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Application of bacteriophage-borne enzyme combined with chlorine dioxide on controlling bacterial biofilm

Zihan Chai, Jing Wang, Suyuan Tao, Haijin Mou*

College of Food Sciences and Engineering, Ocean University of China, Qingdao 266003, PR China

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ABSTRACT

Elimination of bacterial biofilm (BF) in a food processing environment is a difficult process because BFs show great resistance against common disinfectants. In this study, a heat-stable polysaccharide depolymerase that can effectively degrade bacterial exopolysaccharide was prepared from the phage infecting *Klebsiella*. Treatment at 75 °C for 10 min could inactivate the phage entirely, with the titration decreasing from 5.6×10^8 PFU/ml to zero. However, loss of phage enzyme activity was not detected post-treatment. The plate counting showed the phage enzyme could make a rapid decrease in the amount of BF bacteria. The elimination rate approached the maximum (80%) after 4 h of treatment. Enzyme pretreatment could also increase the disinfection effect of chlorine dioxide. Approximately 92% of the BF bacteria were eliminated after treatment with the phage enzyme followed by 30 min of treatment with chlorine dioxide. According to the results of colonies counting and scanning electron microscopy, the phage enzyme could effectively reduce the attachment of bacteria as well as the adhesion of extracellular polymeric substances in the BF. This study has demonstrated that the phage-borne polysaccharide depolymerase enzyme are valuable for eradicating the bacterial BF.

1. Introduction

Microorganisms usually have a natural propensity to attach to wet surfaces, multiply, and embed themselves in glycocalyces predominantly composed of microbially produced exopolysaccharides (EPSs), in addition to proteins, nucleic acids, and presumably lipids, mineral ions, as well as various cellular debris, thereby forming biofilms (BFs) (Coenye & Nelis, 2010; Orgaz et al., 2006). BFs form a complex heterogeneous structure that offers an essential and protective environment for bacteria when they encounter unfavorable conditions, such as shortage of nutrients and high temperatures (Hughes, Sutherland, & Jones, 1998; Sutherland, 2001). BFs have received considerable attention from a broad range of fields of study, especially in the areas of food, environment, and biomedicine (Flint, Bremer, & Brooks, 1997; Maukonen et al., 2003; Sihorkar & Vyas, 2001), since they were first reported nearly 70 years ago (Zobell, 1943). Microorganisms in BFs catalyze chemical and biological reactions causing metal corrosion in pipelines and tanks, reduce the efficacy of heat transfer under conditions in which the BF becomes sufficiently thick, and lead to serious hygiene problems as well as economic losses due to food spoilage and equipment impairment (Bremer, Fillery, & McQuillan, 2006).

An extensively applicable and effective technique able to degrade unwanted BFs without causing adverse side effects currently does not exist. The main strategies available for such purpose include physical methods such as super-high electromagnetic fields, ultrasonic treatment, high-pulse electric fields, low electric fields, air crash, radiation treatment, and freezing method; chemical methods such as cleansing with alkaline or acid disinfectants and use of food packaging materials containing microbicidal components (colistin sulfate and nisin); and biological methods such as phage treatment (Simoes, Simoes, & Vieira, 2010). Generally, conventional chemical treatments, including antibiotics and disinfectants, encounter difficulty in penetrating the BF matrix alone and thus cannot destroy all living cells in the BF (Simoes, Simoes, Machado, Pereira, & Vieira, 2006). BFs usually show great resistance against common antibiotics and disinfectants, the application of which is not safe (Davies, 2003; Midelet & Carpentier, 2004). Chlorine dioxide (ClO_2), a broad-spectrum sterilizer used commonly in food industry, was also proved to be difficult to eliminate the biofilm bacteria formed by *Salmonella* and *Staphylococcus aureus* (Chen, Han, & Li, 2004; Mao, Gao, Chen, Ge, & Chen, 2010). Therefore, new and effective strategies to break up or dissolve the biofilm EPS matrix need to be developed.

* Corresponding author. Tel.: +86 532 82032290; fax: +86 532 82032389.
E-mail address: mousun@ouc.edu.cn (H. Mou).

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Bacteriophages are viruses that may release polysaccharide depolymerase enzymes, which bind to the capsular material of bacterial cells, during infection. Use of these phage enzymes represents a natural, highly specific, and non-toxic approach to controlling microorganisms involved in BF formation. In this study, the BF of *Klebsiella*, a common BF-forming species in the food industry (Houdt & Michiels, 2010; Tang et al., 2009), was obtained by attachment of bacterial cells on sterile glass coupons. A heat-stable phage enzyme exhibiting high-performance hydrolysis of bacterial EPS was extracted from phage lysates and its effects on eliminating the BF of *Klebsiella* were studied.

