CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 The antibacterial effect of chitosan on the growth of food spoilage bacteria

The effect of chitosan on the growth of food spoilage bacteria was studied *in vitro* in the first experiment. The results from this study would be used in the second part of the work where chitosan would be applied in meat. Two types of chitosan, with different molecular weights of 150,000 and 400,000 Da were used. Seven microorganisms used in this work were *B. cereus, M. luteus, E. coli, En. aerogenes, S. marcescen, Ps. fluorescens* and *L. plantarum*. Their typical colonies were shown in Appendix F. The inhibitory effect of chitosan on these seven microorganisms was studied with chitosan solutions at the concentrations of 0.01, 0.02, 0.04, 0.06 and 0.08% (w/v) at pH 6.0. A sample of nutrient broth without any chitosan addition was used as a control. After inoculation, the microbial cultures with the presence of different concentrations of chitosan were incubated by shaking at 180 rpm in a shaker for 24 hr at 37°C or room temperature. The viable cells of each sample were then enumerated. The results shown in Figure 11 indicated that the growth of some bacterial species were decreased with an increase in the concentration of chitosan.

The most sensitive bacteria strain to chitosan was *M. luteus*. Its growth was inhibited by 0.01% (w/v) chitosan with a viable cell number reduced by up to 4 to 5 log CFU/ml. This strain was completely inhibited by chitosan at 0.06% (w/v). However, chitosan at 0.01% (w/v) had no effect on the growth of *B. cereus*. It was found that this strain was inhibited by 0.02% (w/v) of chitosan and the growth was completely inhibited by 0.04% (w/v) of chitosan. At 0.02% (w/v) chitosan, there was a reduction in the viable cell of *B. cereus* count for 5 to 6 log CFU/ml compared to the

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control. For *En. aerogenes* and *E. coli*, the effectiveness of chitosan inhibition increased with increasing chitosan concentrations. Chitosan had a greater inhibition effect on *En. aerogenes* than *E. coli*. It was found that both of these microorganisms was completely inhibited by 0.06% (w/v) chitosan. The other three bacterial strains, *S. marcescen, Ps. fluorescens* and *L. plantarum* had a higher resistance to chitosan. There was only a small decrease in the viable cell numbers with an increase in the chitosan concentrations. They were not completely inhibited at the highest concentration of chitosan tested of 0.08% (w/v). The minimal inhibitory concentration (MIC) was defined as the lowest concentration of chitosan required to completely inhibit the bacteria growth after incubation for 24 hr. The MIC value of *B. cereus* was 0.04% (w/v), while *M. luteus*, *En. aerogenes* and *E. coli* were 0.06% (w/v). However, for the three strains of *S. marcescen*, *Ps. fluorescens* and *L. plantarum*, their MIC values could not be determined by the chitosan concentrations tested in this experiment. The 24 hr incubation time was used in defining MIC because the growth rate of the seven studied bacteria developed to a stationary phase within this time (Appendix C). The results of this experiment are not contrast to those of Sudarshan *et al.* (1992) and Cho (1989) who reported that chitosan is effective against Gram-positive bacteria. In this study, *M. luteus* and *B. cereus* are Gram-positive bacteria that are sensitive to chitosan. However, one Gram-positive bacterium tested, *L. plantarum*, was more resistant to chitosan. Its growth was only inhibited for up to 52-56% (Table 11) by a chitosan concentration of 0.04 %(w/v). In addition, the inhibitory effect of chitosan was also found in some Gram-negative bacteria, such as *En. aerogenes* and *E. coli*, while other Gram-negative bacteria; *Ps. fluorescens* and *S. marcescen* were not inhibited. Further investigation is required to indicate the mechanism of inhibition, as also suggested by Hwang *et al.* (1999). Therefore, the effect of chitosan on microorganisns can not be simply explained in terms of the Gram property.

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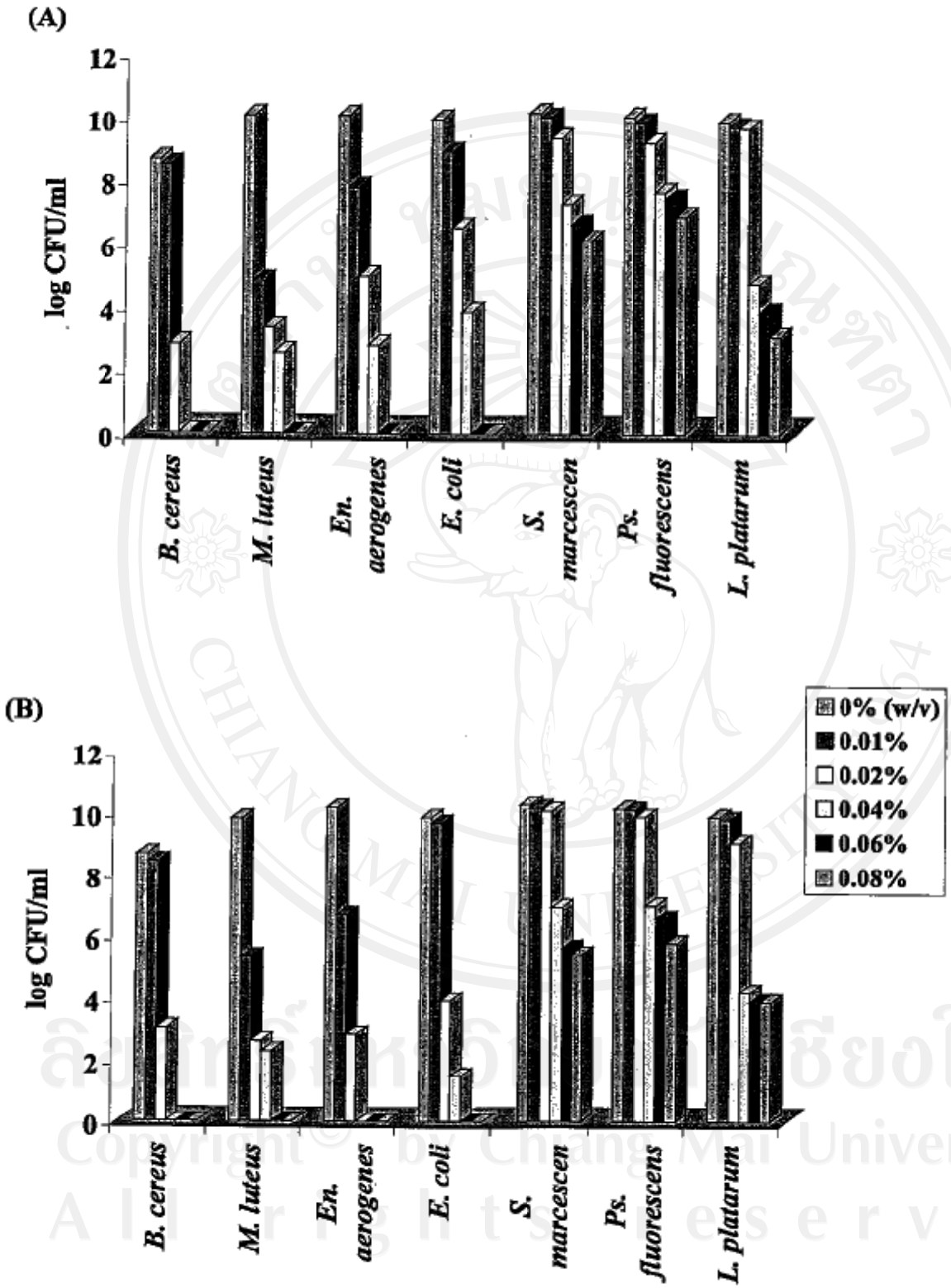

Figure 11 Inhibition effects of chitosan Mw 150,000 Da (A) and chitosan Mw 400,000 Da (B) on growth of food spoilage bacteria.

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No *et al.* (2001) also demonstrated that chitosans and chitosan oligomers showed bactericidal effects and the minimum inhibitory concentration (MIC) values, ranging from 0.05% (w/v) to above 0.1% (w/v), slightly differed with organism tested and chitosan types. Seo *et al.* (1992) tested the effect of chitosan on growth of 11 different bacteria and found that the chitosan MIC ranged from 0.001% (w/v) to 0.1% (w/v). Of the organisms tested, growth of *E. coli, Ps. fluorescens, B. cereus* and *S. aureus* were inhibited by chitosan concentrations of 0.002%, 0.05%, 0.1% and 0.002% (w/v), respectively. Uchida *et al.* (1988) reported the MIC of chitosan oligomers for *E. coli* and *S. aureus* to be 0.025% and 0.05% (w/v), respectively. Joen *et al.* (2001) reported that the MIC values of chitosan were less than or equal to 0.06% against Gram-negative and Gram-positive bacteria. These reported MIC values are comparable to, or lower than the current results. This was probably due to differences in chitosan characteristics or medium pH applied as reported by Wang (1992).

The antibacterial activity of chitosan differed with regard to its molecular weight and type of bacterium was suggested by Rhoades and Roller (2000) and No *et al.* (2001). In this investigation, the inhibition effect of two molecular weight chitosans (150,000 and 400,000 Da) against seven food spoilage bacteria was observed in terms of percentage of growth inhibition of the bacteria tested. The results were shown in Table 11. These two molecular weights chitosan were not significantly different ($p< 0.05$) in *B. cereus, M. luteus, S. marcescen, Ps. fluorescens* and *L. plantarum.* However, it is interesting that the growth inhibition of *En. aerogenes* and *E.coli* by two molecular weight chitosans was significantly different ($p<0.05$) at concentrations of 0.02% and 0.04% (w/v). It was shown that the 400,000 Da chitosan was more effective against *En. aerogenes* and *E. coli* than the 150,000 Da chitosan.

