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Evaluation of zeta potential difference as an indicator for antibacterial strength of low molecular weight chitosan

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ABSTRACT

A set of low molecular weight chitosan (LMWC) products with an intrinsic viscosity-molecular weight (M_V) spectrum from 6.9 kDa to 22.4 kDa, with average nanoparticle sizes from 117 nm to 965 nm, were characterized by their zeta potentials and antibacterial activities. The three strains of *Staphylococcus aureus* and two strains of *Escherichia coli* exhibited differential sensitivities to LMWCs. Both LMWC adsorption test and antibacterial test results indicated that antibacterial activity was mainly contributed by the difference in zeta potential between the surface of LMWC particles and the bacteria. Scanning electron microscopy (SEM) revealed that LMWC with an M_V of 14.8 kDa exerted its antibacterial function by both surface and permeation effects. Once the adsorbed LMWC was washed from the cell wall with an acetate buffer, obvious damages to, and disturbances on, the cell surfaces were observed through SEM.

1. Introduction

Chitosan, obtained by partial de-N-acetylation of chitin, has superior solubility and reactivity than chitin, which gives it enhanced antimicrobial, antitumor, hypolipidemic, hypocholesterolemic, and immunostimulating biofunctionalities (Kim & Rajapakse, 2005; Tharanathan & Kittur, 2003). These biofunctional advantages of chitosan are related to its molecular weight and its degree of acetylation (Tsai, Zhang, & Shieh, 2004). The antibacterial functions of chitosan and its derivatives underlie their primary utility in biological applications (Kim & Rajapakse, 2005). In recent years, growing attention has been paid to natural substances that might function as preservatives. In that respect, there has been considerable interest in the antimicrobial potential of chitosan and its derivatives as a source of safe and natural preservatives for inclusion in pharmaceuticals and food preparations (Raafat & Sahl, 2009).

Regardless of the chitosan source, its antibacterial efficacy is reported to be influenced by a number of factors, including the degree of deacetylation (DD), the degree of polymerization (Park, Kim, & Lee, 2002; Park, Lee, & Kim, 2004b; Tsai et al., 2004), and the microorganism species (Gerasimenko, Avdienko, Bannikova, Zueva, & Varlamov, 2004; Park, Je, Byun, Moon, & Kim, 2004a). The DD value can indirectly represent the protonation degree of chitosan and has a vital role in the adsorption of chitosan to bacteria (Chung et al., 2004) however, the charge density, a directly relevant factor in chitosan's antibacterial activity, has never been quantitatively measured. In the study of Jeon, Park, and Kim (2001), low molecular weight chitosan (LMWC) with a molecular weight of 5–10 kDa produced the highest bactericidal activity against pathogenic bacteria. In addition, a 20 kDa LMWC product showed a higher affinity for lipopolysaccharides than the native chitosan with a molecular weight of approximately 140 kDa (Kondo, Nakatani, Hayashi, & Ito, 2000). Some researchers (Jeon et al., 2001; Raafat, von Bargen, Haas, & Sahl, 2008) even suggested that a Mw of more than 10 kDa is required for antimicrobial activity.

With regard to the specificity of chitosan to various bacteria, it has been shown that, due to the higher negatively charged surface of cell wall, Gram-negative [G(−)] bacteria are more susceptible to chitosan than Gram-positive [G(+)] bacteria (Chung et al., 2004; Lin, Chen, & Peng, 2009a). However, this is contrary to other studies, in similar areas, that show that G(+) strains are more sensitive than G(−) strains (Raafat & Sahl, 2009).

In our previous study, a series of LMWCs with M_V values from 6 kDa to 22.2 kDa demonstrated the presence of a relationship between molecular weight and antibacterial activity (Lin, Lin, & Chen, 2009b). In this study, those same LMWCs were investigated to determine the effects of their charge densities on chitosan's antibacterial activity.

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Table 1
The zeta potentials and minimum inhibitory concentration (MIC) values for two *E. coli* strains and three *S. aureus* strains against 8 different intrinsic viscosity-molecular weight (M_V) of low molecular weight chitosan (LMWC) products prepared in an enzymatic process using chitinase, cellulase, and lysozyme.