2. Material and methods

2.1. Microorganism

BF-forming *Klebsiella* sp. was isolated from the instruments of food processing plant and was stored at Applied Microbiology Lab, Ocean University of China. The bacterial cells were prepared using nutrient broth medium and glucose (5 g/l), which were sterilized at 115 °C separately and then mixed before inoculation.

2.2. Preparation of bacteriophage crude depolymerase enzyme

Bacteriophage infecting *Klebsiella* was isolated from sewage sample and stored at 4 °C before use. *Klebsiella* was incubated in nutrient broth medium under shaking at 32 °C, reaching an optical density at 600 nm (OD_{600}) of approximately 0.4. The OD_{600} of the culture was monitored after the bacteriophage suspension was added at the proportion of 1:10, around 0.1 of the multiplicity of infection (MOI). When the OD was below 0.1, the culture was filtered (pore size, 0.22 μm; Millipore) into a sterile screw cap bottle to remove surviving or intact bacterial cells. The filtrate was sealed in dialysis bags (molecular weight cutoff, 12,000–14,000) and dialyzed at 4 °C for 2 days against deionized water (changed twice each day) to remove low-molecular-weight materials, including glucose from the culture medium. After centrifugation at 1000× *g* for 10 min, the supernatant fluid was collected and further purified.

To purify the phage enzyme, acetone precooled at 4 °C was slowly added into the supernatant fluid, and the mixture was constantly stirred. After 12 h of storage at 4 °C, the solution was centrifuged at 7000× *g* for 5 min to collect the sediment. At 1:10 proportion, the sediment was redissolved in deionized water and served as the source of crude bacteriophage depolymerase enzyme extract.

2.3. Detection of bacteriophage depolymerase enzyme activity

The bacterial EPS was prepared by incubating *Klebsiella* cells in nutrient broth medium (glucose content: 20 g/l) for 50 h. After centrifugation at 7000× *g* for 20 min, the supernatant was collected and precipitated by a twofold volume of acetone. After dialysis at 4 °C for 2 days against deionized water, the bacterial EPS solution was prepared and used to detect the activity of the bacteriophage depolymerase enzyme.

The crude enzyme prepared as the method mentioned above was added to the EPS substrate (5 g/l) with a final proportion of 1:20 and kept at 32 °C for 4 h. Enzyme activity in the hydrolysis of the EPS was determined by measuring the reducing sugars released in the reaction solution according to OD_{520} using the DNS (3,5-dinitrosalicylic acid) (Von Borel, Hostettler, & Deuel, 1952).

After enzymatic degradation, the diffusible oligosaccharide products were examined using HPLC (Agilent 1200, Agilent Technologies, Agilent Technologies, Santa Clara, CA, USA) on a TSK Gel G6000 PWXL column (7.8 mm × 30 cm) eluted with deionized water at 0.6 ml/min and 30 °C. Eluted materials from the column were detected by refractive index monitoring (LaChrom L-7490; Merck-Hitachi Inc., Newark, NJ, USA).

2.4. Effects of heat treatment on enzyme activity

To evaluate the heat stability of the phage enzyme, the enzyme was treated at different temperatures from 60 °C to 90 °C for 10 or 15 min. After it was cooled down in ice bath, enzyme hydrolysis was immediately carried out at 32 °C to analyze the residual activity. The titration of the phage (in plaque-forming units) was determined at the same time according to the sloppy agar method. An aliquot (0.1 ml) of the diluted phage solution was plated in 4 ml of sloppy agar, to which 0.9 ml of mid-exponential-phase phage-susceptible bacteria had been added. After 24 h of incubation, the plates were examined for plaque counting.

2.5. BF Removal tests using phage enzyme

Standard BF was obtained by attachment of bacterial cells on sterile glass coupons (5 cm × 2 cm, Sangge biotechnology Ltd., Shanghai, China) placed inside a plate (9 cm × 10 cm) filled with 15 ml of culture medium at 32 °C for 48 h without shaking. After the culture medium was removed, the glass coupons were submerged into the phage enzyme (1.5 ml), which had been treated with acetone at a volume ratio of 1:2 (crude enzyme solution: acetone) and 75 °C for 10 min, leading to no loss of enzyme activity and zero titration of the phage at different concentrations, and then retained on incubation at 32 °C for 6 h. The group that was not subjected to enzyme treatment served as the control. The coupons were then washed sufficiently with sterile PBS to remove any loosely adherent planktonic cells. After washing, the BF was dislodged from the coupons using a sterile cotton bud, which was then dropped into a capped test tube containing 10 ml of sterile PBS. The test tube was treated with vortex oscillation for 1 min and ultrasonic oscillation for 10 min. The samples were taken out, serially gradient diluted in sterile PBS, and then coated onto nutrient agar plates. The bacterial colonies were counted as colony-forming units per square centimeter after 24 h of culture at 32 °C. The experiments were performed in triplicate, and the mean value was calculated.