The relationship of molecular weight with the antibacterial activity of chitosan had been reported. Joen *et al.* (2001) observed that Mw 10 to 1 kDa of chitosan oligomers was critical for microorganism inhibition and the efficacy increased with Mw. No *et al.* (2001) investigated the antibacterial activity of chitosan (Mw ranging from 1671 to 28 kDa) and chitosan oligomers (Mw ranging from 22 to 1 kDa) for various bacteria. Chitosan of 746 kDa appeared to be the most effective chitosan

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against *E. coli* and *Ps. fluorescens*, compared with chitosan of Mw 470 kDa against *S. typhimurium* and *V. parahaemolyticus*. Chitosan oligomers of 1 kDa showed a relatively greater antimicrobial activity against Gram-negative bacteria while the Mw of 4 and 2 kDa had a better activity against Gram-positive bacteria compared with other Mw. Sekiguchi *et al.* (1994) investigated the antibacterial activity of chitosan oligomer (Mw ranging from 2,350 to 21;600 Da) to various bacteria. Growth of *B. cereus* on agar culture was suppressed by 0.2-0.3% chitosan with Mw 11,000 Da.

Thus, the antimicrobial activity of chitosan has been recognized against several microorganisms and is influenced by a number of factors that including the type of chitosan, the degree of chitosan polymerization and some of its other chemical and physical properties. Uchida *et al.* (1988), in addition, found that chitosan hydrolysate, slightly hydrolyzed with chitosanase, was more active as an antibacterial agent than the native chitosan and chitosan oligomer. Rhoades and Roller (2000) reported that mild hydrolysis of chitosan enhanced its inhibitory activity against some species of spoilage yeast grown in complex laboratory media, whereas highly degraded forms of chitosan showed no antimicrobial acitivity. The antimicrobial effects of native chitosan and its derivatives vary in live hosts. Partial depolymerization of chitosan enhances its antimicrobial activity, whereas chitosan with a short chain length (< 7 of chain length) has a decrease antimicrobial activity. The degree of deacetylation influenced the activity of chitosan but the mechanism of this property of chitosan on antimicrobial activity was not clearly demonstrated (Oh *et al.*, 2001). Cho *et al.* (1998) reported that the bacterial activity of chitosan against *E. coli* and *Bacillus* spp. increased with decreasing viscosity from 1,000 to 10 cP. In conclusion, the antimicrobial activity of chitosan depends on both the type of chitosan and the microorganism used. It could be suggested from several reports that the antibacterial activity of chitosan against microorganism *in vitro* is affected by microorganism strains, cell population, cell age, nature of chitosan used (Mw, %DD, source etc.), condition (temperature, pH), and chitosan solvents (No *et al.*, 2001; Rhoades and Roller, 2000; Roller and Covill, 1999; Oh *et al.*, 2000; Tsai and Su, 1999; Simpson *et al.*, 1997; Chen *et al.*, 1998; Wang, 1992).

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Table 11 Inhibition effect of two molecular weight chitosans on growth of food spoilage bacteria.[a]

Microorganism	Chitosan	% Growth inhibition				
	Mw (Da)	0.01	0.02	0.04	0.06	0.08
B. cereus	150,000	4	68	100	100	100
	400,000	2	65	100	100	100
M. luteus	150,000	58	59	75	100	100
	400,000	49	62	75	100	100
En. aerogenes	150,000	24	51[b]	71[b]	100	100
	400,000	30	61[b]	100[b]	100	100
E. coli	150,000	1	35[b]	64[b]	100	100
	400,000	2	55[b]	82[b]	100	100
L. plantarum	150,000	0	2	52	61	69
	400,000	0	9	56	61	65
Ps. fluorescens	150,000	0	9	24	28	32
	400,000	0	8	30	33	42
S. marcescen	150,000	0	1	34	47	52
	400,000	1	1	32	49	55

Note: [a] Two sample *t*-test was used for % growth inhibition from two molecular weight chitosan in each concentration

[b] significant at level ($p<0.05$)

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4.2 The effect of pH on the antibacterial activity of chitosan

The effect of pH on the antibacterial activity of two molecular weight chitosans was evaluated for *M. luteus* and *B. cereus*, which were sensitive bacteria strains to chitosan from previous experiment. These two molecular weights of chitosan were used at a lowest concentration of 0.01% (w/v) in this experiment in order to evaluate an additional effect of pH on the activity of chitosan. Moreover, several reports about the effect of acidic pH stated that the low pH value contribute to a greater bactericidal effect of chitosan (No *et* a., 2001; Tai and Su, 1999). The pH values in this study was limited up to pH 6.0 because of insolubility of chitosan in organic acid solutions at pH above 6.0 (Muzzarelli, 1973). Two chitosans of Mw 150,000 and 400,000 Da are employed in this study as the result from previous section that showed no significant difference in the percentage of growth inhibition between these two chitosans at 0.01% (w/v) for the two bacteria studied (Table 11).

The antibacterial activity of chitosan on *M. luteus* at different pH values are shown in Figure 12. It can be observed that, at pH 6.0, the maximun pH of chitosan solution used throughout this experiment, chitosan of both molecular weights had an antibacterial effect against *M. luteus*. After 24 hr incubation, the number of living cells in the presence of chitosans are reduced by approximately 2 log CFU/ml. The result at pH 5.5 is similar. However, at pH 5.0, chitosan solutions showed greater antibacterial effect on *M. luteus*. After 24 hr incubation, the number of living *M. luteus* in the presence of chitosans are reduced by approximately 2.5 log CFU/ml. At these pH values (6.0, 5.5, 5.0), it is shown clearly that the observed inhibitory effect was more from the presence of chitosan rather than pH, as the controls continuously displayed growth. At pH 4.5, chitosan of Mw 150,000 Da showed a greater antibacterial effect on *M. luteus* than that of Mw 400,000 Da. However, after 24 hr incubation, the number of living cells in the presence of chitosan did not decrease more than at pH 5.0. It is clear that at pH 4.5, the growth inhibition of *M. luteus* was a combination result of pH and chitosan. By the presence of these two antibacterial factors, the final concentration of living cells after 24 hr incubation is slightly lower than at a higher pH value of 5.0.

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When comparing the effect of pH on the antibacterial activity of each Mw chitosan against *M. luteus*. It was found that the antibacterial activity of both Mw chitosan were not different in each pH condition. However at pH 4.5, the cell numbers of *M. luteus* were affected by the chitosan Mw 150,000 more than the chitosan of Mw 400,000. At pH 4.0 (Figure 12 (E)), chitosan showed no significant effect on *M. luteus*. This figure suggested that the antibacterial activity of chitosan may be affected by such low pH. In general, a combination of antibacterial factors should result in a greater inhibitory effect, due to hurdle effect. In this experiment, this hurdle effect was shown when chitosan was added to a solution with a pH value of 4.5 to be used against *M. luteus*.

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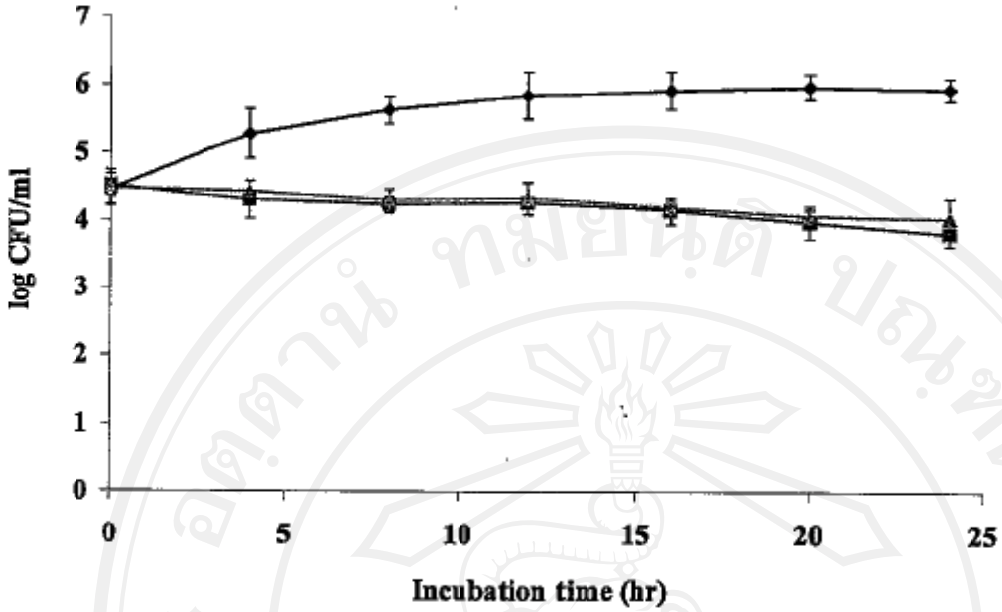
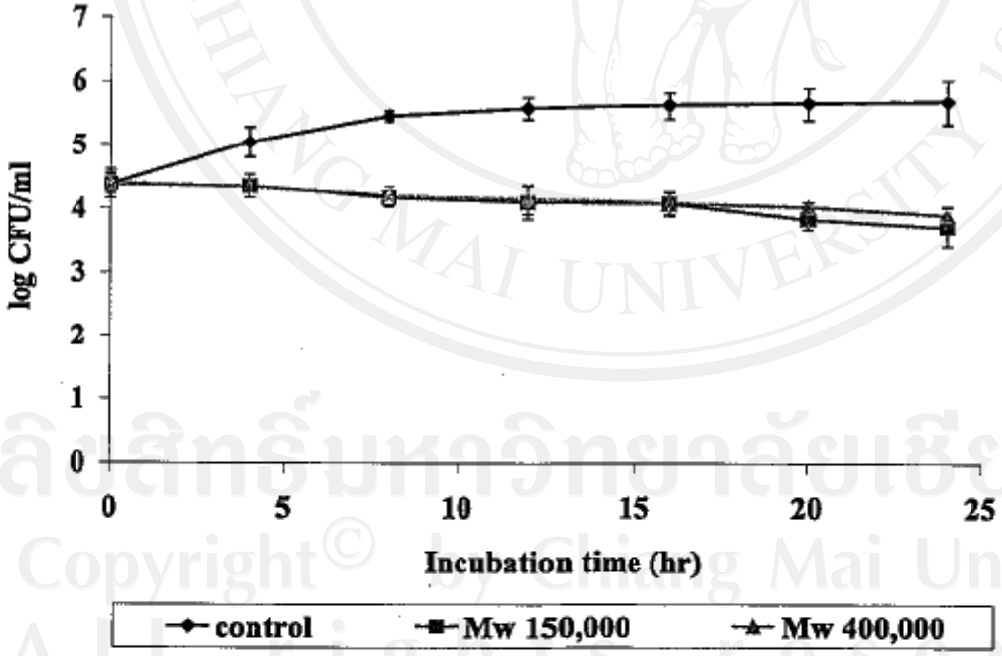


(A) pH 6.0



(B) pH 5.5

Figure 12 The effect of pH on the antibacterial activity of chitosan 0.01% (w/v), pH 6.0 against *M. luteus*.