Applied Enzyme	M_V (kDa)	Bacteria strains :	*E. coli* (BCRC 10314) −13.7 mV[a]	*E. coli* (BCRC 10675) −7.84 mV[a]	*S. aureus* (BCRC 10451) −5.99 mV[a]	*S. aureus* (BCRC 10780) −4.26 mV[a]	*S. aureus* (BCRC 10781) −5.47 mV[a]	Average LMWC m) particle diameter (n
Lysozyme	22.2	81.4	50[a] ± 0.0[a]	70 ± 13.7[a]	800 ± 0.0[a]	NI[b]	880 ± 178.9[a]	965 ± 67.2
	14.8	91.0	50 ± 0.0[a]	100 ± 0.0[b]	400 ± 0.0[b]	800 ± 0.0[a]	720 ± 178.9[a]	601 ± 102.2
	13.9	89.5	70 ± 13.7[b]	120 ± 22.4[c]	880 ± 178.9[a]	1040 ± 219.1[b]	880 ± 178.9[a]	502 ± 137.9
Chitinase	13.5	91.7	50 ± 0.0[a]	50 ± 0.0[d]	480 ± 178.9[b]	1600 ± 0.0[c]	800 ± 0.0[a]	615 ± 119.9
	11.2	87.1	60 ± 11.2[ab]	80 ± 13.7[a]	1120 ± 219.1[c]	NI	1520 ± 178.9[b]	512 ± 10.3
	9.6	74.7	70 ± 13.7[b]	100 ± 0.0[b]	880 ± 178.9[a]	NI	NI	311 ± 77.9
	8.3	65.4	100 ± 0.0[c]	140 ± 27.4[c]	1600 ± 0.0[d]	NI	NI	152 ± 26.2
Cellulase	6.9	27.4	120 ± 22.4[d]	180 ± 22.4[d]	NI	NI	NI	117 ± 5.1

MIC (μg mL^{-1}). Values presented are means ± standard deviations. Means in the same column followed by different letters are significantly different ($P < 0.05$). NI indicates no inhibition, i.e., a MIC > 1600 μg mL^{-1}.
[a] Zeta potential (mV).

2. Materials and methods

2.1. Materials

N-acetyl-D-glucosamine, tri-N-acetylchitotriose, and hexa-N-acetyl-chitohexaose were purchased from Sigma (St. Louis, MO, USA). Lysozyme (EC 3.2.1.17) and cellulase (EC 3.2.1.4, produced by *Trichoderma reesei* ATCC 26921) was purchased from Sigma C2730. A crude chitinase enzyme was prepared by inducing *Trichoderma harzianum* (BCRC 30821) into a chitin-containing medium. This research also made use of Difco culture media (Becton Dickinson, Franklin Lakes, NJ, USA). Chemicals and solvents were all analytical grade and purchased from Merck (Whitehouse Station, NJ, USA). Chitin was purchased from Sigma and chitosans with DDs of 92 and 80 determined by the Fourier Transform Infrared (FTIR) technique were purchased from a local company (Ying Huwa Co., Kaohsiung, Taiwan).

2.2. Microorganisms

Microorganisms used in this study were all purchased from the Bioresource Collection and Research Centre of the Food Industry Research and Development Institute (FIRDI; Hsinchu, Taiwan). The five strains of bacteria used for testing the antibacterial activity of LMWC included three strains of *S. aureus* (BCRC 10451, 10780, and 10781) and two strains of *E. coli* (BCRC 10314 and 10675). *T. harzianum* (BCRC 30821) was used to prepare crude chitinase. *E. coli* was cultured in nutrient broth medium at 37 °C, and *S. aureus* was cultured in trypticase soy broth medium at 37 °C.

2.3. Preparation of crude chitinase enzyme complex and chitinolytic activity assay

Chitinase was prepared according to Felse and Panda (2000). The seed culture medium contained (in g L^{-1}): dextrose, 10; $(NH_4)_2{\cdot}SO_4$, 4.2; NaH_2PO_4, 6.9; KH_2PO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.3; peptone, 1.0; citric acid monohydrate, 10.5; and urea, 0.3. The medium was inoculated with *T. harzianum* spores (approximately 5×10^5 spores per mL) from a 120 h slant. The chitinase induction medium had the following composition (in g L^{-1}): colloidal chitin, 10; $(NH_4)_2{\cdot}SO_4$, 4.2; NaH_2PO_4, 6.9; KH_2PO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.3; Tween 80, 0.2; $FeSO_4{\cdot}7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4$, 0.0014; $CaCl_2{\cdot}2H_2O$, 0.002; pH 5.0. The induction medium was inoculated with 5% (v/v) 48 h seed culture and cultured for 5 d at 30 °C to obtain an optimal chitinase yield.

The chitinolytic activities of enzymes used in this study were assessed by measuring enzyme-released reducing sugar in the hydrolyzing reaction through the dinitrosalicylic acid (DNS) method (Ilyina, Tikhonov, & Varlamov, 2000). An enzymatic hydrolyzing reaction was performed by mixing 1% colloidal chitin solution (pH 4.0) with properly diluted enzyme solutions and reacting in a 42 °C water bath for 1.0 h. Enzymes were then inactivated by heating at 100 °C for 10 min. Supernatant of the reaction mixture, obtained after centrifugation at 10,000 × *g* for 10 min, was mixed with a 2-fold volume of DNS reagent and boiled for 15 min followed by a quick cool down in water. The result was read at OD_{590} using a Biomate-3 spectrophotometer (Thermo Spectronic, Rochester, NY, USA). One unit of enzyme activity represented 1 μmol equiv. of NAG produced per hour.

