifications.



20% w/v Gelatin



22% w/v Gelatin



24% w/v Gelatin

Figure 4.10 Scanning Electron Micrographs of electrospun gelatin-nisin nanofibers which were crosslinked for 5 minutes (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration).

Note: All SEM photographs are in 15,000 x magnification.

4.2.4 Mechanical Properties of Crosslinked Gelatin Nanofibers and Crosslinked Antimicrobial Gelatin Nanofibers Mats

Figures 4.11 - 4.13 show tensile strength, Young's modulus, and elongation of crosslinked gelatin nanofiber mat (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration). From the data obtained, it was found that tensile strength of crosslinked gelatin nanofiber mat decreased when initial nisin concentration was increased. At 0%, 0.6%, 1.2%, 1.8%, 2.4%, and 3.0% w/w initial nisin concentrations, tensile strengths of crosslinked gelatin nanofiber mats were 20.28,

13.31, 13.42, 9.18, 5.60, and 2.59 MPa, and Young's modulus of the mats were 965.9, 776.5, 814.0, 609.4, 540.7, and 162.8 MPa, respectively. Tensile strengths and Young's modulus tended to decrease when initial concentration of nisin increased. As stated, crosslinked nanofibers were bound between nanofibrous membranes. They resulted in an increase in some mechanical properties when compared with non-crosslinked nanofibers mat. However, in this experiment, the sample was crosslinked for 5 minutes which might cause incomplete crosslinking. Therefore, when considering thickness of nanofiber mat in Table D6, the thickness increased when initial concentration of nisin was increased. At 3.0% w/w nisin, nanofiber mat thickness was about 127.8 μm which was more than other nanofibers mats. When crosslinking using saturated glutaraldehyde for 5 minutes, lower fraction of crosslinked nanofibers could; therefore, be obtained in thicker nanofiber mats. This resulted in lower tensile strength and Young's modulus of nanofiber mat. Gelatin and nisin are positively charged molecules that could generate repulsion force between charges when dissolved in the same solvent. When initial concentration of nisin was increased, the size of holes of nanofiber mat was increased and the number of as-spun fibers per unit area decreased. Therefore, it caused a decrease in mechanical properties of the materials. Crosslinked gelatin nanofiber mat (0% w/w nisin) had a higher elongation compared with crosslinked gelatin nanofiber mat which was blended with different initial nisin concentrations. Zhang et al. (2006) reported that crosslinking did not reduce the extension ability of the gelatin fibrous membrane. In contrast, the elongation either remained the same or even higher. Therefore, less thickness of nanofiber mat might provide a complete crosslinked, resulting in higher elongation.

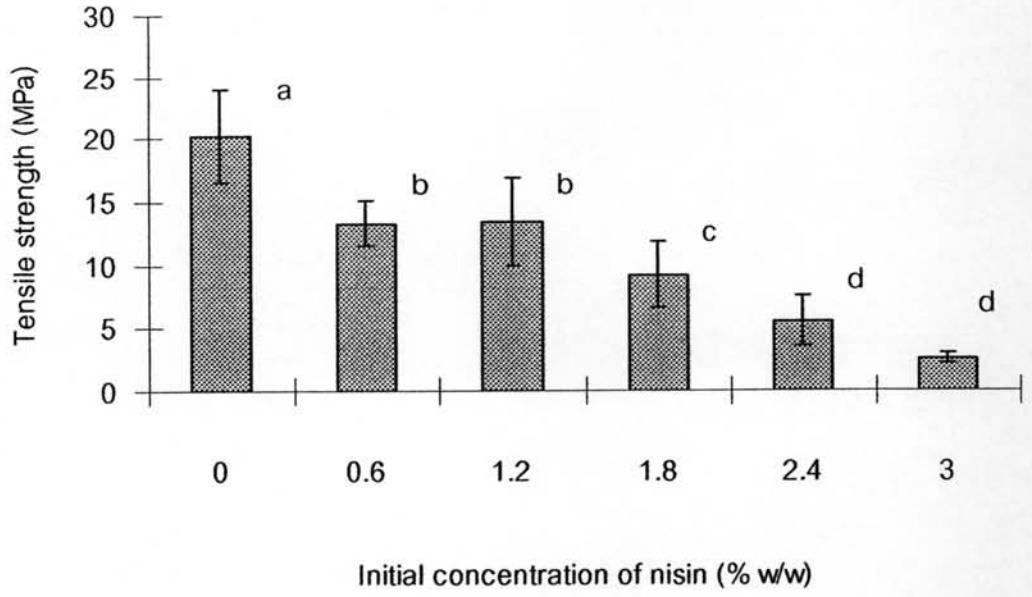


Figure 4.11 Tensile strength of crosslinked gelatin nanofiber mat (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration).

a, b,... shows significant difference(s) ($p \leq 0.05$)

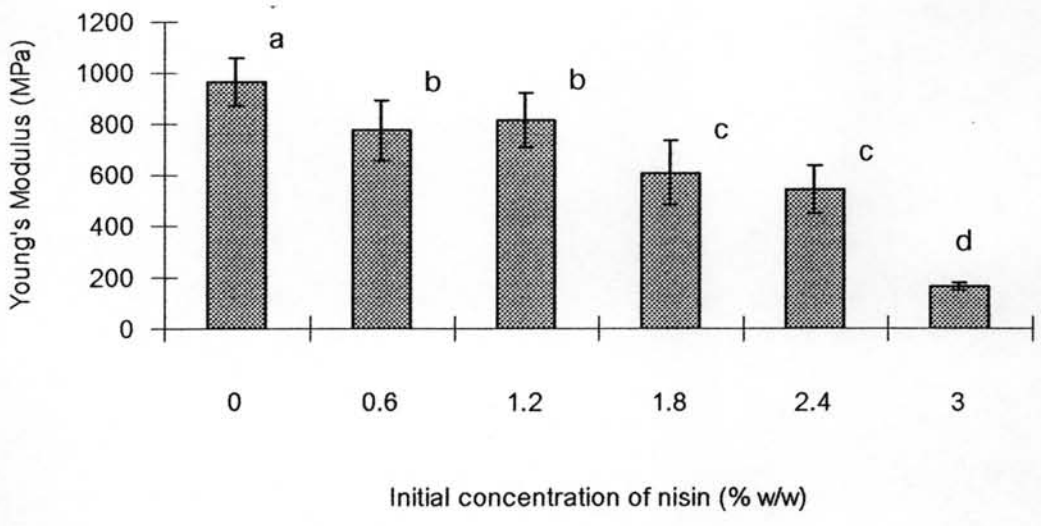


Figure 4.12 Young's modulus of crosslinked gelatin nanofiber mat (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration).

a, b,... shows significant difference(s) ($p \leq 0.05$)