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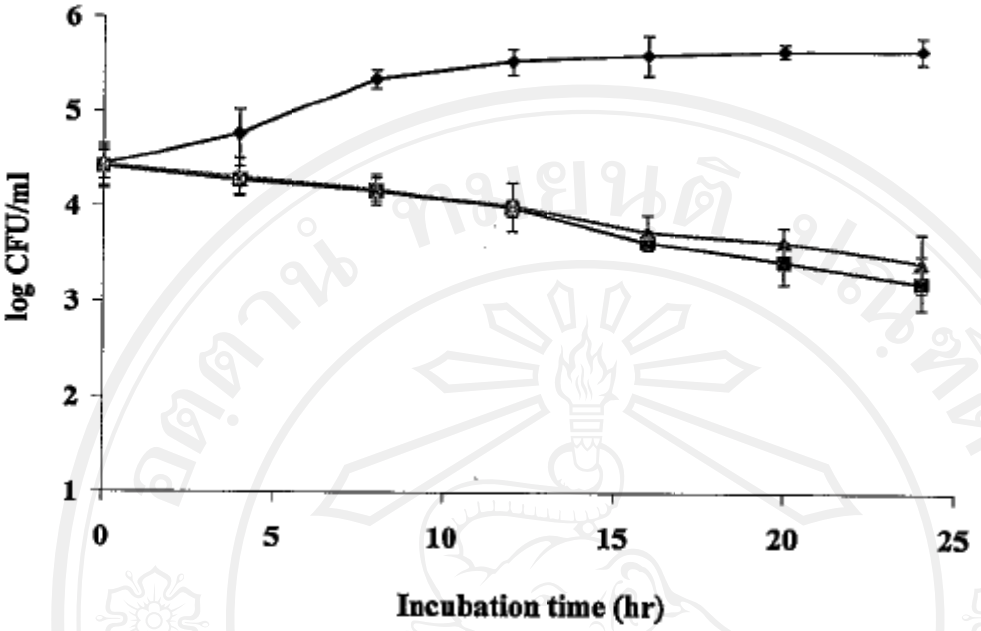
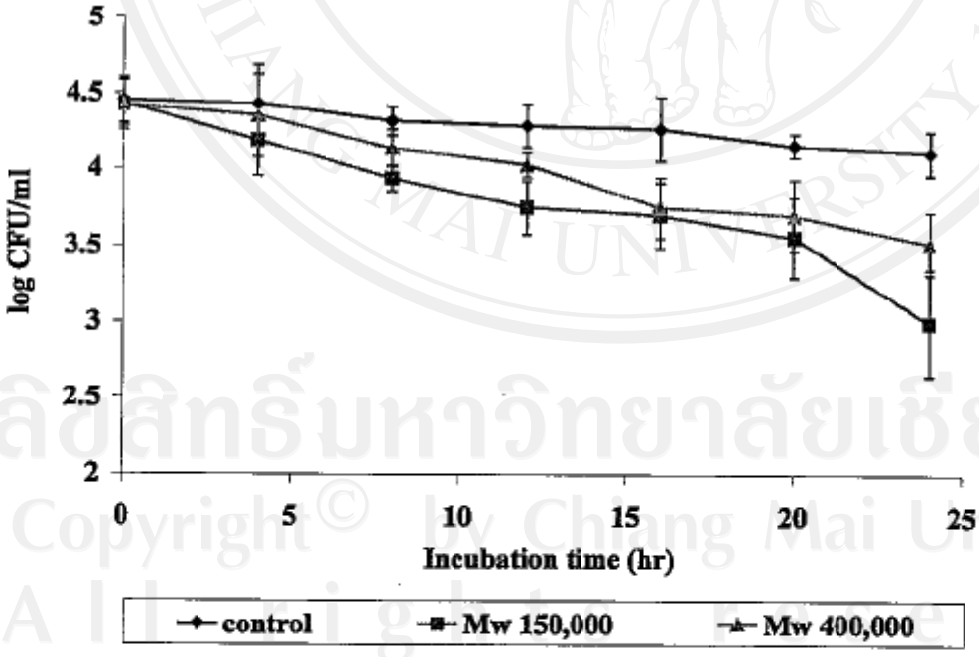


(C) pH 5.0



(D) pH 4.5

Figure 12 The effect of pH on the antibacterial activity of chitosan 0.01% (w/v), pH 6.0 against *M. luteus* (continued).

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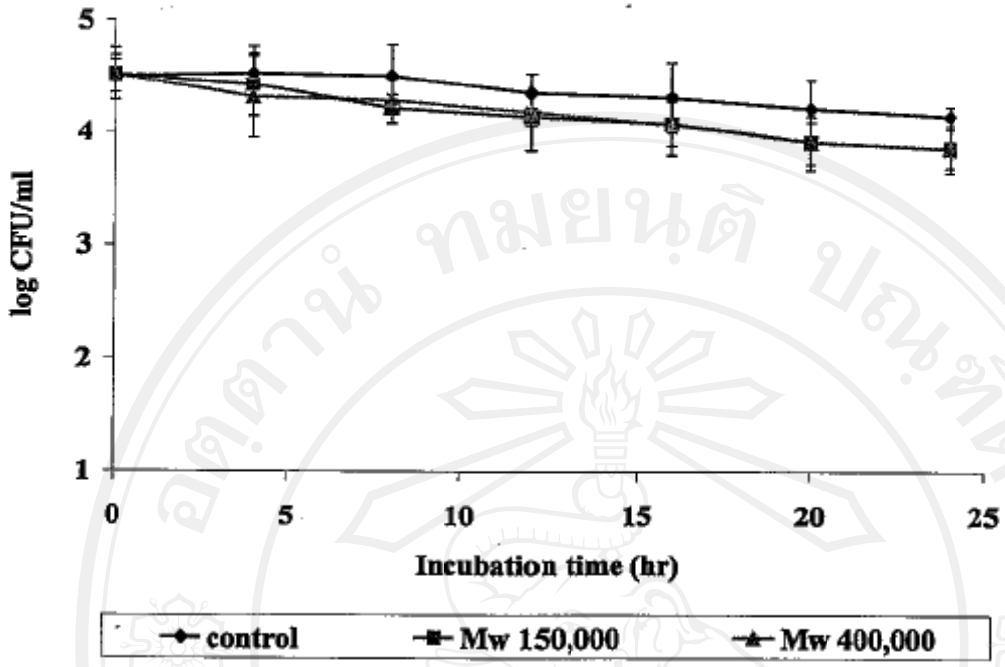

(E) pH 4.0

Figure 12 The effect of pH on the antibacterial activity of chitosan 0.01% (w/v), pH 6.0 against *M. luteus* (continued).

The effect of pH on the antibacterial activity of chitosan against *B. cereus* was shown in Figure 13. At pH 6.0 (Figure 13 (A)), chitosan (0.01% w/v) of both molecular weights did not have any effect against the *B. cereus* culture. A similar result is obtained at pH 5.5 after 24 hr incubation. Especially for the solution at pH 5.5, a longer adaptation phase of the *B. cereus* culture at the beginning of exposure to the chitosan solutions was observed. These results support results from the previous experiment (Figure 11). The effect of chitosan at pH 6.0 and 5.5 on *M. luteus* and *B. cereus* are shown to be different. However, at pH 5.0, 4.5 and 4.0, chitosan showed inhibitory effects against the *B. cereus* culture (Figure 13 (C), (D), (E)). After 24 hr incubation, the cultures with chitosan showed reduction in living cell numbers in the range of 0.75 to 1.5 log CFU/ml, with the most reduction achieved at pH 4.5. Interestingly, at pH 4.0, chitosan did not have an inhibitory effect against the *B. cereus* culture as great as pH 4.5, which is similar to the results from *M. luteus*

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culture. The result from the *B. cereus* culture confirms that the chitosan activity is affected by a low pH value.

An interesting point observed from this experiment is as followed. Although chitosan of 0.01% (w/v) concentration has no inhibitory effect on *B. cereus* culture, a combination with low pH values (5.0 and 4.5) can increase the inhibitory effect of chitosan to this bacterium. Therefore, it can be further concluded that the antibacterial activity of chitosan depends on pH and an optimum pH need to be found when applying chitosan solutions on bacterial cultures for its highest antibacterial activity. The experiment results on different pH values did not show any significant differences between the two chitosan types, except at pH 4.5 and 4.0. Chitosan of Mw 400,000 Da showed a greater reduction on living cells of *B. cereus* after 24 hr incubation. These results, again, are similar to those of *M. luteus*. A further experiment should be carried out to investigate the effect of pH on the activity of chitosan types at different time intervals.

The experiment results in this study are in an agreement with the reports of Tsai and Su (1999) and No *et al.* (1999). They reported that acidic pH increased the bactericidal effect of chitosan against food spoilage bacteria. Moreover, these results are in an agreement with Wang (1992), who found that the antibacterial activity of chitosan against five species of food-borne pathogens (*S. aureus, E. coli, Y. enterocolitica, L. monocytogenes* and *S. typhimurium*) was highest at a certain pH value (5.5). It can be suggested that an application of chitosan to lower acidic food would enhance its effectiveness as a natural preservative.