2.4. Preparation of enzymatic chitosan hydrolysates

A set of LMWC products, comprising an M_V spectrum from 6 kDa to 22.2 kDa was prepared according to Lin et al. (2009b). Two hundred milliliters of 2.2% chitosan, dissolved in 100 mM sodium acetate buffer of pH 4.0, was mixed with 20 mL crude chitinase, cellulase, or lysozyme at 100 U mL^{-1}. This mixture was then incubated at 42 °C. An aliquot (10 mL) of the mixture was withdrawn at the appropriate time and then boiled for 10 min to stop the enzymatic reaction.

Table 2
Pearson correlation analysis results between log M_V and difference of zeta potential (D_{ZP}) of low molecular weight chitosan (LMWC), prepared from chitinase and lysozyme, and the resultant minimum inhibitory concentrations (MIC).

	MIC		
	E. coli BCRC10314	*E. coli* BCR10675	*S. aureus* BCR10451
Log M_V	$r_p^2 = -0.70$	$r_p^2 = -0.63$	$r_p^2 = -0.45$
D_{ZP}[a]	$r_p^2 = 0.83$	$r_p^2 = -0.69$	$r_p^2 = -0.63$

r_p^2, Pearson correlation coefficient ($P < 0.001$).
[a] Difference of zeta potential between LMWC and respective bacterial strain.

2.5. The assay of degree of LMWC polymerization

Chitosan hydrolysates were sampled along the reaction time and measured for viscosity. The M_V of the LMWC was determined based on its intrinsic viscosity, obtained in 0.25 M HAc/0.25 M NaAc at 25 °C, according to the equation of Mark–Houwink–Sakurada (MHS) (Kasaai, Arul, & Charlet, 2000) stated as: $[\eta] = 1.49 \times 10^{-4} M_V^{0.79}$. An LMWC with an M_V of 14.8 kDa was designated as LMWC_148.

2.6. Minimal inhibition concentration (MIC) test

One hundred microliters of each 16 h old $\sim 5 \times 10^6$ CFU mL^{-1} tested microorganism was mixed with 100 μL sterilized chitosan hydrolysate samples, which were 2-fold serially diluted in advance with a corresponding medium buffered with 100 mM phosphate buffer (pH 7.0) to the designated concentration. A corresponding mixture containing no bacteria was used as a control. The mixture was then applied to each well of a 96-well microplate and incubated for 48 h at a temperature suited for each microorganism. Growth of the tested microorganism was monitored at OD_{590} every 6 h for 48 h by an ELISA reader (Biolog Inc., Hayward, CA, USA). All treatments were conducted five times. MIC was ultimately defined as the lowest concentration of the sample required to inhibit bacterial growth for over 48 h beyond that of the control sample.

2.7. Zeta potential and particle size measurements

Particle size and zeta potential were measured using a Zetasizer Nano-ZS-90 (Malvern Instruments, Malvern, UK). The analysis was