The BF bacteria elimination effects were studied by treatment with the phage suspension as control group. The phage suspension, a mixture of phage particles with the titration of 5.6×10^8 PFU/ml and phage enzyme, was directly collected by phage lysis. The treatment of the disinfectant chlorine dioxide (ClO_2; 100 mg/ml effective chlorinum) was also carried out after the pretreatment with the phage enzyme. The biofilm grown on glass coupons was exposed to phage enzyme with different concentrations for 4 h and followed by the treatment of disinfectants ClO_2. The residual bacterial colonies were counted within 30 min as the method mentioned above.

2.6. Analysis by scanning electron microscopy (SEM)

BF-covered discs were washed sufficiently with sterile PBS and fixed in 2.5 ml/100 ml glutaraldehyde for 12 h. The discs were washed again with sterile PBS and immersed in 1 g/100 ml osmic acid for further fixing. Specimens were dehydrated in an ethanol series (15 min each in 50, 70, 80 and 90 ml/100 ml, 30 min in 100 ml/100 ml twice), exchanged with isopentyl acetate, and then critical point dried for 20 min. The sample was sputter coated with gold using a Hummer instrument (IB-3; Eiko Inc., Kansas, KS, USA) and examined using a scanning electron microscope (JSM-840;

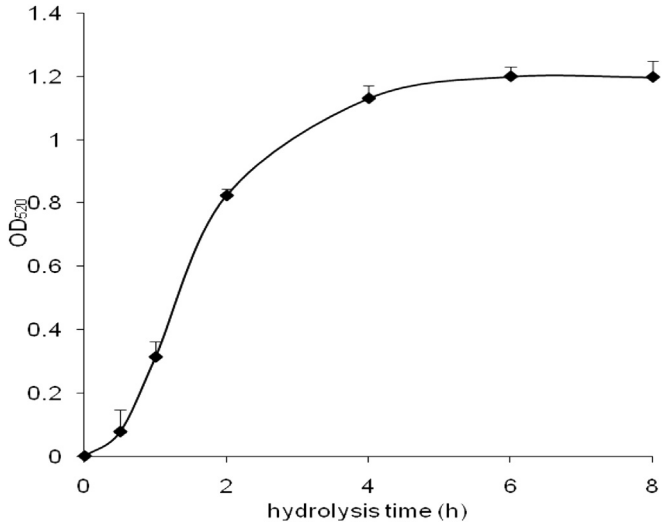

Fig. 1. Hydrolysis of bacterial extracellular polysaccharide using crude bacteriophage depolymerase enzyme analyzed by the reducing sugar released. The experiments were performed in triplicate.

JEOL Ltd., Japan). The micrograph under SEM illuminated the effects of the phage enzyme at different time points on the structure as well as the bacterial number of the BF.

3. Results

3.1. Hydrolytic properties of phage enzyme

Analysis of the release of reducing sugars showed that the EPS-hydrolyzing activity of the phage crude depolymerase enzyme was high. The amount of reducing sugars produced by EPS hydrolysis significantly increased, reaching the peak after approximately 4 h (Fig. 1), as reaction time increased. The OD_{520} did not change much even if the reaction was extended to 24 h.

3.2. Acetone precipitation on phage enzyme

With the addition of acetone into the phage lysate solution, the precipitated protein showed a marked increase in the activity of the phage enzyme and a rapid drop in phage titration, both of which nearly reached the maximum values at the volume ratio of 1:2 (crude enzyme solution/acetone). The enzyme activity at this volume ratio was 24% higher than that at 1:1 (Fig. 2). The addition of acetone could also effectively eliminate the active phage particles from the enzyme. In comparison with that at the volume ratio of 1:1, more than 99% of the active phage particles were eliminated at 1:2. In the following tests, a 1:2 volume ratio was adopted to prepare the phage enzyme.

3.3. Heat stability of phage enzyme

As the pretreatment temperature increased from 60 to 75 °C, no loss of phage enzyme activity was detected, but phage titration decreased sharply until it reached zero when the temperature was 75 °C (Fig. 3). The enzyme activity partially disappeared at 80 °C and higher, showing that the phage enzyme was a heat-stable protein. Treatment for 10 or 15 min showed minimal differences in enzyme activity and phage titration. As treatment at 75 °C for 10 min could inactivate the phage entirely while keeping the enzyme activity above 95%, these conditions were adopted to prepare the phage enzyme in the BF removal tests.