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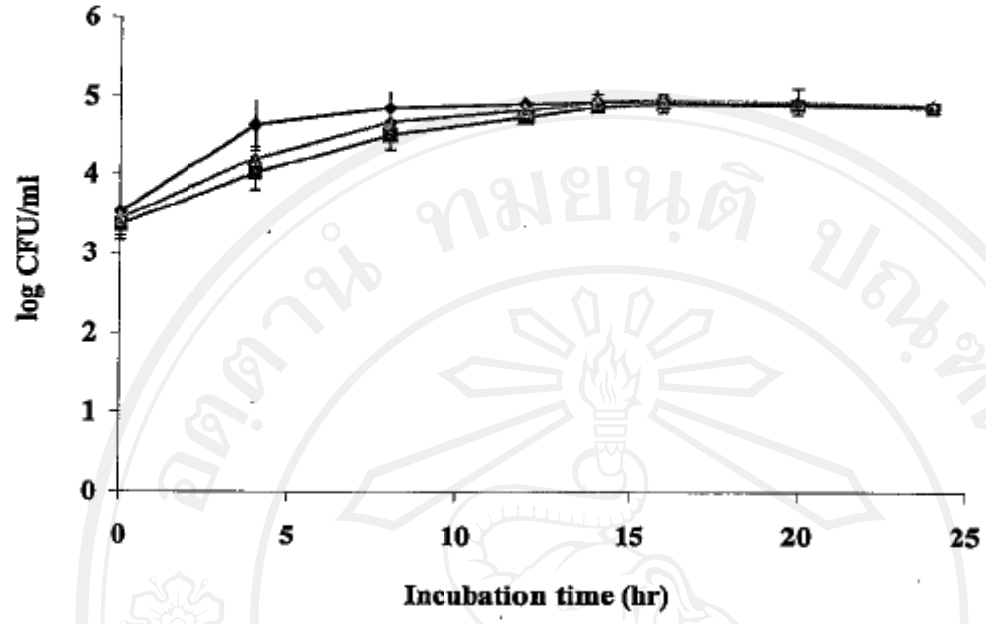
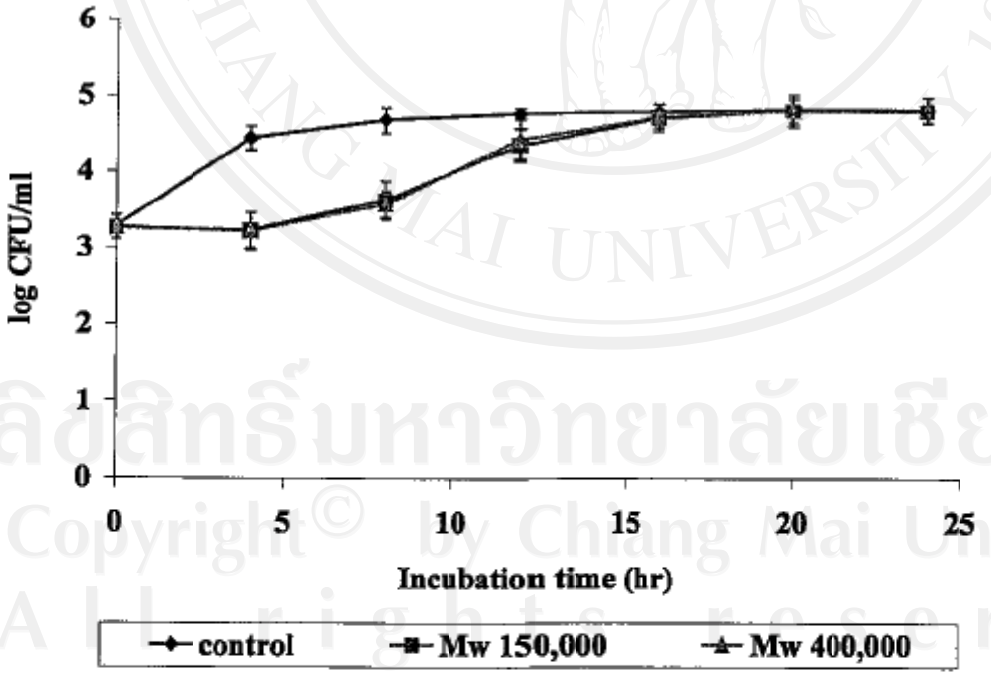


(A) pH 6.0



(B) pH 5.5

Figure 13 The effect of pH on the antibacterial activity of chitosan 0.01% (w/v), pH 6.0 against *B. cereus*.

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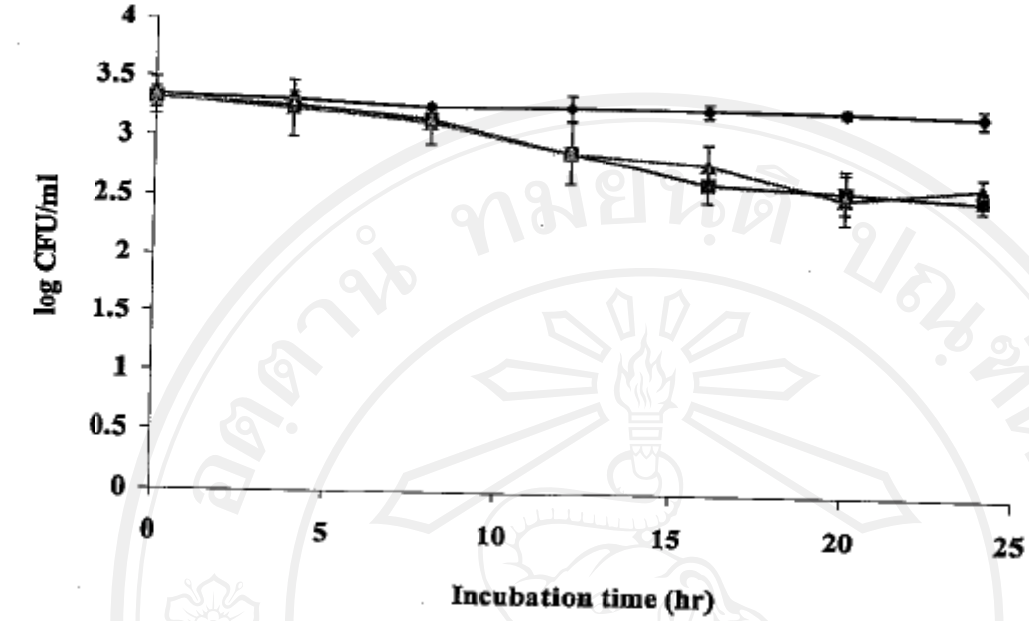
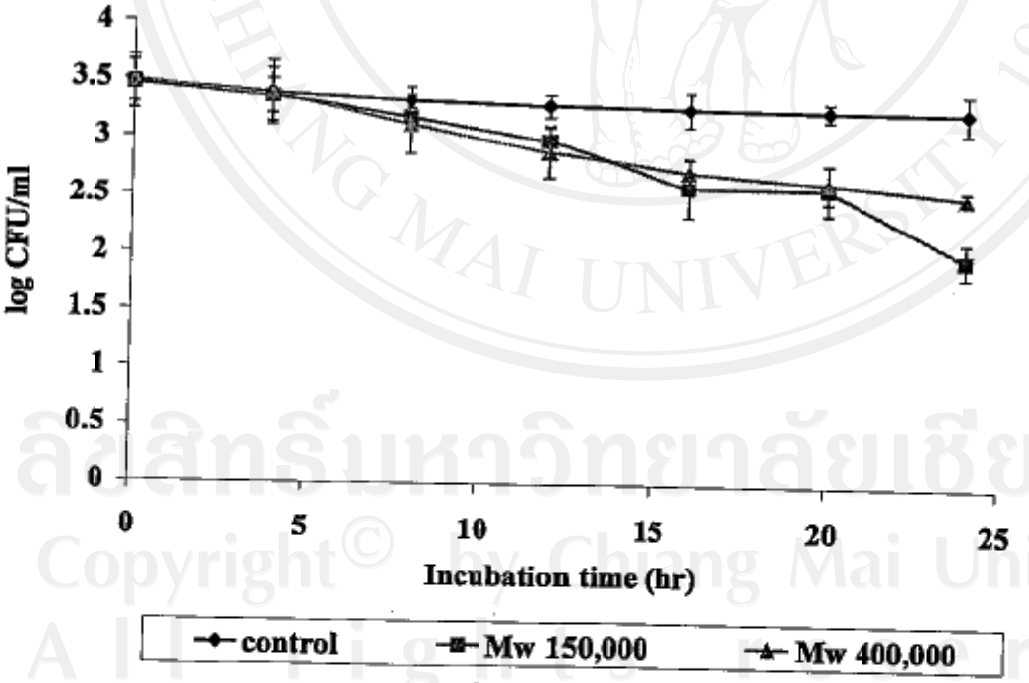


(C) pH 5.0



(D) pH 4.5

Figure 13 The effect of pH on the antibacterial activity of chitosan 0.01% (w/v), pH 6.0 against *B. cereus* (continued).

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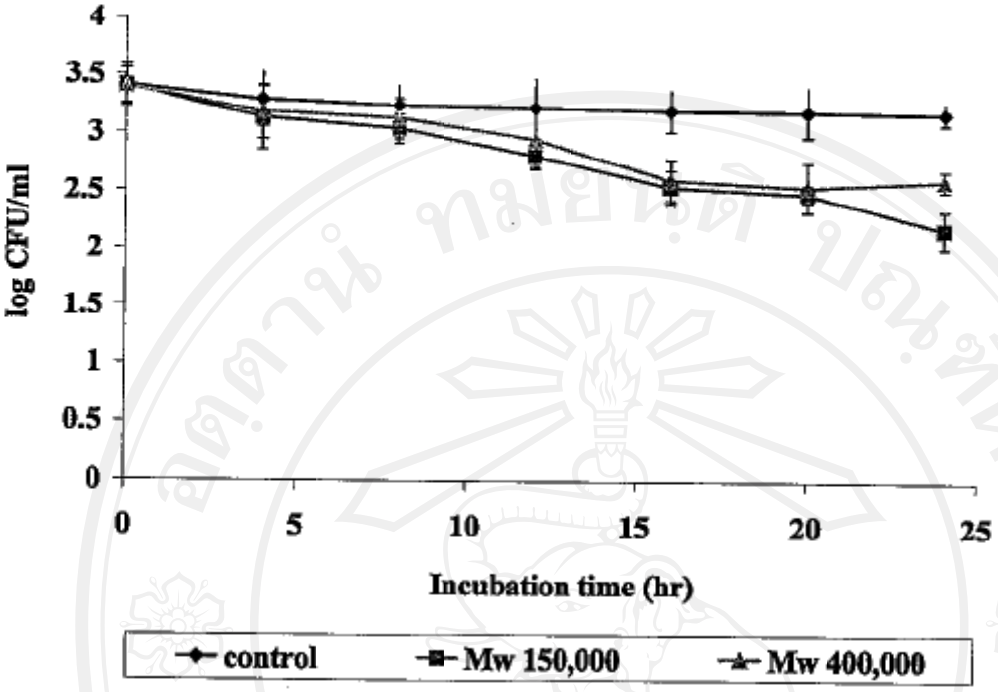

(E) pH 4.0

Figure 13 The effect of pH on the antibacterial activity of chitosan 0.01% (w/v), pH 6.0 against *B. cereus* (continued).