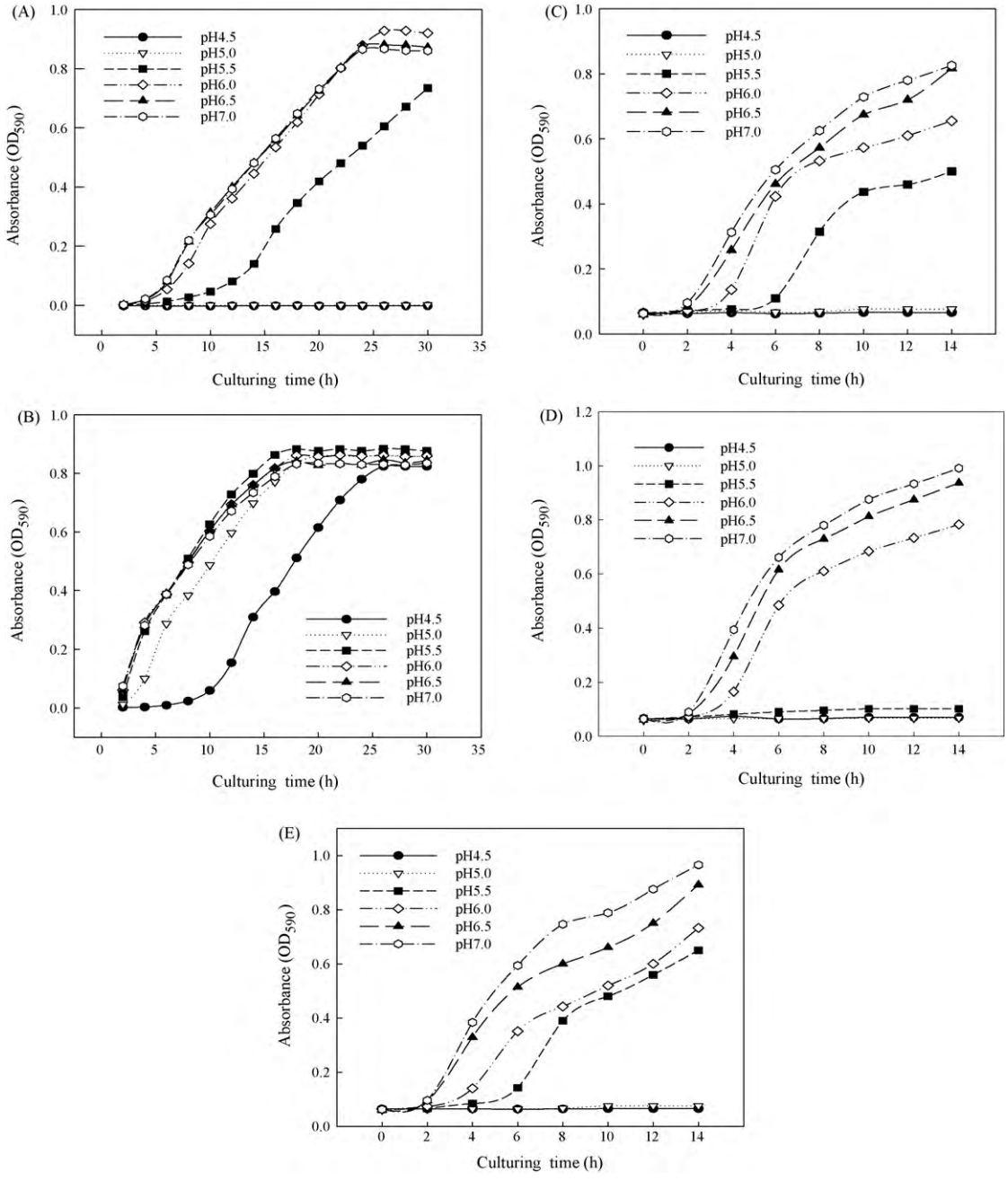

Fig. 1. Growth curves of two *E. coli* strains, BCRC 10314 (A), and BCRC 10675 (B), and three *S. aureus* strains, BCRC 10451 (C), BCRC 10780 (D), and BCRC 10781 (E) under different culturing medium pH conditions.

performed at a scattering angle of 90° at 25 °C. For zeta potential measurements, LMWC samples were dispersed in 100 mM sodium acetate buffer and bacteria samples ($\sim 1 \times 10^7$ cells mL^{-1}) were suspended in 0.1% peptone solution and measured under the automatic mode. The polydispersity index (PDI) describes the homogeneity of the samples. Low values indicate homogenous vesicles, PDI values larger than 0.5 indicate a higher heterogeneity.

2.8. Bacterial growth curve

Growth curves of the bacteria strains cultured under the interfering factors, pH and LMWC, were recorded by an Advantec TVS062CA Bio-photorecorder (Advantec Toyo Co. Ltd., Tokyo, Japan). To perform this test, a 5 mL medium in a 1 L form tube was inoculated with 20 μL of 16 h bacterial culture. To determine the pH effect on the five tested bacterial growth, a series of media, adjusted with acetated buffer (100 mM) to different pH levels from 4.5 to 7.0, was used. In addition, to reveal the bacterial susceptibility to LMWC, tested bacteria were cultivated with various concentrations of LMWC_148 at pH 6.5.

2.9. Flocculation test

The flocculation assay was performed according to Strand, Varum, and Ostgaard (2003) with slight modification. To 9 mL of a 16 h culture of bacterial suspension ($\sim 5 \times 10^9$ CFU mL^{-1}), 1.0 mL of LMWC_148 solution (in 100 mM acetate buffer, pH 7.0) was added under stirring on a vortex mixer (2000 rpm, 5 s) to final concentrations of 0 μg mL^{-1}, 200 μg mL^{-1}, 400 μg mL^{-1}, 800 μg mL^{-1}. The tubes were incubated on a rotary shaker at 200 rpm for 1.0 h and then allowed to stand for 30 min at room temperature. Subsequently, 0.1 mL of supernatant sample was pipetted from the middle of the tube and spread on the suitable medium agar plate. The bacterial count was measured after 24 h of culture.