3.4. BF Removal tests using phage enzyme

As shown in Fig. 4, the phage enzyme that had been precipitated by a twofold volume of acetone and treated at 75 °C for 10 min was essential to eliminating BF bacteria compared with the control. The number of BF bacteria significantly decreased at each tested concentration of the phage enzyme as treatment progressed from 0 to 6 h. The most effective elimination appeared after 4 h of treatment with the enzyme solution (no dilution), with more than 80% of the BF bacteria having been eliminated. The BF removal rate did not change much as treatment time increased to 6 h. In comparison,

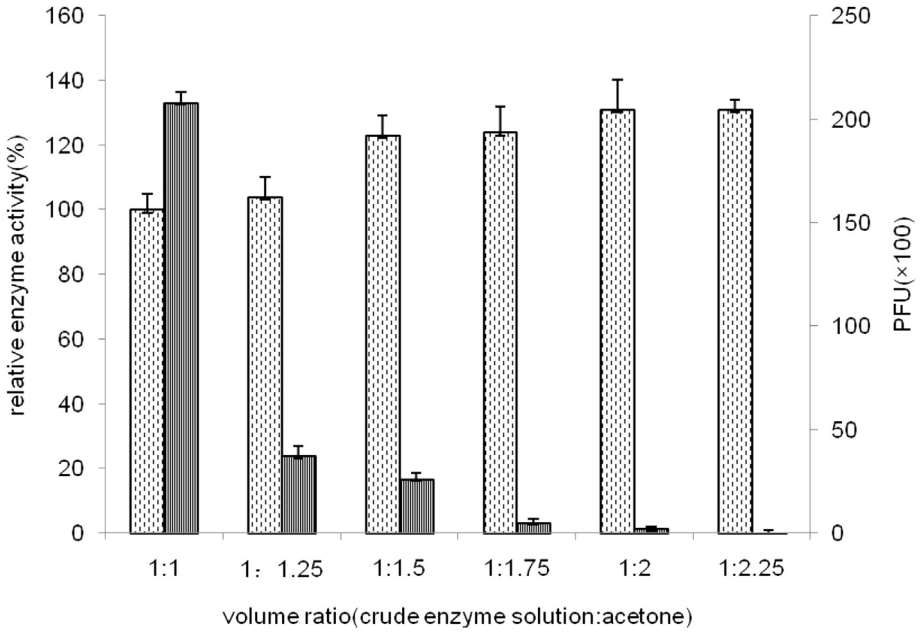

Fig. 2. The relative enzyme activity (%) and titration of phage (PFU) after acetone precipitation with different volume ratios (crude enzyme solution: acetone), ▭, relative enzyme activity; ▨, titration of phage. Supposing the enzyme activity was 100% at volume ratio of 1:1. The experiments were performed in triplicate.

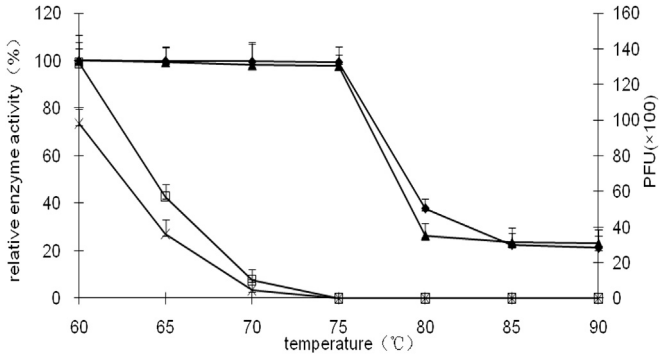

Fig. 3. Heat-stability test of the phage and its polysaccharide depolymerase enzyme. The crude phage enzyme solution was treated at different temperatures for 10 min or 15 min to analyze the loss of the enzyme activity (◆, 10 min; ▲, 15 min) and the residual phage titration (□, 10 min; × , 15 min). Supposing the enzyme activity was 100% at 60 °C. The experiments were performed in triplicate.

phage suspension, which was a mixture of phage particles with the titration of 5.6×10^8 PFU/ml and phage enzyme, could reach a significant elimination of the BF bacteria of about 88% after treatment for 4 h.

This study has confirmed that the phage enzyme is an effective and important tool for cleaning extracellular polymeric substances before BF bacteria are sterilized by disinfectants unable to penetrate the BF matrix alone. After the BF was pretreated with the phage enzyme at different concentrations for 4 h, treatment with the disinfectant ClO_2 could effectively decrease the number of bacteria in the BF. Approximately 92% of the BF bacteria were eliminated after treatment with the phage enzyme (no dilution) followed by 30 min of treatment with ClO_2 (Fig. 5). In comparison, stand-alone ClO_2 treatment for 30 min could only eliminate 75% of the BF bacteria.