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4.3 The effect of temperature on the antibacterial activity of chitosan

The effect of temperature on the antibacterial activity of chitosan against *B. cereus* and *M. luteus* was observed at temperatures of 25 and 37°C. These temperatures are chosen because the bacteria could develop and grow at these temperatures. Chitosan (0.01% (w/v)) of Mw 150,000 and 400,000 Da were used in this experiment. The pH of chitosan solutions was adjusted to 6.0. The results (not shown) indicated that there is no differences in the inhibitory effect of these chitosans at these two incubation temperatures. In fact, chitosan of both types showed no inhibitory effect at all against *B. cereus* after 24 hr incubation, which is similar to the results obtained from the previous parts (section 4.1, Figure 11; section 4.2, Figure 13 (A)). A further experiment should be carried out to investigate the effect of temperatures on the antibacterial activity of chitosan by expanding the range of growth temperatures for the bacteria, before any conclusion is made.

Tasi and Su (1999) demostrated the effect of temperature on the antibacterial activity of chitosan in *E. coli*. They reported that the bactericidal activity of chitosan increased with increasing temperatures. At lower temperatures (4°C and 15°C), the number of *E. coli* declined quickly within the first 5 hr and then stabilized. They suggested that the results maybe due to changing in the reaction rate between chitosan and cells which were affected by low temperatures. The stress of low temperature may change cell surface structures in ways that decrease the number of surface binding sites or electronegativity for chitosan. More research on the effect of temperature on the cell surface structures is necessary before a conclusive explanation can be made. However, from the pH and temperature experiments, it could be suggested that the culture condition had an effect on the growth of microorganisms because the culture condition would affect the ratio of chitosan-cell interaction. The inhibition effect of chitosan was not found when the cell of microorganisms was not developed and small grown.

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4.4 The effect of chitosan on a mixed culture *in vitro*

The inhibitory effect of chitosan with a Mw of 150,000 Da on a mixed culture of *M. luteus* and *B. cereus* at a chitosan concentration of 0.01% (w/v) after 24 hr incubation was investigated. The result was shown in Figure 14. It was found that chitosan had a greater inhibitory effect to *M. luteus* than *B. cereus* in the mixed culture. Viable cells of *M. luteus* were decreased by 4.5 log CFU/ml when compared to the control. Whereas, the cells of *B. cereus* were inhibited by a slightly decrease in the numbers for up to 2 log CFU/ml in the mixed culture containing chitosan. In the control of the mixed culture, both organisms were growth and multiplied to increase their cell numbers to reach a level of 7 log CFU/ml after 24 hr incubation. From this result, it can be suggested that chitosan had a greater inhibition effect against *M. luteus* because in the single culture, the bacteria was also a more sensitive strain to chitosan at 0.01% (w/v) concentration compared to *B. cereus*. Compared with the previous result in section 4.1 (Figure 11), it was interesting to find out that the viable cells of *B. cereus* in the mixed culture was reduced lower than in the single culture when the 0.01% (w/v) chitosan solution was present (4% and 26%). There was not any simple explanation for this results. A possibility that the presence of *M. luteus* affected the viability of *B. cereus* could not be ruled out. There was a probability that extracellular compounds excrete by *M. luteus* or intracellular compounds of the bacteria that was released after the cells damaged by chitosan may become toxic compounds for the growth of *B. cereus*. A further research in this area would be useful in explaining these results and also helping to have a better understanding about interactions that occurred between different microorganisms in food.

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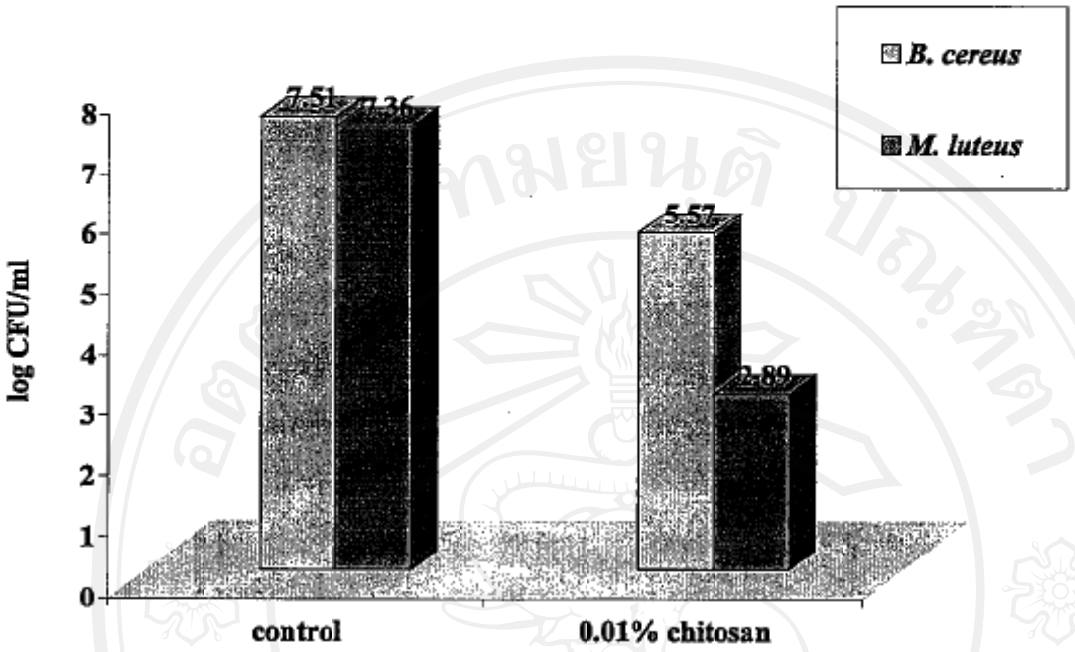

Figure 14 The inhibitory effect of chitosan 0.01% (w/v), pH 6.0 on a mixed culture of *M. luteus* and *B. cereus* after 24 hr incubation at 37°C.

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4.5 The effect of cell population on the antibacterial activity of chitosan

The antibacterial effect of chitosan with a Mw of 150,000 Da in liquid medium with different cell inoculum, which was prepared a serial dilution was observed. The bacteria cell numbers were obtained from a standard graph between OD at 660 nm versus cell counts (Appendix D). The growth of bacteria was measured as turbidity by a spectrophotometer at 660 nm because the turbidity at the optical density of 600 nm could not be used for low dilution series. The result for *M. luteus* was shown in Figure 15. In media containing chitosan without diluting the cell inoculum ($3.9x10^{10}$ cells/ml), it was shown that chitosan had no inhibitory effect. There was not any differences between the chitosan treatment and control. When an initial cell inoculum of $9.1x10^{9}$ cells/ml was added into the chitosan broth, the growth rate was delayed at the beginning of incubation. However, after 24 hr incubation, chitosan did not show any inhibitory effect. It could be explained that if the cell numbers of *M. luteus* presence in an excess number, then the presence of chitosan did not affect the microorganism because the high cell density and the rapid growth of the bacteria may overshadow the effectiveness of chitosan. However, chitosan showed its effectiveness when the cell inoculum was reduced. It was interesting that the growth of *M. luteus* was inhibited by chitosan when the inoculum was $1.5x10^{9}$ cells/ml or below. It is clear that the antibacterial action of chitosan depends on the population of microorganisms. Chitosan would have a better antibacterial effect when the number of living microorganisms does not exceed a certain level.

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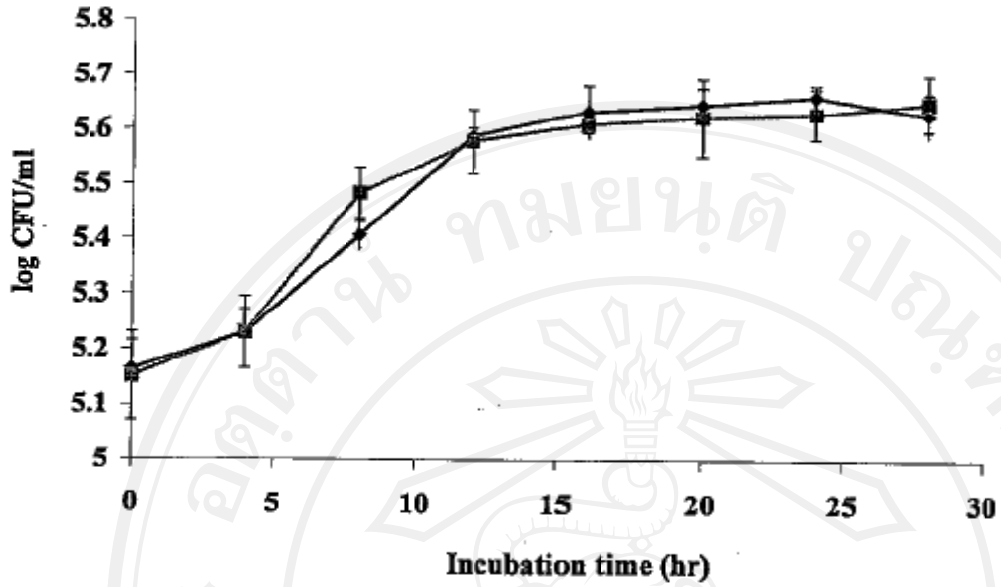
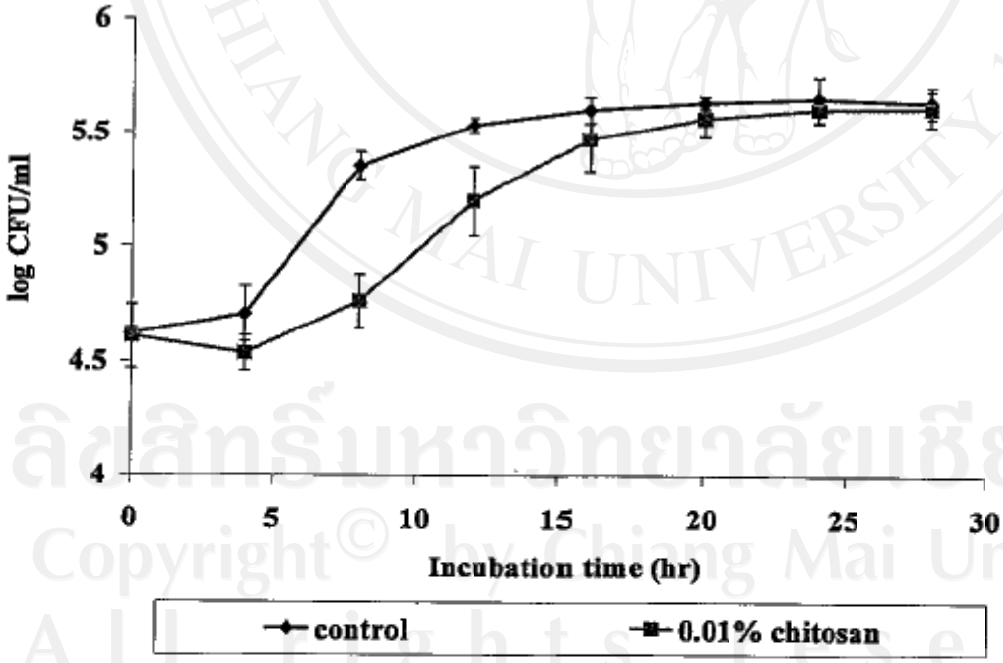