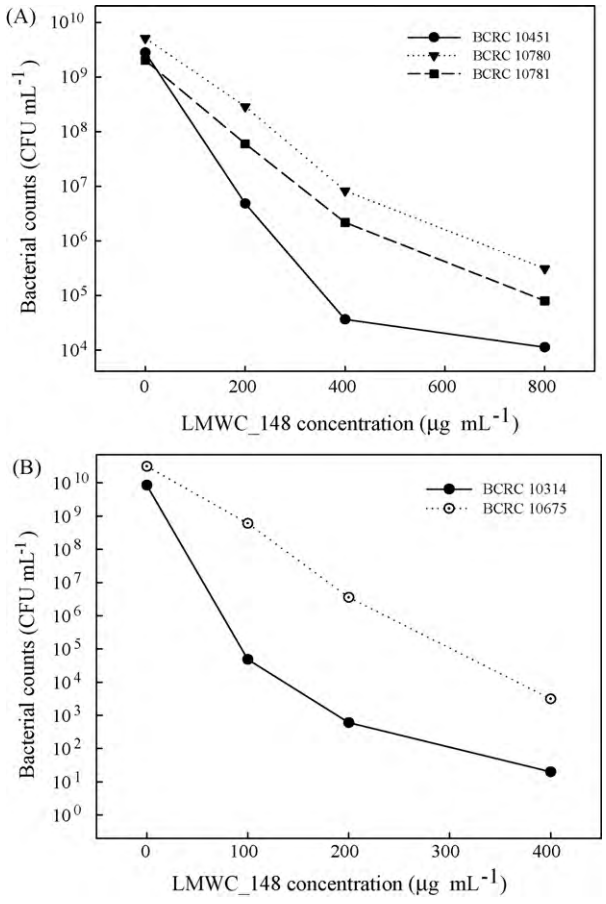

Fig. 2. Bacterial residue levels in the supernatant of flocculation tests of three strains of *S. aureus* (A) and two strains of *E. coli* (B) after treatment with increasing concentrations of LMWC_148.

2.10. Morphology

A 16 h bacterial culture of *S. aureus* and *E. coli* was treated with 800 μg mL^{-1} and 100 μg mL^{-1} of LMWC_148, respectively, as mentioned in flocculation test section. The precipitated part was collected and washed once with 20 mM sodium acetate buffer by centrifugation at 3000 × *g* for 5 min to remove the LMWC coating on bacterial surface. The bacterial samples were fixed in a glutaraldehyde/paraformaldehye solution and dried stepwise with 50–100% ethanol solution. The dried samples, mounted on aluminum studs and coated with gold palladium under vacuum conditions, were then examined with scanning electron microscopy (SEM, Vega TS5136MM, Tescan, Brno, Czech Republic).

2.11. Statistical analysis

Mean values and standard deviations were calculated using the data from five tests per sample. Results were compared by the least significant difference test and with Pearson correlation analysis using SAS Version 8.01 (SAS Institute, Cary, NC, USA).

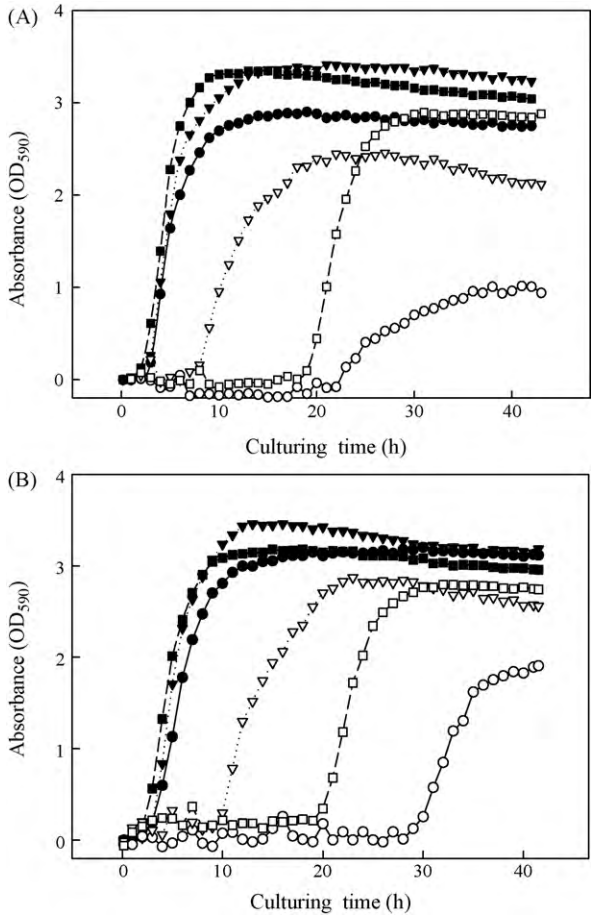

Fig. 3. Growth curves of three strains of *S. aureus* grown at 400 μg mL^{-1} (A) and 800 μg mL^{-1} (B) of LMWC_148. Solid and open symbols represent control and LMWC-treated groups, respectively. Circles indicate strain BCRC 10451; triangles, BCRC 10780; rectangles, BCRC 10781.