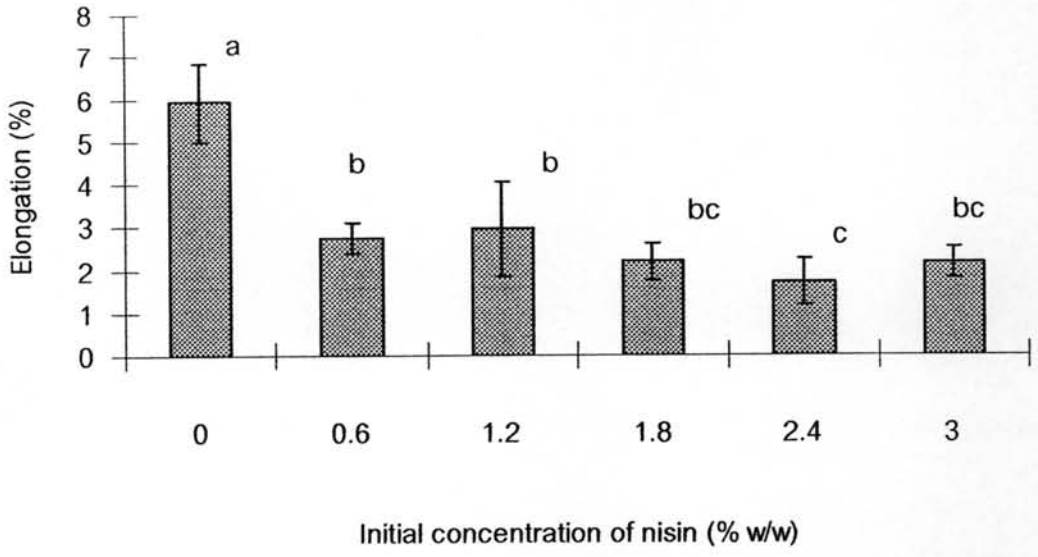


Figure 4.13 Elongation of crosslinked gelatin nanofiber mat (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration).
a, b,... shows significant difference(s) ($p \leq 0.05$)

Figures 4.14 - 4.16 show tensile strength, Young's modulus and elongation of crosslinked antimicrobial gelatin nanofiber mat (3% w/w nisin concentration and 20% - 24% w/v gelatin concentrations). Tensile strengths of crosslinked antimicrobial gelatin nanofibers mats were not significantly difference ($p > 0.05$) while their Young's moduli significantly increased ($p \leq 0.05$) when increasing gelatin concentration from 22% w/v to 24% w/v. Elongation (%) of crosslinked antimicrobial gelatin nanofibers mats were not significantly difference ($p > 0.05$). Huang et al. (2004) reported that the highest mechanical performance of the nanofiber membrane did not correspond to the lowest or the highest mass concentration of gelatin. In an earlier work (Huang et al., 2004) the finest nanofiber generally exhibited higher tensile strength. However, if there were beads on the fiber surface, the situation would definitely be different. The nanofiber material (with the smallest fiber diameter but having beads on the fiber surface) even exhibited poorer mechanical performance than the one which had the largest fiber diameter without any beads. However, the present study showed that tensile strength and Young's modulus of crosslinked antimicrobial gelatin nanofibers mats produced

from solution of 20% w/v gelatin concentration were significantly higher ($p \leq 0.05$) than that from 22% w/v to 24% w/v gelatin solutions. This could be due to ambient parameters which affected the size and the shape of the electrospun antimicrobial gelatin nanofiber, resulting in the varieties of mechanical properties (Mit-uppatham et al., 2004).

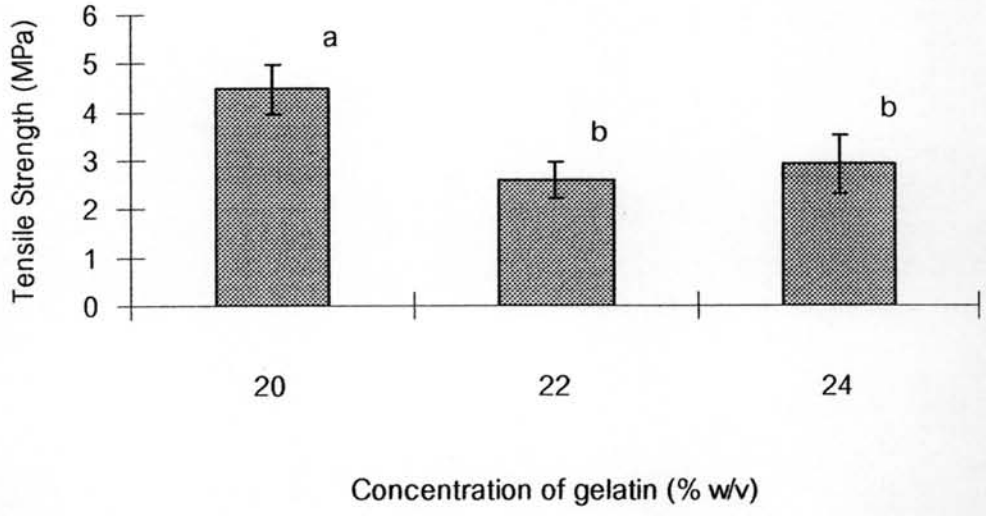


Figure 4.14 Tensile strength of crosslinked antimicrobial gelatin nanofiber mat (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration).
a and b shows significant difference(s) ($p \leq 0.05$)

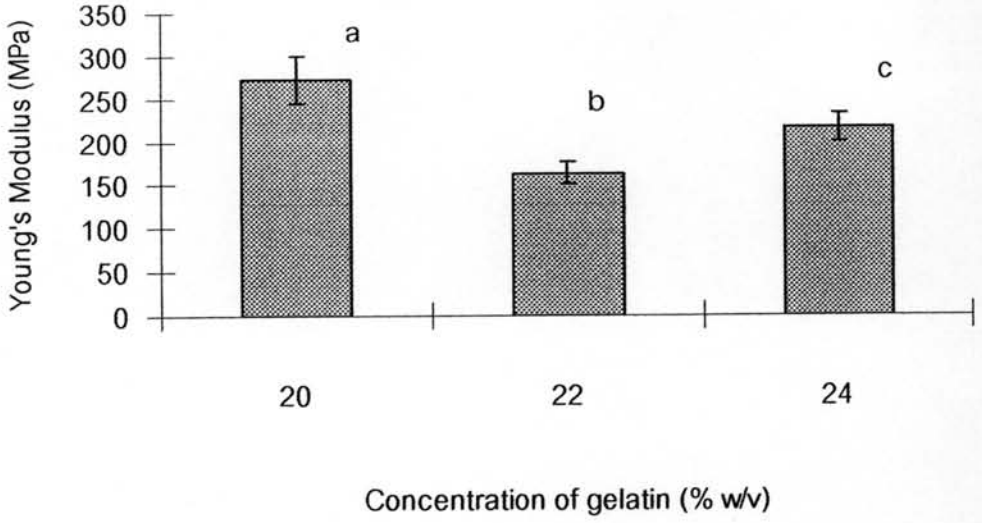


Figure 4.15 Young's modulus of crosslinked antimicrobial gelatin nanofiber mat (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration).

a, b,... shows significant difference(s) ($p \leq 0.05$)