(A) cell inoculum 3.4×10^{10} cells/ml



(B) cell inoculum 9.1×10^{9} cells/ml

Figure 15 The effect of cell population on antibacterial activity of chitosan (0.01% w/v) against *M. luteus* at 37°C for 24 hr.

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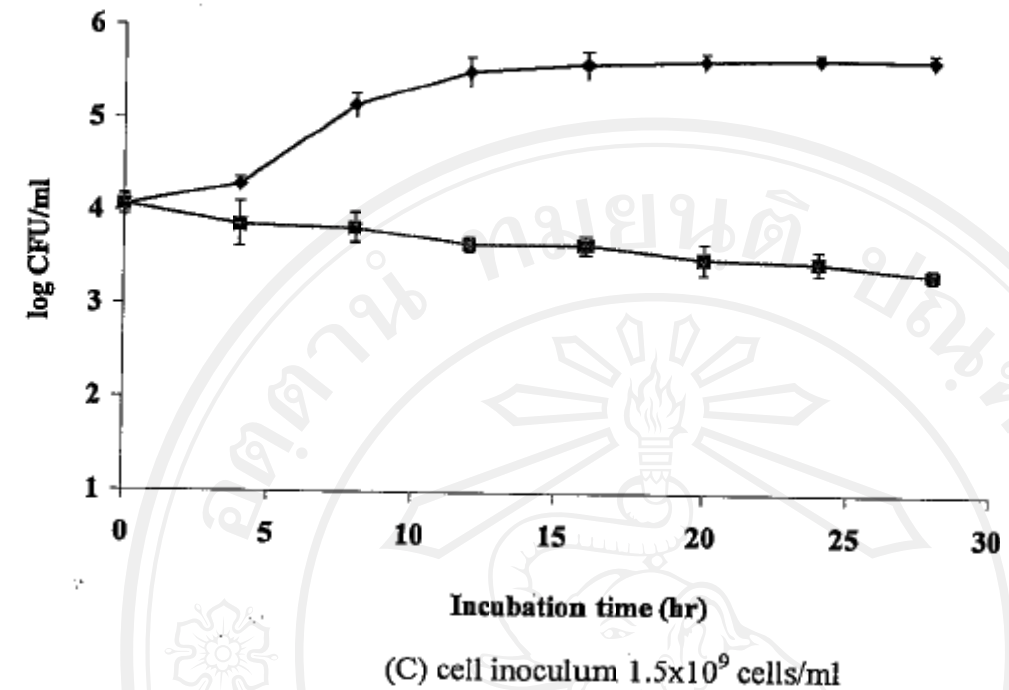
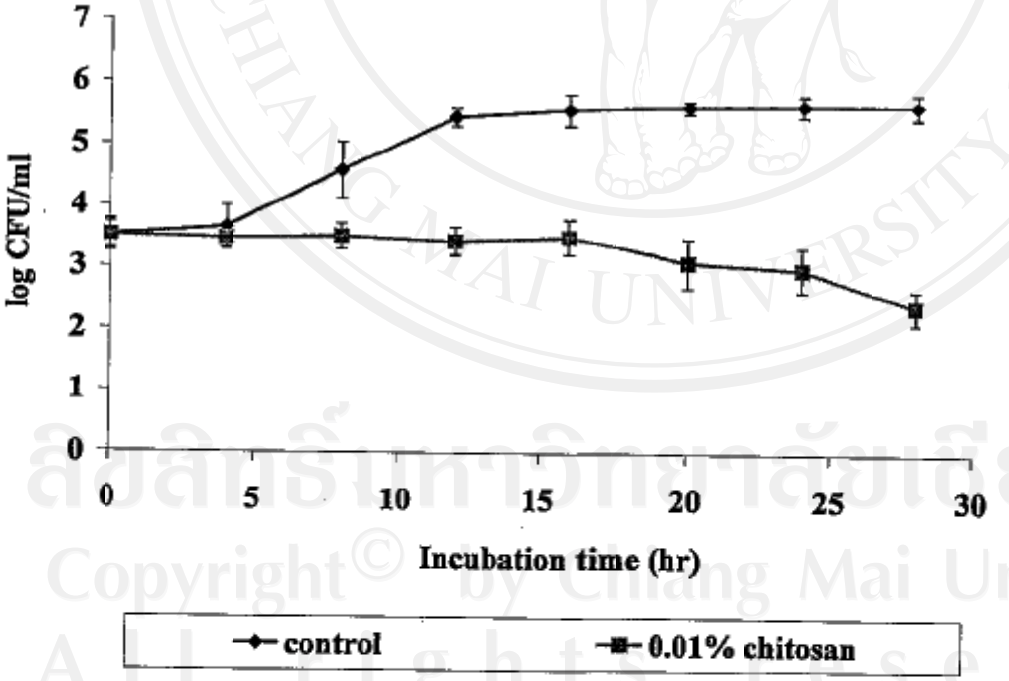


(C) cell inoculum 1.5×10^9 cells/ml



(D) cell inoculum 3.2×10^8 cells/ml

Figure 15 The effect of cell population on antibacterial activity of chitosan 0.01% (w/v) against *M. luteus* at 37°C for 24 hr (continued).

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Figure 16 showed the results of *B. cereus*, which was similar to the results of *M. luteus*. No inhibitory effect of chitosan in the culture was observed when the cell inoculum was present at a number of $3.4x10^9$ cells/ml. However, when the initial cell inoculum were reduced to $6.2x10^8$ and $1.1x10^7$ cells/ml, the growth rate of *B. cereus* in the chitosan medium was delayed at the beginning. After 24 hr incubation, no difference was observed between these cultures and the controls. It is shown that chitosan can prolong the lag phase of the cultures at these initial concentrations. If the initial cell inoculum was $3.2x10^7$ cells/ml, chitosan could inhibit the growth of *B. cereus* during 24 hr incubation. The cell numbers were decreased about 2 log CFU/ml when compared to the control. It is interesting that chitosan had an effect on the growth of microorganisms in the lag phase when the cell inoculum was lower. Similarly, it can be suggested that the antibacterial activity of chitosan depends on the inoculum size, as was found with *M. luteus*.

It can be concluded that a chitosan concentration of 0.01% w/v had an antibacterial activity only when the cell populations were not greater than $9.1x10^9$ and $6.2x10^8$ cells/ml for *M. luteus* and *B. cereus*, respectively. The actual initial cell number for different microorganisms that chitosan can have a significant antibacterial effect should be investigated in a further study.

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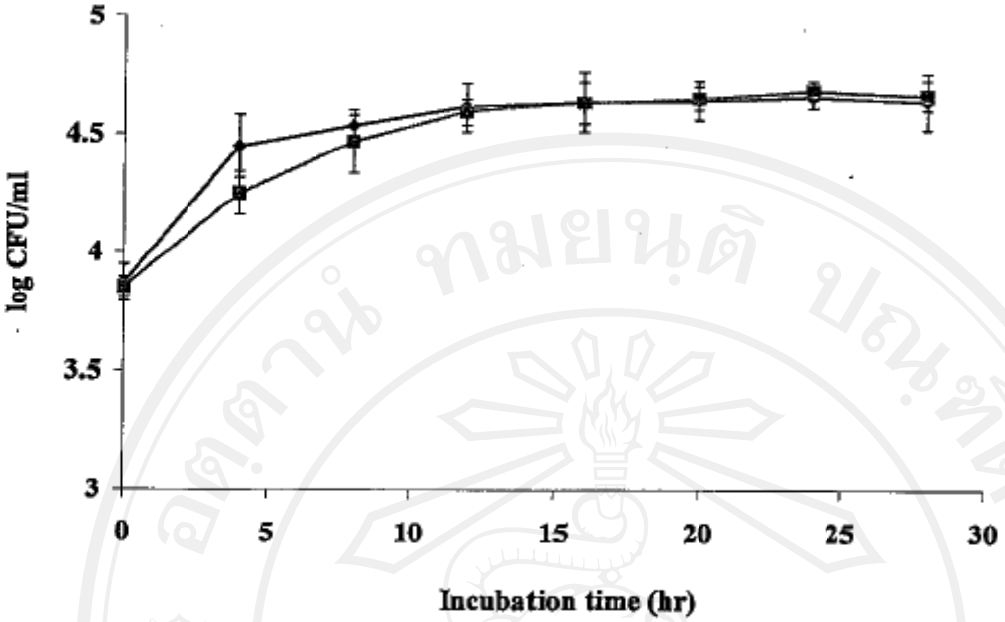
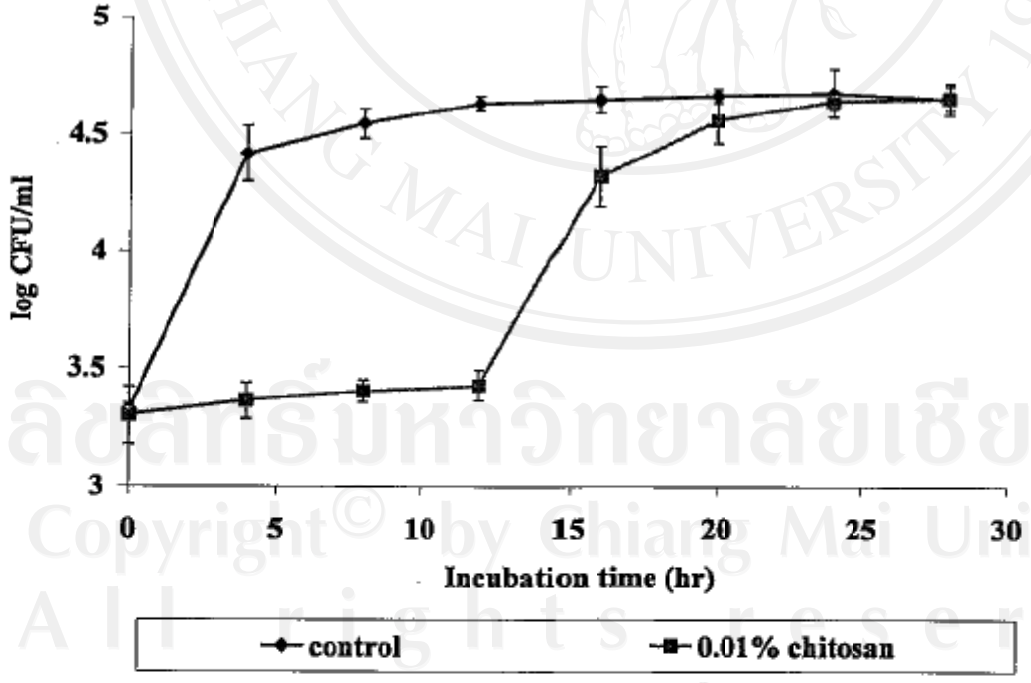