3. Results and discussion

The cationic nature of chitosan has been suggested the key factor contributing to its interaction with the negatively charged microbial cell surface and producing an impairment of bacterial activity (Chung et al., 2004; Raafat et al., 2008). Due to a lack of proper tools, total charge density has not been previously available for both chitosan and bacteria, however, the net surface charge amounts can now be assessed on the basis of their zeta potential (Saito, Takatsuka, Kato, Ishihara, & Okuda, 1997). Table 1 shows that the LMWC zeta potentials decreased as M_V decreased, as did their antibacterial activity as indicated by the changes in MIC values. When we reviewed the zeta potentials of all bacteria strains tested in this study, it was found that the zeta potential difference (D_{ZP}) between the LMWC and the bacteria was an indicator of the strength of the LMWC's antibacterial activity. The sensitivity of the tested bacterial strains to LMWC increased with their zeta potential in the following order: *E. coli* (BCRC 10314, −13.7 mV) > *E. coli* (BCRC 10675, −7.84 mV) > *S. aureus* (BCRC 10451, −5.99 mV) > *S. aureus* (BCRC 10781, −5.47 mV) > *S. aureus* (BCRC 10780, −4.26 mV). In addition, correlation analysis results presented in Table 2 demonstrated a higher significant correlation coefficient between D_{ZP} and MIC in strains BCRC 10314, BCRC 10675, and BCRC 10451 when compared to the correlation coefficients between log M_V and MIC. Even though M_V-related effects were not negligible, the D_{ZP} between LMWC and bacteria had the predominant relationship with the antibacterial activity of LMWC.

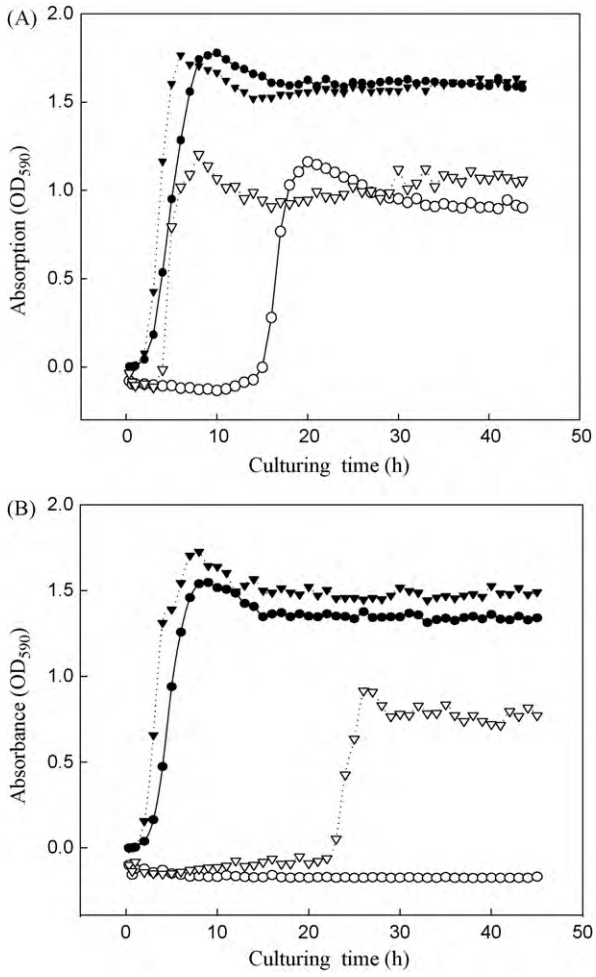

Fig. 4. Growth curves of two strains of *E. coli* grown at 100 μg mL^{-1} (A) and 200 μg mL^{-1} (B) of LMWC_148. Solid and open symbols represent control and LMWC-treated groups, respectively. Circles indicate strain BCRC 10314; triangles, BCRC 10675.

Besides, according to Table 1, the average diameters of LMWCs, as determined by dynamic light scattering, fell into the nanoparticle size range with PDI between 0.25 and 0.33 (data not shown) and were related to their corresponding M_V. The roles of chitosan nanoparticles in the antibacterial activity (Du, Niu, Xu, Xu, & Fan, 2009; Qi, Xu, Jiang, Hu, & Zou, 2004) have been investigated. It was previously suggested that chitosan nanoparticles produce excellent inhibitory effects on microorganisms were attributable to the enhanced zeta potential (Qi et al., 2004). This hypothesis was partially supported by our results, in which LMWCs with better antibacterial activity had M_V of 11.2–22.2 kDa and particle sizes of 502–965 nm. The antibacterial activity of the two LMWCs with particle sizes of 311 nm or less and M_V of 9.6 kDa or less was low and the MIC values increased with particle size. Thus, it appears that smaller nanoparticle sizes do not maintain the same level of antibacterial efficacy of larger LMWCs.