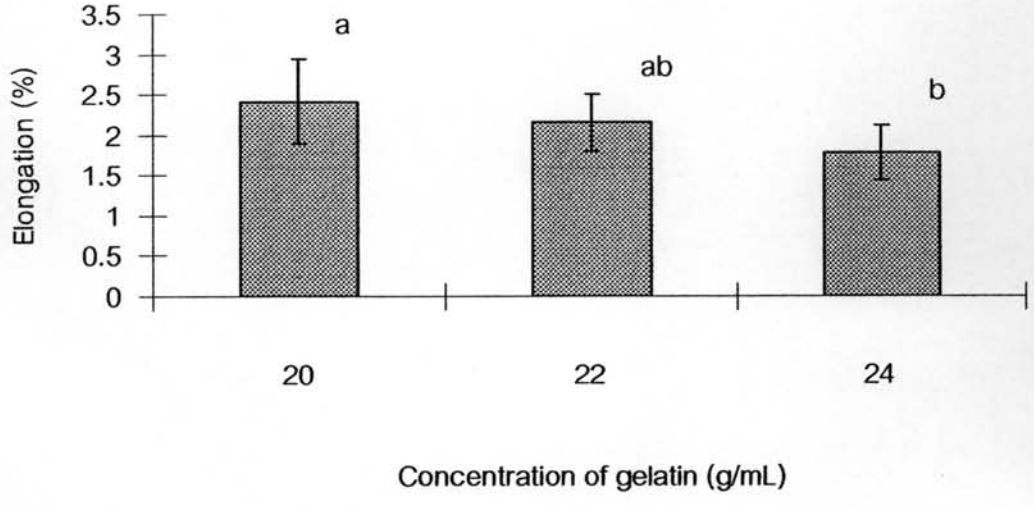


Figure 4.16 Elongation of crosslinked antimicrobial gelatin nanofiber mat (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration).

a, b,... shows significant difference(s) ($p \leq 0.05$)

4.2.5 Concentration of Nisin in Antimicrobial Gelatin Nanofibers Mats

After nisin was extracted from antimicrobial gelatin nanofiber mat, it was tested for inhibition against *Lactobacillus plantarum* by agar diffusion technique. Figure 4.17 depicts the width of inhibition zone of nisin from antimicrobial gelatin nanofiber and crosslinked antimicrobial gelatin nanofiber mat. Antimicrobial gelatin nanofiber mat containing higher initial nisin concentration increased the width of inhibition zone. Gelatin nanofiber without nisin could not inhibit the growth of *Lactobacillus plantarum*. At 0%, 0.6%, 1.2%, 1.8%, 2.4%, and 3.0% w/w initial nisin concentrations, the width of inhibition zone were 0, 1.576, 1.735, 1.834, 1.853, and 1.984 cm, respectively. This result agreed with that of Padgett et al. (1998) who reported that when concentration of nisin in heat-press soy protein films increased from 0.1 to 6.0 mg nisin/g film, the inhibition of *Lactobacillus plantarum* NCDO 1752 increased. Comparing the inhibition zone between antimicrobial gelatin nanofiber mat and crosslinked antimicrobial gelatin nanofiber mat, efficiency of nisin release from crosslinked antimicrobial gelatin nanofiber mat was lower. Crosslinked antimicrobial gelatin nanofiber mat containing higher nisin concentration increased the width of inhibition zone significantly ($p \leq 0.05$). At 0%, 0.6%, 1.2%, 1.8%, 2.4%, and 3.0% w/w initial nisin concentrations, the width of inhibition zone generated by crosslinked antimicrobial gelatin nanofiber mat were 0, 1.343, 1.586, 1.744, 1.831, and 1.916 cm, respectively. Crosslinking between nanofibers resulted in increased stability of gelatin nanofiber mat. Therefore, gelatin nanofiber mat could not be dissolved by heating and using acid solution during extraction. Besides, crosslinking process might decrease the activity of nisin which is a polypeptide. Therefore, the width of inhibition zone was smaller than non-crosslinked nanofiber.

Figure 4.18 displays the width of inhibition zone of nisin from antimicrobial gelatin nanofiber and crosslinked antimicrobial gelatin nanofiber mat. At fixed nisin concentration, the width of inhibition zone caused by antimicrobial gelatin nanofiber mat at 20% w/v gelatin concentration was less than that at 24% w/v gelatin concentration but was higher than that at 22% w/v gelatin concentration. At 20%, 22%, and 24% w/v gelatin concentrations, the width of inhibition zone caused by non-crosslinked

antimicrobial gelatin nanofiber mat were 2.000, 1.984, and 2.056 cm, respectively. After crosslinking, the inhibition zones of 20%, 22%, and 24% w/v gelatin concentrations were 1.900, 1.916, and 1.943, respectively. The width of inhibition zone is shown in Figure 4.19 and 4.20. Crosslinking caused a reduction in inhibition zone. The widths of inhibition zone at 22% w/v and 24% w/v gelatin concentration were not significantly different ($p>0.05$) because they contained the same amount nisin and the thickness of the nanofiber mat was not significantly different (127.8 μm and 132.8 μm, respectively). Therefore, antimicrobial gelatin nanofiber at 22% w/v and 24% w/v gelatin concentrations were approximately equally crosslinked. At 20% w/v gelatin concentration, the thickness of gelatin nanofiber mat was 69.6 μm which is thinner than 22% w/v (127.8 μm) and 24% w/v (132.8 μm) gelatin concentration. This could result in more complete crosslinking by saturated glutaraldehyde vapor. Therefore, less amount of nisin could be extracted from the nanofiber mat.

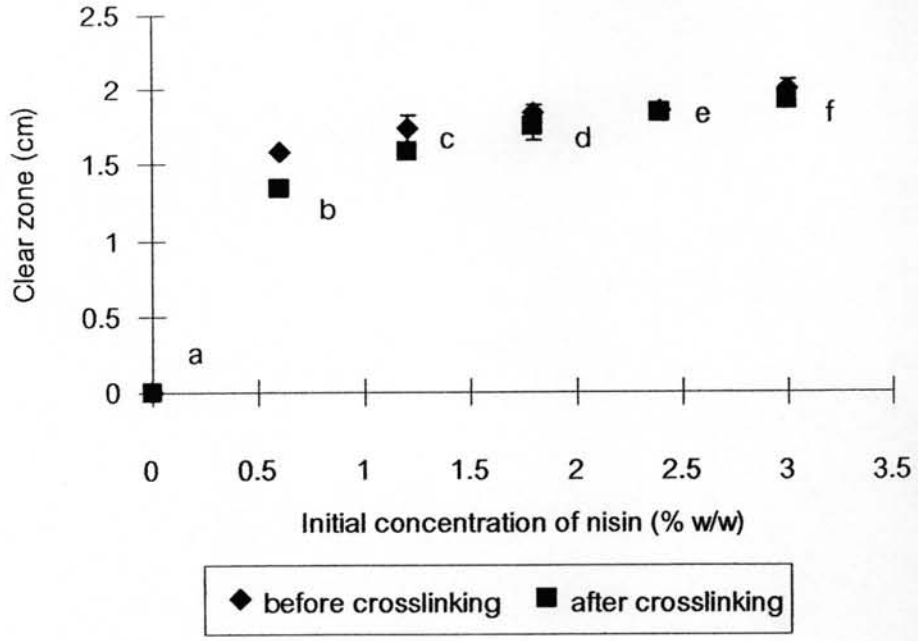


Figure 4.17 The width of inhibition zone of nisin from antimicrobial gelatin nanofiber mat (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration) against *Lactobacillus plantarum*.

a, b,... shows significant difference(s) ($p \leq 0.05$)