(A) cell inoculum 3.4×10^9 cells/ml



(B) cell inoculum 6.2×10^8 cells/ml

Figure 16 The effect of cell population on antibacterial activity of chitosan 0.01% (w/v) against *B. cereus* at 37°C for 24 hr.

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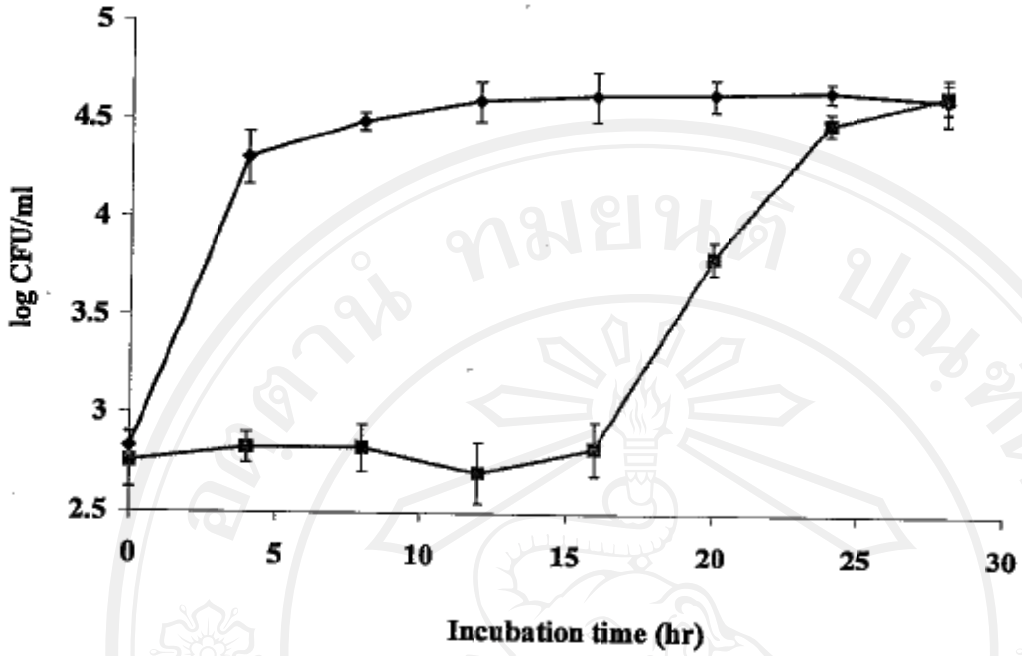
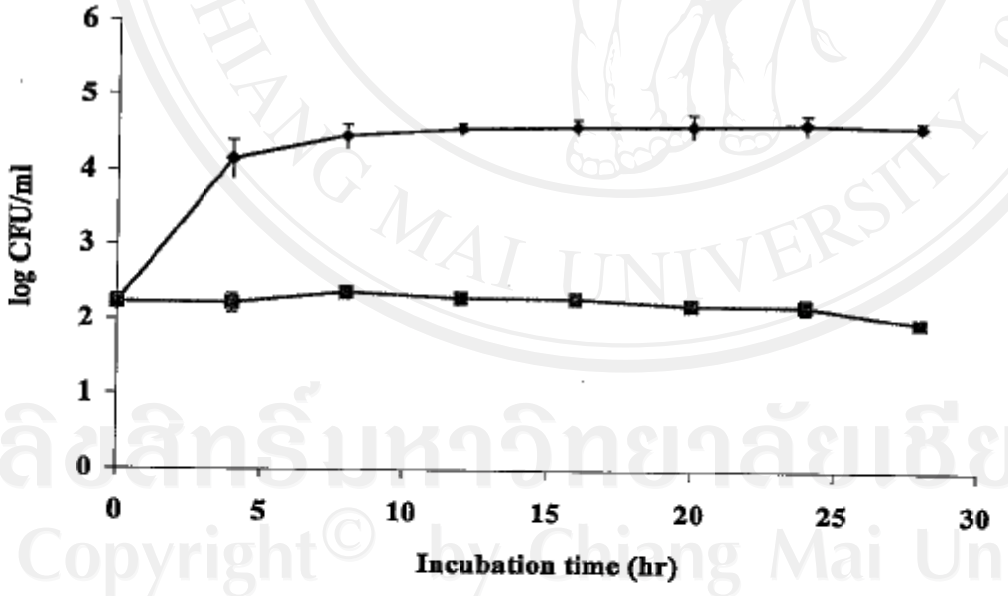


(C) cell inoculum 1.1×10^8 cells/ml



(D) cell inoculum 3.2×10^7 cells/ml

Figure 16 The effect of cell population on antibacterial activity of chitosan 0.01% (w/v) against *B. cereus* at 37°C for 24 hr (continued).

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4.6 Effect of chitosan on microbial survival and growth in minced pork

The antimicrobial activity of chitosan against certain types of bacteria in minced pork during storage at 4°C for 10 days was investigated. This product was selected as a food model because at present, meat products are produced and sold in an increasing number. It may be possible to apply a natural preservative in meat products without any addition of chemical preservatives. Figure 17 showed the effects of added chitosan with a Mw of 150,000 Da to microoganisms propagated in the minced pork. Chitosan had a different inhibition effect to reduce the overall bacteria counts (aerobic bacteria, lactic acid bacteria, *Salmonella* and *Shigella* spp. and *E. coli*) in the minced pork. The initial viable cell counts of aerobic bacteria in the control was 6 log CFU/g, and it was gradually increased to 7 log CFU/g on the 10^{th} day of storage. When the sample was immediately added with chitosans, the viable cell counts of aerobic bacteria were decreased by 0.5 to 1.0 log CFU/g and remained in the range of 5 to 6 log CFU/g throughout 10 days of storage. When comparing the antibacterial activity of two chitosan concentrations, the differences of the two chitosan concentrations, 0.025 and 0.05% (w/w) only found in the first 4 days of storage at 4°C.

In the case of lactic acid bacteria in the minced pork, the lower chitosan concentration of 0.025% (w/w) had no effect on the growth of lactic acid bacteria during storage at 4°C (Figure 17 (B)). The viable cell counts were remained about 5 log CFU/g and only had a slightly change during 10 days storage. On the other hand, the higher chitosan concentration of 0.05% (w/w) was more effective. The lactic acid bacteria were reduced by 0.5 to 1.0 log CFU/g immediately after an exposure to chitosan as compared with the control. However, the viable cell counts of lactic acid bacteria were decreased in a small extent in the presence of this chitosan concentration because the lactic acid bacteria could grow well at an acid condition. Therefore, the organism would be less affected by a reduction in the pH values, which was another factor that could increase the antibacterial activity of chitosan.

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For the results of the antibacterial effect of chitosan on *Salmonella-Shigella* spp. and *E. coli*, it was found a completely different result than the results of two previous organisms. The chitosans could effectively inhibit the growth of the two targets organism, no detected growth was observed after the 2th day of storage. For *Samonella-Shigella* spp., 0.05% (w/w) chitosan concentration showed a greater inhibition effect than at a concentration of 0.025% (w/w). Cell numbers were also different on the 2th day of storage because it could not be detected any viable cells when chitosan 0.05% (w/w) was present. While for *E. coli,* chitosan at the concentration of 0.025% (w/w) had a same inhibitory effect as with 0.05% (w/w) chitosan. This was consistent with the first study (4.1), the result of the antibacterial activity of chitosan against food spoilage bacteria *in vitro*. The result indicated that the MIC of chitosan against *E. coli* was 0.04% (w/v) and its growth was completely inhibited at chitosan concentration of 0.06%. In this study, the inhibitory effect of chitosan on the growth of some isolated food spoilage bacteria was significantly different from that of the native microorganisms present in the meat. Some bacteria were inhibited at higher chitosan concentrations compared to the result from the *in vitro* experiment. However, from these experiments, it could be suggested that chitosan had a different level of inhibition against microorganisms in food, and this may be due to the genera of microorganisms in that particular food, the initial cells that presence in food and the physicochemical properties of food.