3.5. Analysis of BF by SEM

The BF grown on glass coupons was exposed to the phage enzyme, ClO_2, and crude phage solution to analyze its configuration under SEM. As shown in Fig. 6A, an extensive BF presented as a clear aggregate of microorganisms in which bacterial cells adhered to one another, connected by self-produced extracellular polymeric substances. It appeared to have a stereo configuration in which a group of bacteria colonized and was enclosed within the extracellular matrix. Fig. 6B shows another micrograph of this sample of BF containing a mass of extracellular polymeric substances, which were significantly degraded by the phage enzyme after treatment for 6 h (Fig. 6C). Fig. 6D illustrates that the cell structure was severely damaged by ClO_2. After the crude phage suspension (without its self-produced polysaccharide-degrading enzyme removed) came in contact with the BF, further interactions leading to considerable cell lysis occurred, dependent on the susceptibility of the BF bacteria to the phage and the availability of receptor sites based on partial polysaccharide elimination by the phage enzyme (Fig. 6E).

Counting of BF bacterial cells treated with the phage enzyme and disinfectant was also illustrated by SEM (Fig. 7A–E). Treatment with phage-borne polysaccharide depolymerase enzyme could effectively decrease the attachment of bacteria and the extracellular polymeric substances in the BF. Only a few bacterial cells remained in the visual field, and almost no extracellular polymeric substances surrounded them after the BF was treated with the enzyme for 6 h. The synergistic effects of the phage enzyme and disinfectant on the BF were also evaluated. The surface of the glass discs was treated with ClO_2 for 30 min after exposure to the phage enzyme for 4 h. BF materials were almost completely removed from the glass discs, and only a few bacterial cells remained on the glass, most of which were distorted (Fig. 7E). These outcomes differ from those of treatment with the phage enzyme alone, which kept the bacterial cells intact.

4. Discussion

Bacteriophages, which possess the potential ability of producing polysaccharide-degrading enzymes, widely occur in nature. During infection of bacteriophages against a host cell, they may produce polysaccharide-degrading enzymes to destroy the associated capsular EPSs, which delay or prevent phages from gaining access to receptors on the cell wall of each infected bacterium (Mah & O'Toole, 2001). Bacteriophage enzymes have been used to

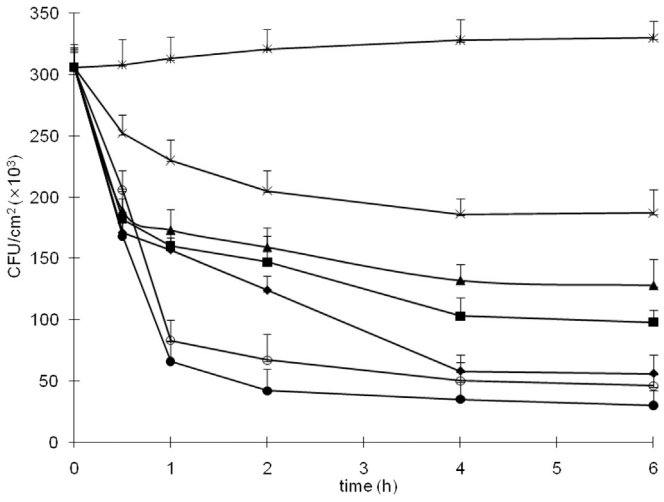

Fig. 4. Elimination of biofilm bacteria by the phage suspension or phage enzyme at the range from 0 to 6 h of treatment. *Klebsiella* biofilm grown on glass coupons was exposed to phage suspension or phage enzyme with different concentrations (phage suspension: ● 5.6×10^8 PFU/ml, ◇4 5.6×10^5 PFU/ml; phage enzyme: ◆ no dilution, ■ 2 times dilution, ▲ 4 times dilution, × 8 times dilution, * control). The experiments were performed in triplicate.

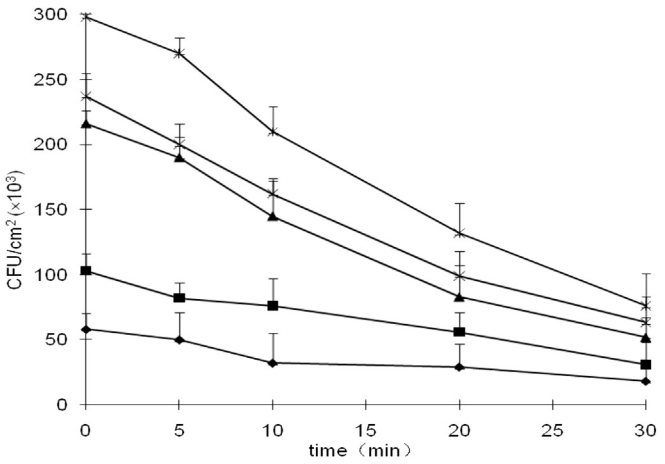

Fig. 5. Elimination of biofilm bacteria by the disinfectant ClO_2 after pretreatment by phage enzyme. The biofilm grown on glass coupons was exposed to phage enzyme with different concentrations (◆ no dilution, ■ 2 times dilution, ▲ 4 times dilution, × 8 times dilution, * control) for 4 h and followed by the treatment of disinfectants ClO_2 (100 mg/ml effective chlorinum). The experiments were performed in triplicate.