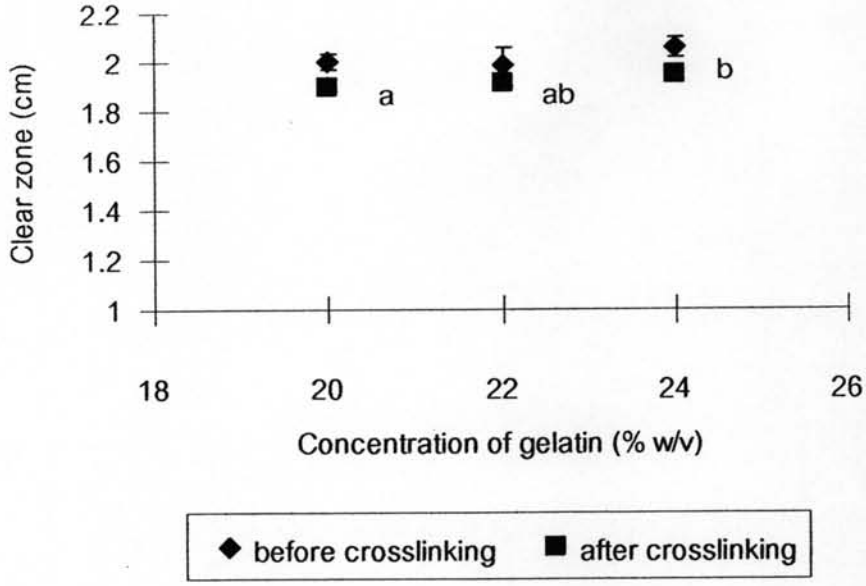


Figure 4.18 The width of inhibition zone of nisin from antimicrobial gelatin nanofiber mat (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration) against *Lactobacillus plantarum*.

a, b,... shows significant difference(s) ($p \leq 0.05$)

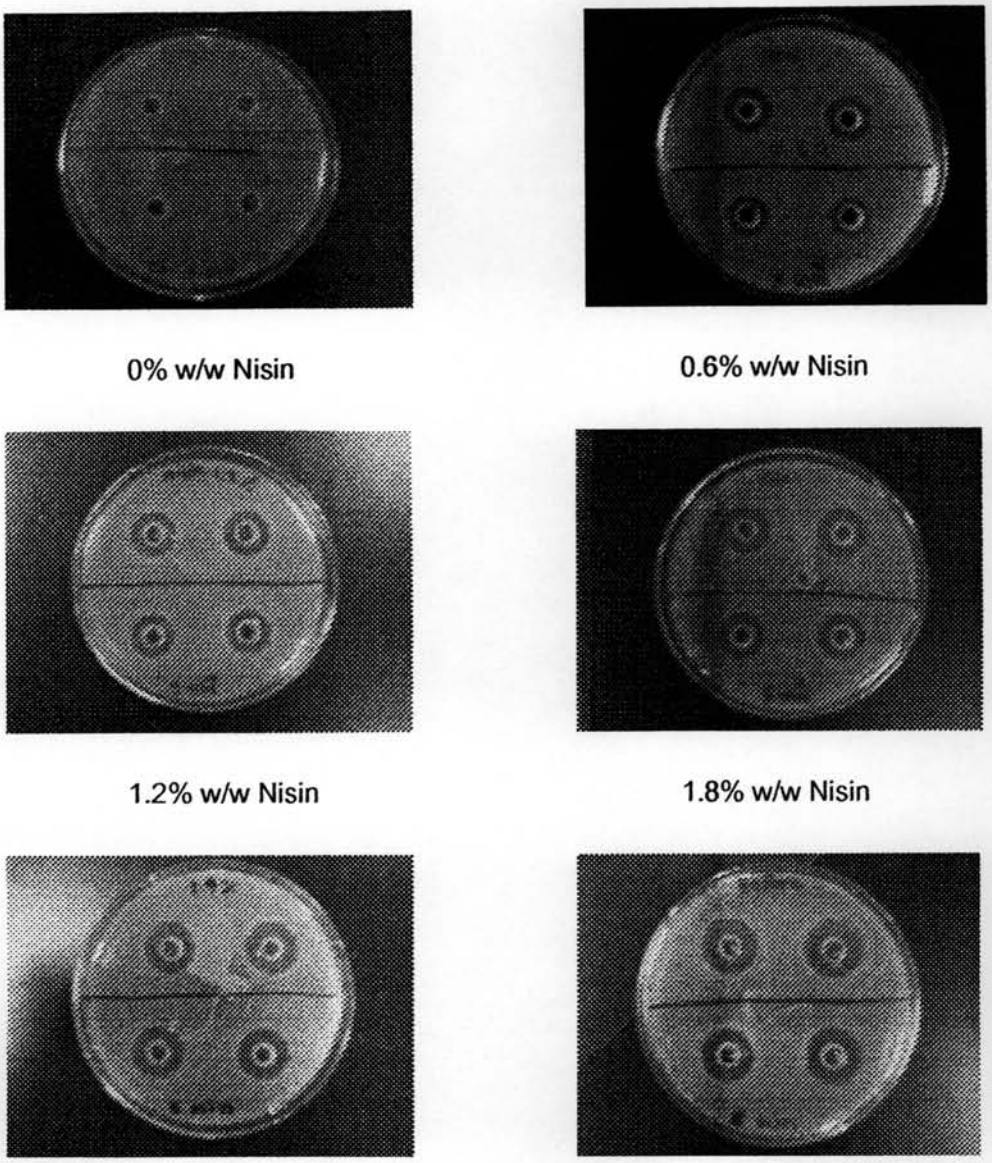
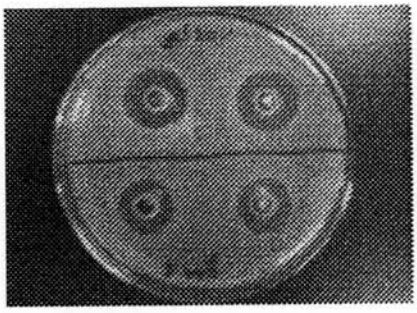
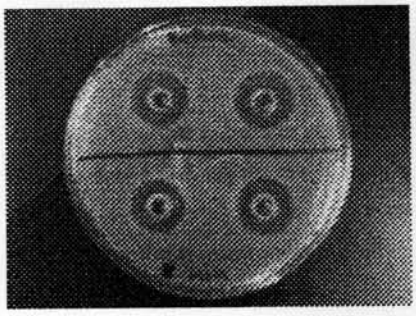


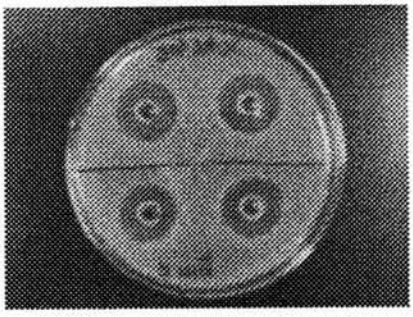
Figure 4.19 The width of inhibition zone (cm) in plates of nisin from antimicrobial gelatin nanofiber (upper zone) and crosslinked antimicrobial gelatin nanofiber mat (lower zone) (22% w/v gelatin concentration and 0% - 3% w/w initial nisin concentration) against *Lactobacillus plantarum.*



20% w/v Gelatin



22% w/v Gelatin



24% w/v Gelatin

Figure 4.20 The width of inhibition zone (cm) in plates of nisin from antimicrobial gelatin nanofiber (upper zone) and crosslinked antimicrobial gelatin nanofiber mat (lower zone) (3% w/w nisin concentration and 20% - 24% w/v gelatin concentration) against *Lactobacillus plantarum*.

It could be seen from above that inhibition zone was not affected by gelatin concentration (nanofiber size) but by nisin concentration. Crosslinking also reduced inhibition zone.