Table 1 also shows that both of the *E. coli* strains were much more sensitive than *S. aureus* to LMWC. This result supports the observations in the study of Chung et al. (2004). However, this is opposite to other studies (Jeon et al., 2001; No, Park, Lee, & Meyers, 2002). We suggest pH effect is the possible reason for such conflicting observations. The native form of chitosan can only be steadily dissolved at a pH of 6, a pH level that can interfere with bacterial growth by altering the electronegativity of the cell surface and should be avoided in antibacterial activity assays. However, chitosan does not exhibit antimicrobial activity at pH 7.0, probably due to deprotonation of amine groups, as well as poor solubility in water at this pH (Liu, Guan, Yang, Li, & Yao, 2001). For this purpose, the pH tolerance of the bacterial strains used in this study was analyzed before evaluating chitosan's antibacterial activity. Fig. 1(A) and (B) shows that growths of both *E. coli* strains were not affected at pH 6–7; however, the growth of BCRC10314 was affected at and below pH 5.5, whereas BCRC10675 was not noticeably affected until pH 4.5. According to Fig. 1(C)–(E), as the pH decreased below pH 6.5, growths in all three *S. aureus* strains markedly slowed, but especially in strains BCRC 10451 and BCRC 10781, both of which also show greater sensitivity to LMWC than BCRC 10780 (Table 1). These pH results clearly point out that the antibacterial assays must be performed under suitable culture conditions. All antibacterial assays in this study were performed at pH 7, a level at which the LMWCs were completely soluble and at which growth in the tested bacteria was not affected. Despite *S. aureus* strains being more sensitive to lower pH conditions, those strains were still more resistant to LMWC than *E. coli* strains at pH 7.

Many researchers have suggested that chitosan performs its antibacterial action through adsorption of bacterial cell (Raafat et al., 2008; Vishu Kumar, Varadaraj, Gowda, & Tharanathan, 2005). In order to determine if an LMWC exert its antibacterial action in the same way, adsorption tests were carried out. Fig. 2 shows that all strains' count were markedly reduced by the addition of LMWC_148, especially, *E. coli* BCRC 10314 (8 log reduction), 10675 (5 log) and *S. aureus* BCRC 10451 (5 log), and were the most vulnerable strains to LMWC_148 addition. The adsorption of LMWC may temporarily inhibit bacterial growth, which can then be reversed over time (Raafat et al., 2008; Rhoades & Roller, 2000). To determine if the inhibition of bacteria growth is temporary or permanent in the presence of LMWC, a biorecorder device, by which optical density indications of bacterial growth can be recorded under shaking conditions, was used to monitor the growth of LMWC-treated bacteria. Fig. 3 shows the growth curves of the three *S. aureus* strains in the presence of LMWC_148. All of the LMWC-treated strains, but especially the most vulnerable strain BCRC 10451, demonstrated a longer lag phase before growth resumption. The figure also shows that the lag period was extended when LMWC concentration was