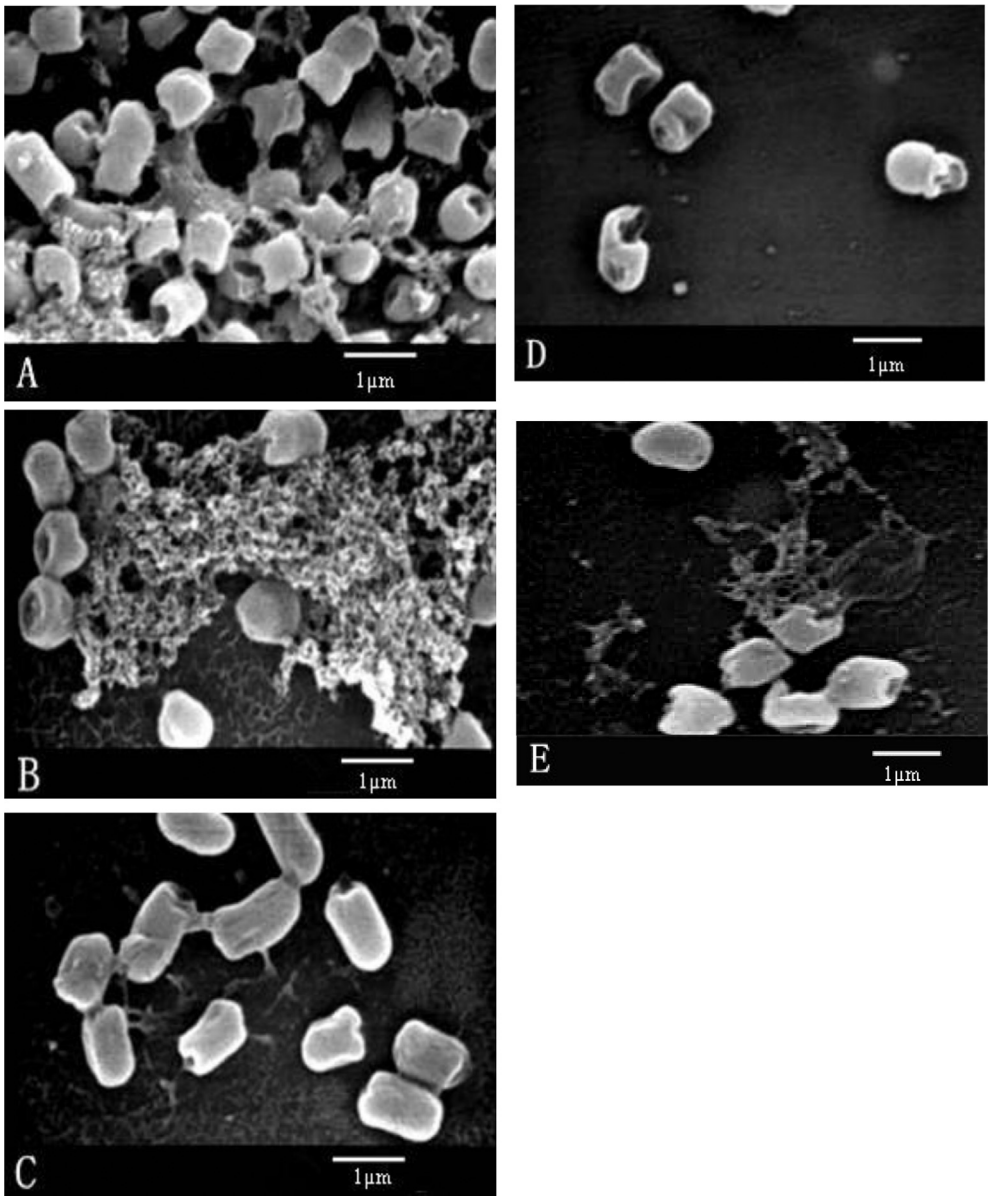

Fig. 6. Structural details of biofilm and bacterial cells under SEM. The biofilm was constructed by growing the *Klebsiella* cells on the glass coupons for 24 h, showing the intercellular materials combining the cells together (A) and massive extracellular polymeric substances (B). To analyze the structural changes after different treatments, biofilm was exposed to phage enzyme for 6 h (C), phage enzyme for 4 h followed by treatment of disinfection ClO_2 (D), and crude phage suspension for 1 h (E), respectively.

hydrolyze the EPSs of bacteria since 1956 (Adams & Park, 1956). Sustaining studies have been reported on the chemical structure of bacterial EPSs for many years, mainly taking advantage of the phage enzyme's specificity acting on closely related polysaccharide chains (Dutton, Parolis, Joseleau, & Marais, 1986; Sutherland, 1976). In addition, owing to their varying EPS compositions, phage enzymes represent a very useful and highly specific tool for the preparation of novel oligosaccharides with particular physiological activities (Dutton et al., 1981).