Comparing the width of inhibition zone with nisin standard curve (see appendix C), crosslinked antimicrobial gelatin nanofiber mat production by electrostatic spinning technique and crosslinking process caused a reduction in nisin contained in nanofiber as shown in Table 4.3. After electrostatic spinning and crosslinking, the retention of nisin in crosslinked antimicrobial gelatin nanofiber mat was in the 1.08% - 1.22% w/w range. The decrease in nisin might be caused by the high voltage (15 kV)

current which was used to produce antimicrobial gelatin nanofiber by electrostatic spinning. High voltage probably deteriorated nisin which was a polypeptide. Dawson et al. (2003) reported that the film-formation method had an effect on the retention of nisin activity ($p<0.05$) with casting retaining greater activity than heat-pressing. Based on Hoffman et al. (1997), in absence of water, nisin was stable at 100 $^{\circ}C$. However, there was a loss of nisin antimicrobial activity at 149 $^{\circ}C$. Besides, the process of crosslinking by saturated glutaraldehyde vapor for increasing insolubility and mechanical properties of nanofibers mats might result in some degree of inability of dissolution of nisin from nanofiber mat. Further, crosslinking process might decrease antimicrobial activity of nisin due to reaction between glutaraldehyde and nisin.

Table 4.3 Initial nisin concentration, inhibition zone, nisin retention, and % nisin retention in crosslinked antimicrobial gelatin nanofiber mat.

Sample	Initial nisin concentration ($*10^{-2}$ mg /mL)	Inhibition zone width (cm)	Nisin retention ($*10^{-2}$ mg/mL)	% Nisin Retention
22% w/v gelatin	0	-	0	0
22% w/v gelatin 0.6% w/w nisin	132	1.34	1.52	1.15
22%w/v gelatin 1.2% w/w nisin	264	1.59	2.98	1.13
22%w/v gelatin 1.8% w/w nisin	396	1.74	4.62	1.17
22%w/v gelatin 2.4% w/w nisin	528	1.83	5.89	1.12
22%w/v gelatin 3.0% w/w nisin	660	1.92	7.45	1.13
20%w/v gelatin 3.0% w/w nisin	660	1.90	7.13	1.08
24%w/v gelatin 3.0% w/w nisin	660	1.94	8.03	1.22

Remark : "-" represents no inhibition zone

4.3 Effects of Temperature and Water Activity (a_w) on Nisin Release from Crosslinked Antimicrobial Gelatin Nanofiber Mat

According to the previous experiment, when initial nisin concentration was increased, the width of inhibition zone increased. However, there was nisin loss during

electrostatic spinning and crosslinking process. Therefore, the maximum nisin concentration (3% w/w) was chosen in this experiment. The diameter of nanofiber did not depend on concentration of nisin but depended on gelatin concentration. Increasing concentration of gelatin from 20% w/v to 24% w/v, the diameter of nanofiber was significantly increased ($p \leq 0.05$). At 22% w/v and 24% w/v gelatin concentration, nanofibers were continuous without beads, while nanofiber at 20% w/v gelatin concentration were not smooth and had beads on string. Although 24% w/v gelatin concentration could result very fine fibers without beads, when comparing the amount of gelatin to be used, 22% w/v gelatin concentration was more economical. Moreover, at fixed concentration of nisin and varied gelatin concentrations, there was no significant difference ($p > 0.05$) in nisin retention. Besides, 22% w/v gelatin concentration provides smaller fiber diameter, higher surface area per volume, as compared to 24% w/v gelatin concentration. Therefore, antimicrobial gelatin nanofibers mats were produced from 22% w/v gelatin concentration and 3% w/w nisin concentration for subsequent studies.

4.3.1 Effect of Temperature on Nisin Release

Effect of temperature and time on the release of nisin from crosslinked antimicrobial gelatin nanofiber mat which was shown by the width of inhibition zone of *Lactobacillus plantarum* TISTR 850 is presented in Figures 4.21 - 4.24. Nisin release from crosslinked antimicrobial gelatin nanofiber mat was observed from 1 minute onwards at 25, 35 and 45 $^{\circ}$C, and from 4 hours onwards at 5 $^{\circ}$C. The results showed that release of nisin increased with increasing time and increasing temperature accelerated nisin release. The width of zone of nisin inhibition against *Lactobacillus plantarum* at the maximum release level at 5, 25, 35, and 45 $^{\circ}$C were 1.75, 1.94, 1.97, and 2.03 cm, respectively. The result indicated that when nanofiber containing nisin was in contact with water, nisin rapidly released. Nisin is bacteriocin which has positive charged (+4) and its structure processes amphipathic properties (Breukink and de Kruijff, 1999; Cha et al., 2002). Therefore, electrostatic reaction between nisin and gelatin could occur. Nisin was released because of the repulsion between positive

charges of nisin and gelatin. An increase in temperature caused mobility of molecules to increase and, thus increased nisin release. Food surfaces are diverse and provide small cavities and convoluted locations to "hide" bacteria from direct package contact. In many processed foods, microorganisms locate mostly on the food surface. For this reason, antimicrobials that do not migrate from a food contact film are successful in preventing growth on the film but not as effective in preventing growth on the food surface (Dawson et al., 2003). When considering crosslinked gelatin nanofiber mat containing nisin that can also permit nisin release, it has an advantage of controlled releasing nisin from nanofiber allowing nisin to diffuse into the food. Therefore, it could inhibit the growth of microorganisms which grow both on food surface and inside.

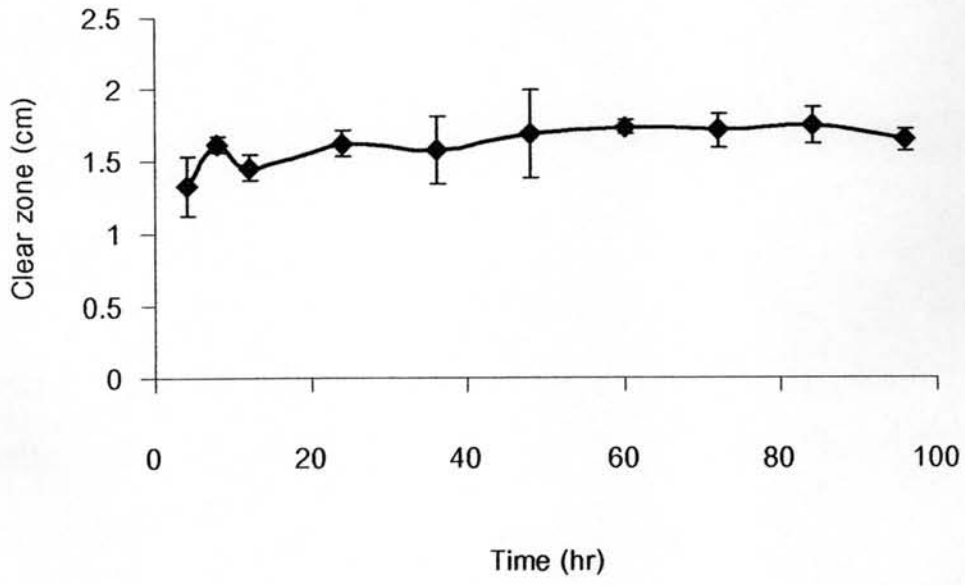


Figure 4.21 Inhibition zone of *Lactobacillus plantarum* caused by nisin released from crosslinked antimicrobial gelatin nanofiber mat in distilled water at 5 °C.