For the calculation of the cost of chitosan to be applied in food, the cost of 1 g chitosan used as a food preservative in the minced pork is 48 baht. In this study we used a technical chitosan that had a price of 1200 baht/25 g. If a food grade chitosan are used, the lower cost of chitosan will be needed, which is 0.70 baht/g because the price of a food grade chitosan is about 700 baht/kg. However, this study showed that chitosan could be a potential compound to be used as a natural food preservative in the future.

In the recent years, several researchers tried to investigate the application of chitosan on the food surfaces by dipping, mixing or spraying. Chitosan showed to delay and decrease spoilage of prawns in a mayonnaise-based salad (Roller and

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Covill, 2000 and Oh *et al.*, 2001), pork sausage (Sagoo *et al.*, 2002), raw shrimp (Simpson *et al.*, 1997), apple juice (Roller and Covill, 1999), beef (Darmadji and Izumimoto, 1994) and oyster (Chen *et al.*, 1998).

Chitosan has recently been affirmed as Generally Recognized as Safe (GRAS) by the US FDA, thus removing some of the regulatory restrictions on its use in food (Sagoo *et al.*, 2002). Chitosan has been approved as a food additive in Korea and Japan since 1995 and 1983, respectively. Significance results have been for the use of chitosan with a high antibacterial activity as a preservative to prevent health hazards associated with consumption of food contaminated with pathogenic bacteria or to extend the shelf-life of food by inhibiting growth of spoilage bacteria (No *et al.*, 2001). However, a successful application of any novel antimicrobial agent as a food preservative depends on a number of factors. Adequate control of microbial growth in food using chitosan would require extensive additional knowledge of the factors that determine chitosan performance, including the effects of pH, temperature, specific strain, other preservatives, food components, and degree of contamination. These factors can notably influence the effectiveness chitosan as an antimicrobial agent (Roller and Covill, 2000 and Wang, 1992).

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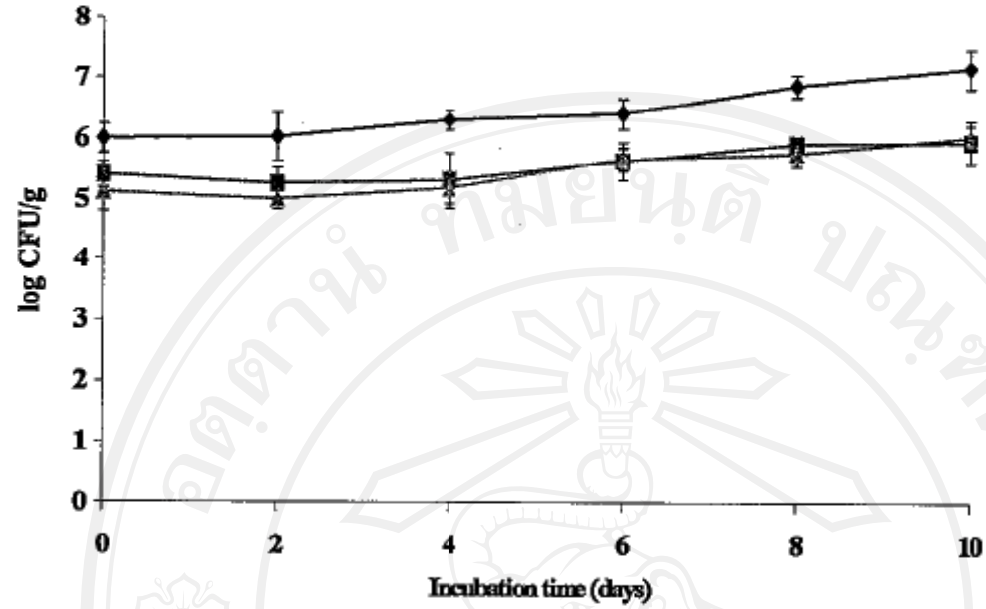
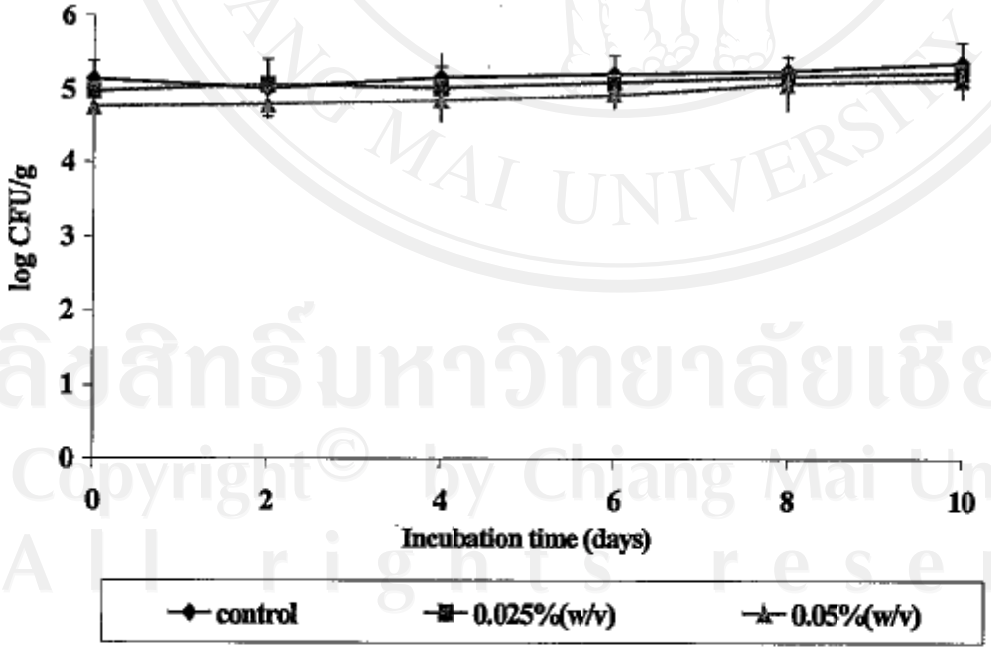


(A) Aerobic bacteria



(B) Lactic acid bacteria

Figure 17 Viable cell counts in minced porked stored at 4 °C for 10 days.

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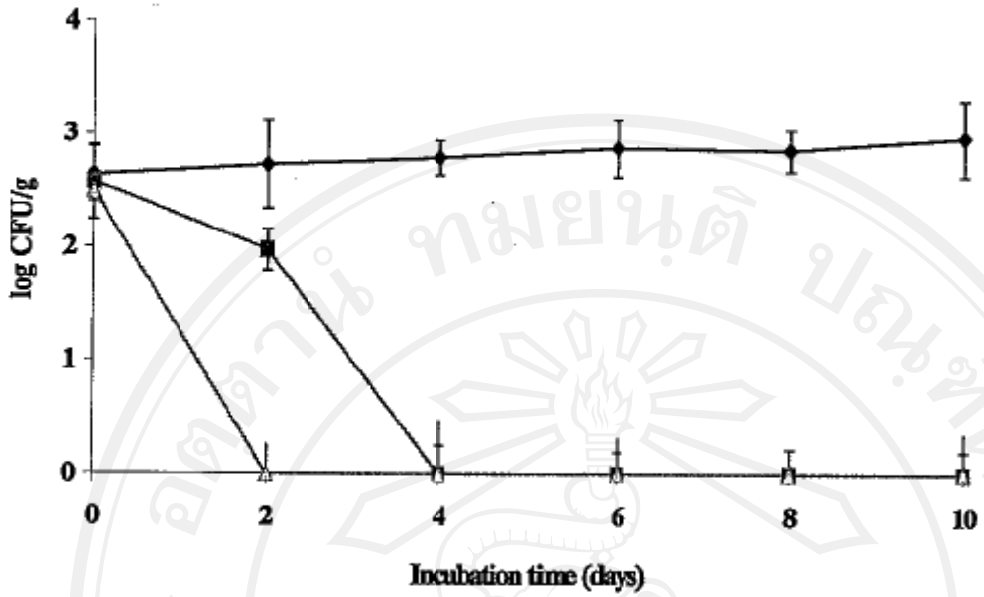
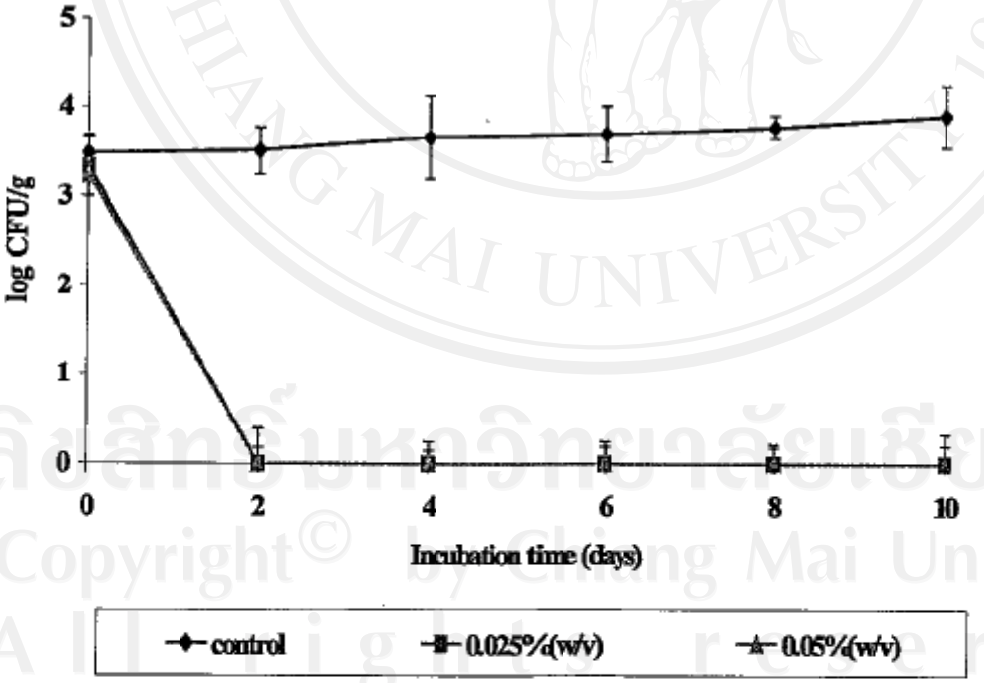


(C) *Salmonella-Shigella* spp.



(D) *E.coli*

Figure 17 Viable cell counts in minced porked stored at 4 °C for 10 days (continued).

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