In this study, the phage enzyme we prepared was highly efficient and specific for EPS hydrolysis. The results showed that the reaction was nearly complete after enzymatic hydrolysis for 4 h at 32 °C. The recovery rate of crude bacteriophage enzyme extract reached the maximum, and the titration of the phage decreased sharply from 5.6×10^8 to less than 2×10^2 PFU/ml after it was treated with acetone at the volume ratio of 1:2 (crude enzyme solution/acetone). Treatment at 75 °C for 10 min could kill the phage entirely, whereas no loss of phage enzyme activity was observed. The application of the heat-stable phage enzyme can effectively inhibit bacterial contamination during oligosaccharide preparation and exclude the influence of the phage on the BF. Sutherland (1967) also demonstrated that the enzyme extracted from the phage F31 showed great degradation activity above 55 °C against the EPS from *Klebsiella aerogenes*.

In food processing environments, BFs have been implicated as a source of persistent contamination. The contaminating microorganisms in BFs adhere to various food processing settings, including equipment, table boards, water pipes, and other food industrial systems, making it difficult to eradicate them and further leading to corrosion (Gram, Bagge-Ravn, Ng, Gymoese, & Vogel, 2007). Moreover, BFs provide substrates for other microorganisms that are less prone to form BFs, increasing the probability of pathogen survival and further dissemination in food (Lapidot, Romling, & Yaron, 2006). Currently, disinfectants and other antimicrobial