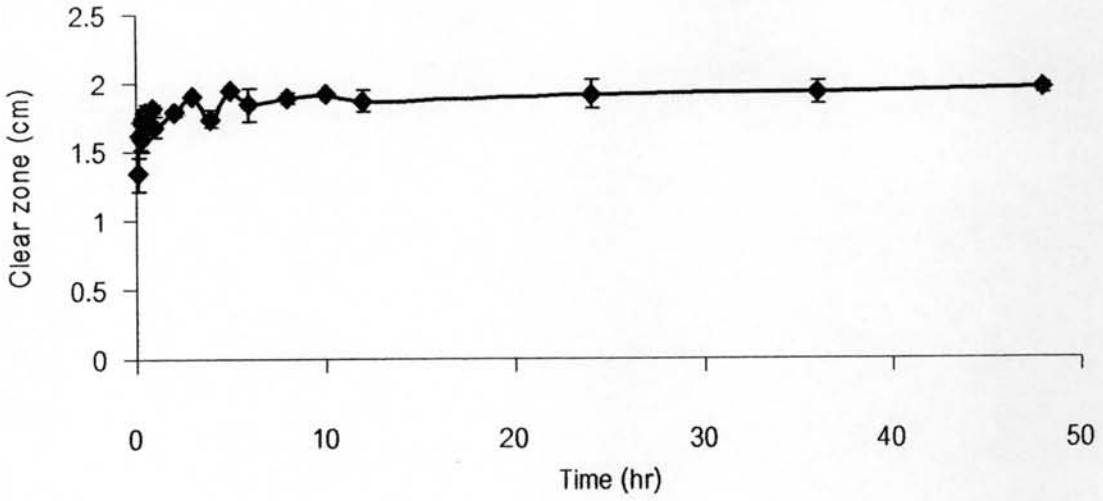


Figure 4.22 Inhibition zone of *Lactobacillus plantarum* caused by nisin released from crosslinked antimicrobial gelatin nanofiber mat in distilled water at 25 °C.

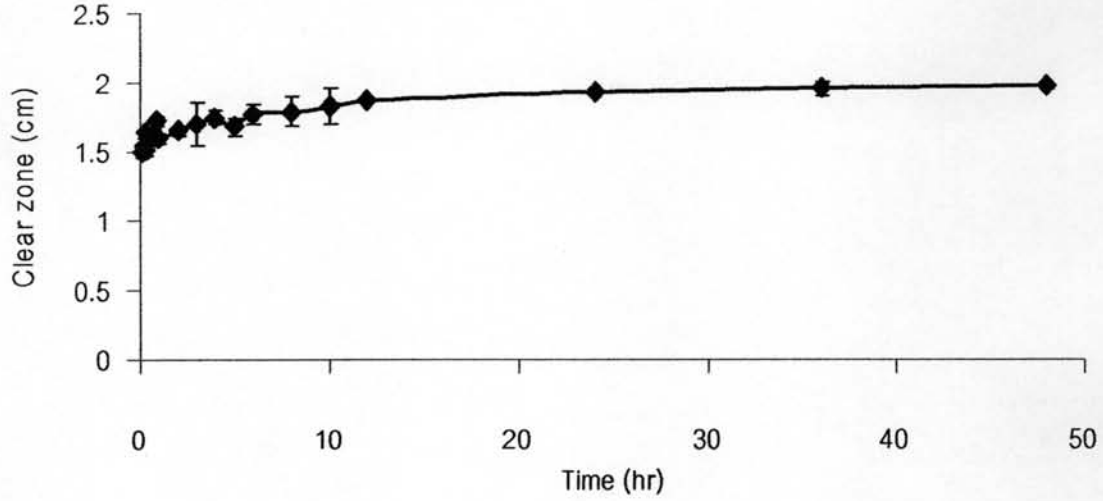


Figure 4.23 Inhibition zone of *Lactobacillus plantarum* caused by nisin released from crosslinked antimicrobial gelatin nanofiber mat in distilled water at 35 °C.

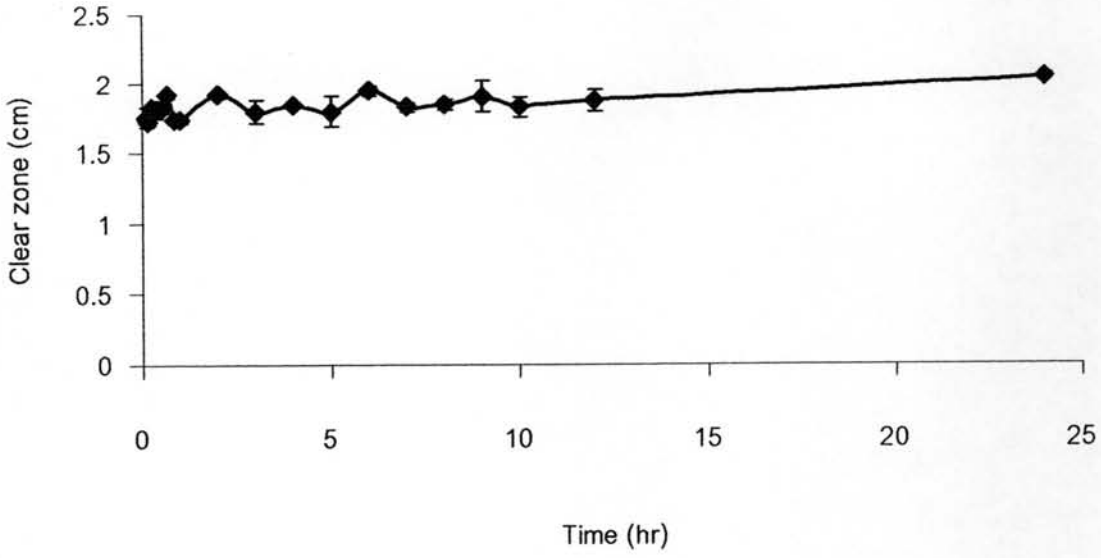


Figure 4.24 Inhibition zone of *Lactobacillus plantarum* caused by nisin released from crosslinked antimicrobial gelatin nanofiber mat in distilled water at 45 °C.

4.3.2 Effect of Water Activity on Nisin Release

Effect of water activity and time on the release of nisin from crosslinked antimicrobial gelatin nanofiber mat which was shown by the width of inhibition zone of *Lactobacillus plantarum* TISTR 850 is presented in Figure 4.25. Nisin release from crosslinked antimicrobial gelatin nanofiber mat was observed from 1 minute onwards at 0.955, 0.975, and 0.992 water activity (25 °C). This water activity range represents the water activity in foods such as fresh meat and fish (a_w = 0.99), cheese (a_w = 0.97), and bread (a_w = 0.95) (The Dairy Research & Information Center, 1996; Chaplin, 2007). The release of nisin increased with increasing time. Due to the fact that narrow water activity levels were investigated, no significant difference in nisin release was observed. The width of inhibition zone of nisin against *Lactobacillus plantarum* at the maximum release level at water activity 0.955, 0.975, and 0.992 were 1.97, 2.09, and 1.94 cm, respectively.