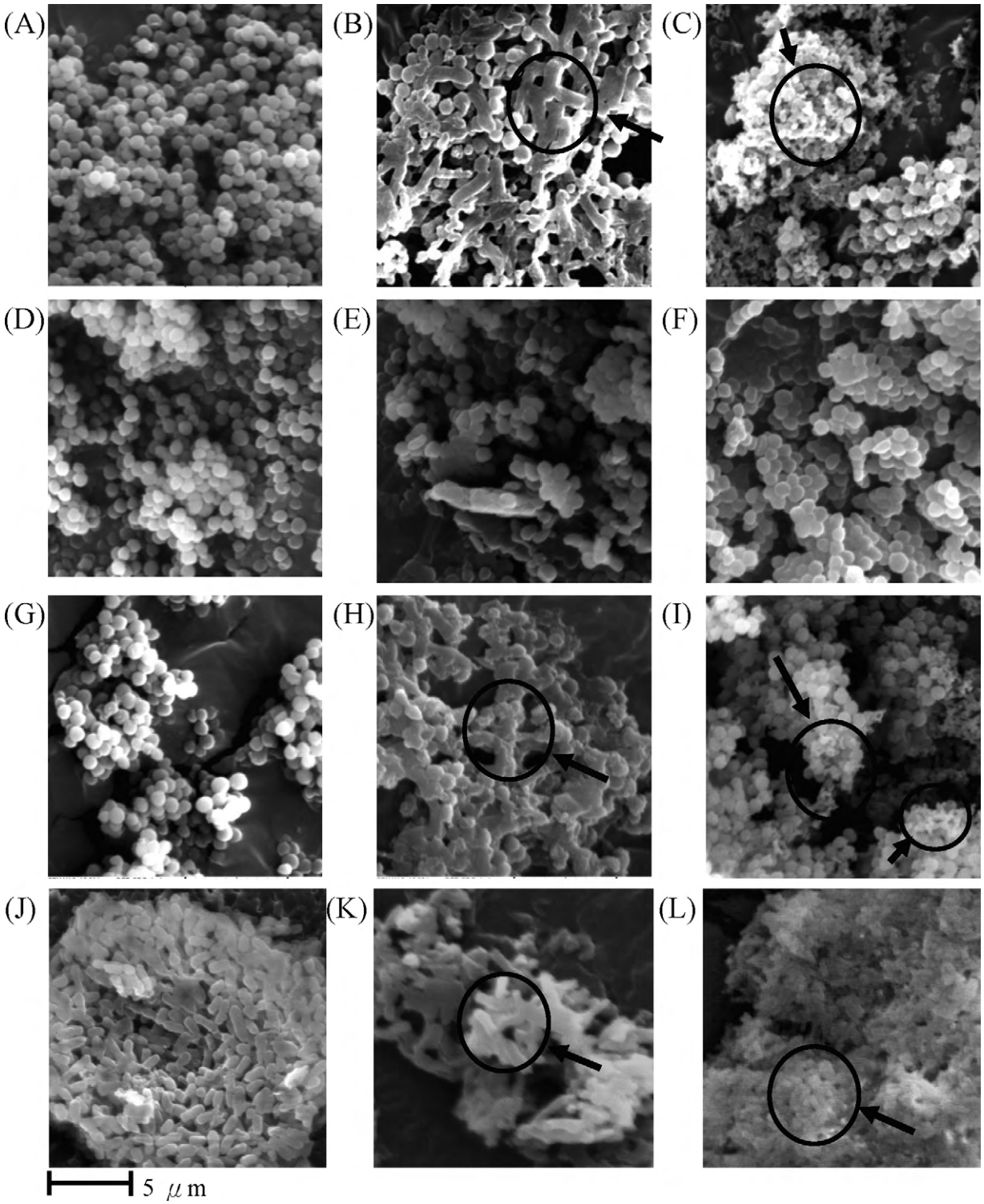

Fig. 5. Scanning electron microscope photographs of three *S. aureus* strains, BCRC 10451, BCRC 10780, and BCRC 10781 (A, D, G, respectively) and an *E. coli* strain, BCRC 10314 (J), treated with LMWC_148 (800 μg mL^{-1} for *S. aureus* strains and 100 μg mL^{-1} for *E. coli* strain) for 1 h, then precipitated (B, E, H, K, respectively) and washed with 20 mM acetate buffer, pH 6.0 (C, F, I, L, respectively). The circles in (B), (H), and (K) indicate LMWC coating; the circles in (C), (I), and (L) indicate damaged cell surfaces.

increased. Furthermore, in contrast to the other two strains, growth in BCRC 10451 did not recover from the stationary phase to the control level (Fig. 3). Similar results were obtained for the two LMWC-treated *E. coli* strains (Fig. 4), but both strains did not recover and return to the control level; in particular, growth did not occur in BCRC 10314. This cessation or slowing of growth is assumed to result from permanent damage to the cells caused by LMWC treatment. Two most acceptable hypotheses for the antibacterial activity of LMWC concluded by several researchers (Strand et al., 2003; Vishu Kumar et al., 2005) include a surface effect, through which chitosan inhibits bacteria by adsorption to the cell surface, and a permeation effect, by which chitosan disturbs membrane integrity by insertion into the cell membrane. To closely discern the possible effects of chitosan on cell surface, SEM was performed

to observe morphology changes in LMWC-treated strains. *S. aureus* BCRC 10451 was almost completely coated with LMWC (Fig. 5A), while BCRC 10781 was less coated (Fig. 5D), and BCRC 10780 was least coated (Fig. 5G). These results paralleled their respective susceptibility to LMWC. Although those images support the suggestion that bacteria growth inhibition by LMWC may arise from the surface effect, we investigated whether the permeation effect had a role by using a weak acetate buffer to remove the LMWC coating on the cell' surface. Acetate buffer treatment noticeably removed the LMWC coated on BCRC 10781 (Fig. 5F) and BCRC 10451 (Fig. 5C); however, the latter demonstrated badly damaged cell surfaces, which may partially be explained by permeation effect. In addition, the cell surfaces in the two less LMWC-sensitive strains were not markedly affected. In the two LMWC-sensitive *E. coli* strains (Fig. 5J–L, here only BCRC 10314 was shown), the cell surface was heavily damaged after acetate buffer treatment. These results suggest that the interaction effect of LMWC with cell surface polymers is proportional to the corresponding D_{ZP} and may interfere with dynamic processes within the cytoplasmic membrane, alter cell integrity.

4. Conclusion

The results reported here demonstrated that the antibacterial activity strength of LMWC is mainly decided by D_{ZP} between LMWC molecules and cell surface. The nanoparticle nature of LMWC, with proper molecular size, may enhance their antibacterial activity. The pH effect on the tested bacterial strains must be taken into consideration in determining antibacterial activity of LMWC. The inhibition of bacterial growth by LMWC may involve surface effect and, to some extent, permeation effect. We suggest that the set of LMWCs used in this study may be ideal for use in further exploration of bactericidal mechanisms inside and on the cell.

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