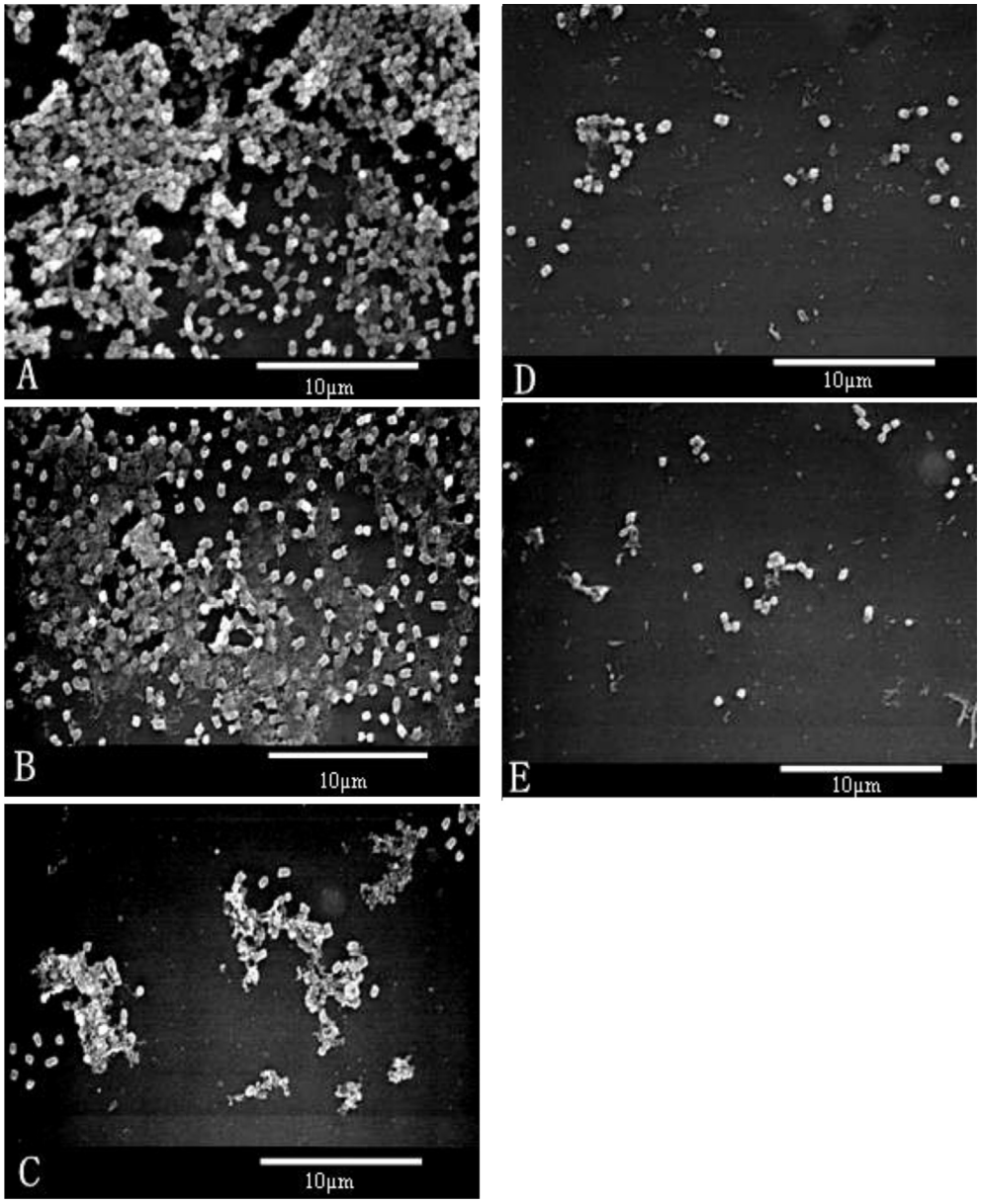

Fig. 7. Counting of biofilm bacterial cells treated by phage enzyme and disinfectant under SEM. The biofilm grown on glass coupons was exposed to phage enzyme for different times, 0 h (A), 1 h (B), 4 h (C) and 6 h (D), respectively, or exposed to phage enzyme for 4 h followed by treatment of disinfection ClO_2 (E) to analyze the residual cells in the biofilm.

products that can only take effect at high concentrations are the main tools for controlling BFs. Generally, disinfectants cannot penetrate the BF matrix after an ineffective cleaning procedure and thus cannot destroy all living cells in the BF (Simoes et al., 2006). Moreover, cells in BFs likely possess a degree of natural resistance and physiological plasticity through mutation or genetic exchange, which allows microorganisms to survive and grow when they encounter high concentrations of disinfectants or antimicrobial products (Gilbert & McBain, 2003).

Phage-based detergents, such as phage particles, endolysin, and phage-borne glycanase, are known as bio-cleaners and serve as a natural option to overcome the problem with BFs in the food industry. Phage particles have been previously used to degrade BFs (Sutherland, Hughes, Skillman, & Tait, 2004), but research on the effects of bacteriophage enzymes on BFs is limited. Phages are capable of infecting bacterial cells in different BF layers by penetrating the polymeric matrix and transporting within the BF water channels (Sillankorva, Pospiech, Azeredo, & Neubauer, 2007). The phage-borne glycanase and endolysin play key physiological roles in the process of phage invasion through the EPS matrix and release of progeny phage particles from the infected host cells, respectively. The BF formed by *Streptococcus suis* could be dispersed by a bacteriophage endolysin designated as LySMP, with more than 80% removal compared with treatment with either antibiotics or bacteriophage alone. In addition, *S. suis* cells themselves were found to be inactivated by LySMP (Meng et al., 2011). Evidence of the ability of bacteriophage enzymes to degrade bacterial EPSs has been recorded for approximately 50 years (Simoes et al., 2010). Some precedents of using phage-borne glycanase for facilitating the BF removal process have been presented. Bacteriophage migration through a BF of *Pseudomonas aeruginosa* is reportedly promoted by the reduction in alginate viscosity resulting from EPS degradation by phage enzymes (Hanlon, Denyer, Ollif, & Ibrahim, 2001). An enzymatic bacteriophage that had the ability to produce an EPS depolymerase enzyme to

attack the BF matrix, substantially reducing the BF cell count, was engineered to enhance the BF elimination effect of the bacteriophage (Lu & Collins, 2007). In the present study, the phage enzyme exhibited EPS-hydrolyzing activity, which was valuable for the safe and effective control and eradication of the bacterial BF. The results of the plate counting indicated that the BF treated with the phage enzyme for 4 h could reach a significant elimination rate of 80%, higher than that with the disinfectant ClO_2. Approximately 92% of the BF bacteria were eliminated after pretreatment with the phage enzyme followed by ClO_2 treatment for 30 min. The phage suspension worked as the combination of phage particles and phage enzyme also showed notable degradation on the BF. The findings implied that the phage enzyme can act as an effective cleaning procedure to break up or dissolve the EPS matrix associated with the BF so that disinfectants can gain access to the target cells and further destroy the BF. A positive correlation between the results of SEM and those of plate counting was observed. Furthermore, the bacterial cell distortion due to the destruction of cell membrane by the action of ClO_2, was observed in the SEM micrograph. In comparison, the phage enzyme almost had no influence on the shape of the bacterial cells, suggesting that the phage enzyme clearly has potential applications on the control and elimination of bacterial BFs in food processing and other industrial fields.

However, this technology has not yet been widely developed. The contradiction between the specificity of the enzyme action mechanism and the complexity of the BF matrix limits the application of phage enzymes in the food industry. Further investigation into different alternatives for enzymatic removal in combination with other enzymes or green eliminating detergents is needed. In addition, future studies should be in parallel with high-efficiency phage enzyme production in response to the limitation of the low prices of chemicals used today.

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