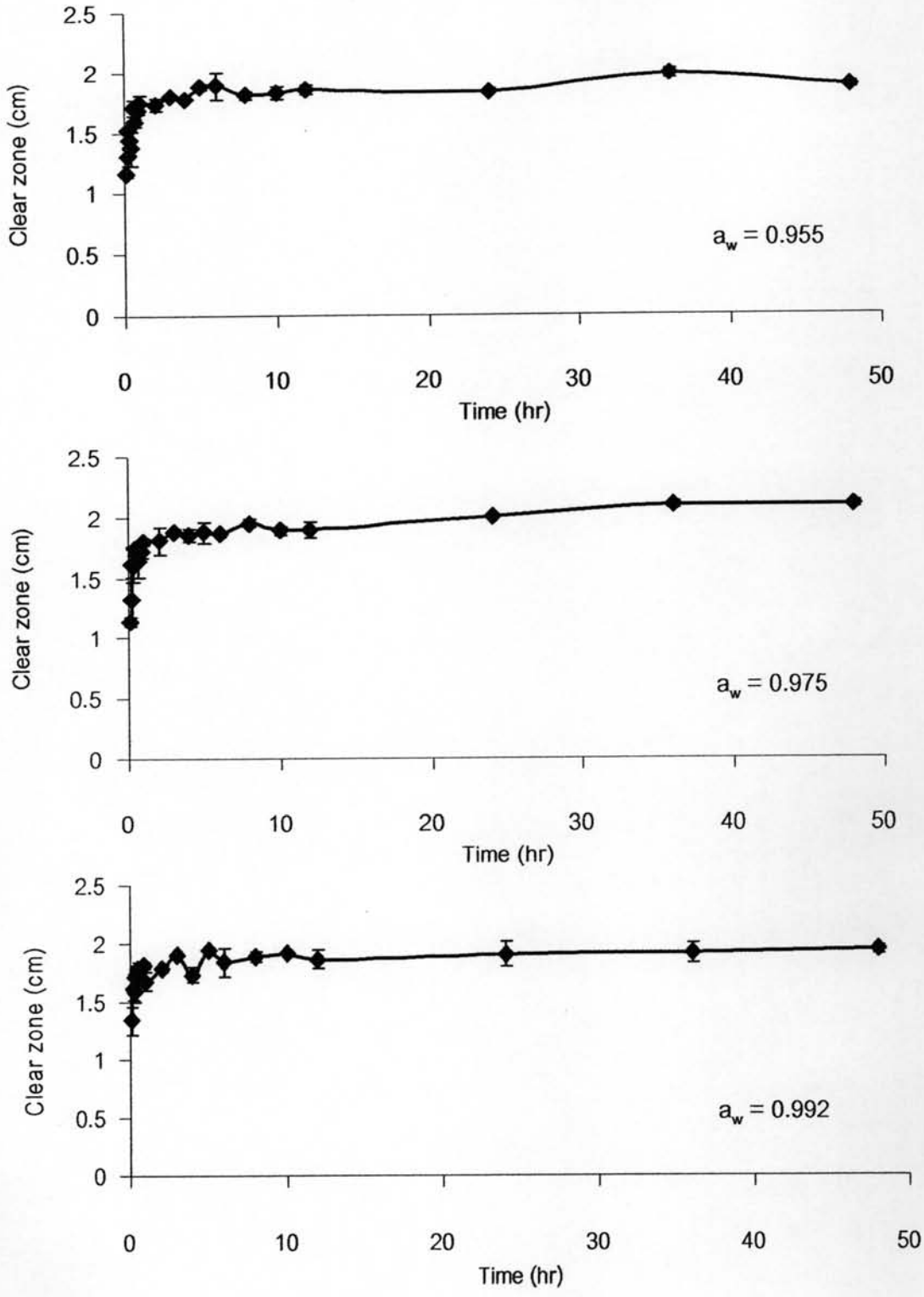


Figure 4.25 Inhibition zone of *Lactobacillus plantarum* caused by nisin released from crosslinked antimicrobial gelatin nanofiber mat in distilled water-glycerol that had 0.955, 0.975 and 0.992 water activities (25 °C).

4.4 Effects of Crosslinked Antimicrobial Gelatin Nanofiber Mat to Inhibiting Growth of Bacteria

Crosslinked antimicrobial gelatin nanofiber mat (22% w/v gelatin concentration and 3% w/w nisin concentration) was chosen for this experiment. Effect of nisin from crosslinked antimicrobial gelatin nanofibers mats on the inhibition of *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* DMST 17303, and *Salmonella* Typhimurium ATCC 13311 was compared with crosslinked gelatin nanofiber mat without nisin (control sample) (Figures 4.26 - 4.28). Crosslinked gelatin nanofiber mat without nisin could not inhibit the growth of the three microorganisms, while crosslinked antimicrobial gelatin nanofiber mat could inhibit *Staphylococcus aureus* and *Listeria monocytogenes* but not *Salmonella* Typhimurium. Crosslinked antimicrobial gelatin nanofiber mat could reduce *Staphylococcus aureus* count from 5.68 log to 3.14 log at 2 hours, to 2.66 log at 8 hours, to 2.10 log at 24 hours, and to 0.41 log at 48 hours. This is consistent with Millette et al. (2007) who reported that nisin reduced *Staphylococcus aureus* count in beef product, which was wrapped by palmitoylated alginate-based film that contained 500 and 1000 IU/mL nisin, from 4 log CFU/cm^2 to 3.09 log CFU/cm^2 and 2.14 log CFU/cm^2 after storage at 4 ^{o}C for 7 days. For *Listeria monocytogenes*, the microbial count decreased from 6.27 log to 0.65 log at 2 hours. After 8 hours onwards, crosslinked antimicrobial gelatin nanofiber mat could completely inhibit the growth of *Listeria monocytogenes*. According to the report of Orr et al. (1998), nisin reduced 2 log of *Listeria monocytogenes* in cast corn zein films containing nisin after refrigerated storage for 48 hours. Hoffman et al. (2001) reported that film containing nisin reduced *Listeria monocytogenes* population by 1.0 log at 2 hours and 5.5 log after 48 hours. Ko et al. (2001) reported the maximum reduction in *Listeria monocytogenes* counts by nisin in soy protein films occurred at 60 minutes. They reported a reduction of *Listeria monocytogenes* from 5.24 log to 3.0 log after 60 minutes of incubation. There was no further reduction in bacterial counts from 60 minutes to 120 minutes of incubation. *Salmonella* Typhimurium counts decreased from 6.01 log to 4.54 log in the first 4 hours and the microbial counts increased to 7.95 log after 48 hours. The resulted showed that crosslinked antimicrobial gelatin nanofiber mat could not inhibit *Salmonella* Typhimurium. Nisin could not inhibit *Salmonella* Typhimurium with increasing time

because *Salmonella* Typhimurium is gram-negative which has different cell wall composition from gram-positive which could be easily inhibited by nisin. Nisin can inhibit gram-positive bacteria by binding of nisin to the cytoplasmic membrane, which has an outside cell wall consisting of thick peptidoglycan with complete peptide-bridge, inducing pore formation causing the cell to become permeable to small ions and molecules. The formation of this pore induces a drop in proton motive force and causes ATP reduction (Winkwoski et al., 1991). In contrast, gram-negative microorganism has thinner peptidoglycan layer with incomplete peptide-bridge but has an outside cell wall which is composed of lipoprotein, lipopolysaccharide, and phospholipids (Stevens et al., 1992a; R&D of AllVet Co. Ltd., 2007) that can prevent nisin from binding with the cytoplasmic membrane (Stevens et al., 1992a).

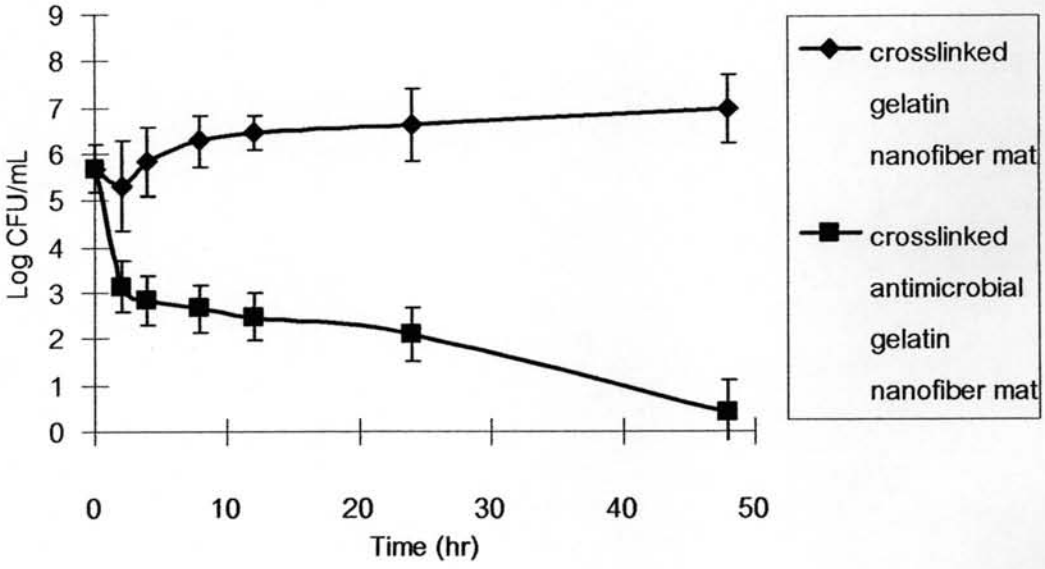


Figure 4.26 Effect of nisin in crosslinked gelatin nanofiber mat on the inhibition of *Staphylococcus aureus* ATCC 25923 (10^6 CFU/mL).

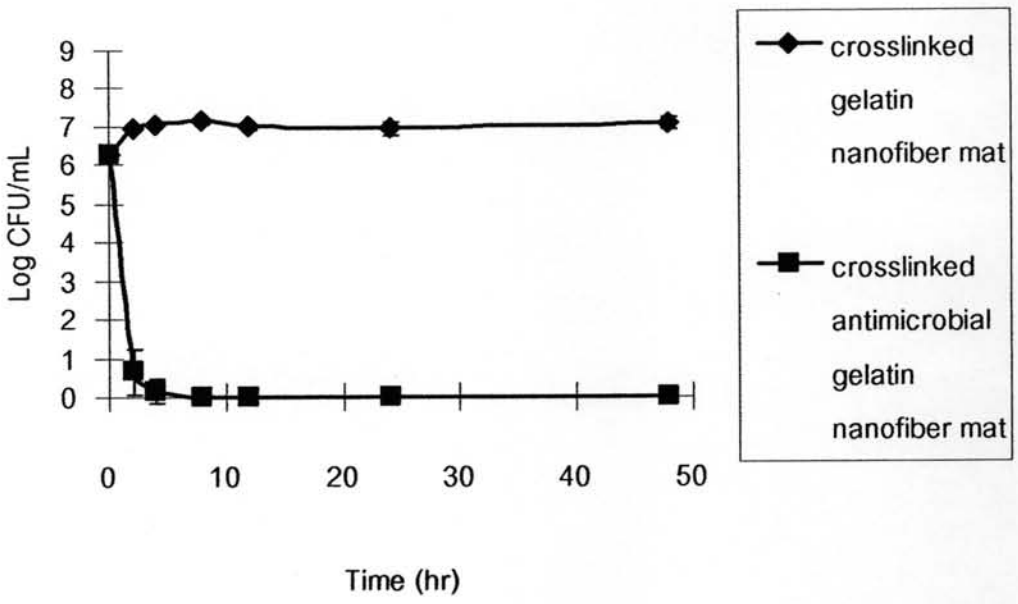


Figure 4.27 Effect of nisin in crosslinked gelatin nanofiber mat on the inhibition of *Listeria monocytogenes* DMST 17303 (10^6 CFU/mL).

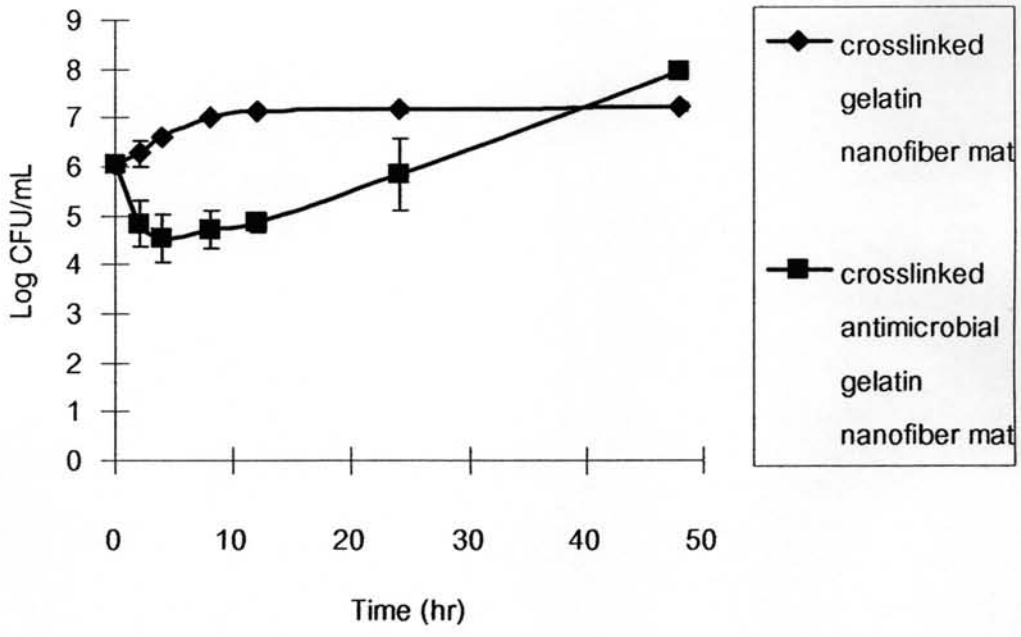


Figure 4.28 Effect of nisin in crosslinked gelatin nanofiber mat on the inhibition of *Salmonella* Typhimurium ATCC 13311 (10^6 CFU/mL).

4.5 Antimicrobial Activity of Crosslinked Antimicrobial Gelatin Nanofibers Mats after 5 Months Storage

Crosslinked antimicrobial gelatin nanofiber mat was stored in a desiccator at room temperature (~ 25 °C) for 5 months and tested for its antimicrobial activity against *Lactobacillus plantarum* TISTR 850. After storing for 0, 1, 3, and 5 months, crosslinked nanofibers morphology did not change. Further, the crosslinked antimicrobial gelatin nanofiber mat retained its ability in inhibiting *Lactobacillus plantarum* TISTR 850 (Table 4.4).

Table 4.4 Inhibition zone of crosslinked antimicrobial gelatin nanofiber mat of *Lactobacillus plantarum* TISTR 850 at different storage times.

Time (month)	Average width of the inhibition zone (cm)
0	1.814±0.048
1	1.848±0.024
3	1.896±0.033
5	1.859±0.017