ENHANCING THE ANTIBACTERIAL EFFECT OF BACTERIOCIN FROM LACTOCOCCUS LACTIS SUBSP. LACTIS USING CHITOSAN NANOPARTICLES

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ABSTRACT

Chitosan, a cationic polymer derived from the hot alkali deacetylation of chitin, has numerous biological applications with non-toxicity, biocompatibility and biodegradability. Chitosan nanoparticles were prepared and encapsulated with bacteriocin extracted from Lactococcus lactis subsp. lactis to produce chitosan nanoparticles conjugate bacteriocin using ionic gelation method. This formulation was examined for its antibacterial activity representing food bio-preservative against Salmonella typhimurium, Escherichiae coli, Bacillus cereus and Staphylococcus aureus, compared with chitosan nanoparticles, crude chitosan and free bacteriocin. Agar diffusion method was applied to evaluate the in-vitro drug release, effect of pH and temperature on its stability. The results revealed that the In-vitro release within 24 hours of chitosan nanoparticles conjugate bacteriocin was controlled by about (79%) with cumulative and sustained effect when compared with free bacteriocin (94%). Chitosan nanoparticles conjugate bacteriocin exhibit the highest antibacterial activity (with significant difference p ≤ 0.05) followed by free bacteriocin and chitosan nanoparticles, while crude chitosan was the lowest representing thermal stability (≤ 70ºC) when subjected to low pH. Gram-positive bacteria were more susceptible than Gram-negative bacteria to all of the components. Chitosan nanoparticles contribute successful safe food preservative enhancement when incorporated with bacteriocin against the common food-borne pathogenic bacteria.

Keywords: Bacteriocin, Chitosan nanoparticles, Nanoparticles conjugate bacteriocin

INTRODUCTION

Chitosan is a non-toxic biodegradable copolymer consists of D-glucosamine and N-acetyld-G-glucosamine units from chitin deacetylation in the presence of hot alkali (Zaghoul et al., 2015). It contains amino, primary and secondary hydroxyl as relative function groups in C2, C3 and C6 positions, respectively (All and Toliba, 2018). Ionic gelation method with tripolyphosphate (TPP) ion was investigated by Kahdestani et al. (2021) to prepare chitosan nanoparticles (CSNps) from crude chitosan. Chitosan reveal antimicrobial activity against many spoilage and pathogenic microorganisms representing Gram-positive and Gram-negative bacteria, molds and yeasts. Its antimicrobial effect is depending on the deacetylation degree, type of microorganism, molecular weight and pH value (Eldaly et al., 2018). The antimicrobial properties of CSNps and its derivatives were proven in previous literature studies (Acay et al., 2020). It encapsulates bioactive formulations in micro or nanoparticles form in addition to its antimicrobial properties (Correa-Pacheco et al., 2018). The low toxic and ecological safe biocompatibility and admirable biodegradability with antimicrobial activity provided ample opportunities for further applications (Chawla et al., 2014). CSNps are effective for sustained bioactive release because it mitigates the bioactive release (Kahdestani et al., 2021). Lactic acid bacteria (LAB), commonly used in food preservation, exhibit antagonistic activity and inhibiting pathogenic and spoilage microbiota in food and food products via bacteriocins and other metabolites which have vital antimicrobial capacities (Akbar et al., 2019).

Bacteriocins, antimicrobial peptides synthesized by ribosomes, display bacteriostatic or bacteriocidal effect toward target specificity closely related and/or broad range bacterial strains (Abdehamei et al., 2015; Woraprayote et al., 2016). These bacteriocins have small cationic molecules containing 30 to 60 amino acids which form amphiphilic helices, have heat stability when subjected to temperature of 100ºC for 10 min and differ from each other in mode of action, spectrum of activity, biochemical properties, genetic origin and molecular weight (Mokoena, 2017).

Among bacteriocinogenic LAB, Lactococcus spp produced bacteriocins (Yusuf and Abdul Hamid, 2013). Lactococcus lactis generally isolated from fermented raw milk and known as Generally Recognized As Safe (GRAS) strain. This strain prevent pathogenic bacterial growth in the fermented products by converting lactose to lactic acid as a result of its proteolytic activity. The produced lactic acid considers an important role in the final taste and texture of fermented products (Tena and Suárez, 2020). The most common bacteriocin produced by Lc. lactis is nisin A and its variants. It represents Class I bacteriocin, Ripps, post-translationally modified peptides, heat stable, lanthionine and methyl-lanthionine containing peptides (<5 KDa) with members consisting lacticins (Meade et al., 2020). Whereas, LAB produce one bacteriocin, L. lactis produce 2 synergistically Class II bacteriocins named LsbA and LsbB (Duhan et al., 2013). Bacteriocins inhibit only Gram-positive bacteria including Staphylococcus aureus and Listeria monocytogenes because Gram negative bacteria exhibit high resistance to these compounds (Moreno et al., 2000).

Where bacteriocins exploitation to be applied as preservatives showing slow pace moving is yet to be addressed for various limitations, bacteriocin-nanoconjugates utilization in food industry are basically focused to overcome the challenges of using bacteriocins alone. To combat the direct addition of bacteriocin and its susceptibility to storage conditions, changes in temperatures and production process, nanoparticles provides an efficient technology to protect and deliver their potential antibacterial effect (Sidhu and Nehra, 2019). Nanoparticles are known as particles with dimensions ranged from 1 to 100 nm with unique properties thanks to the reduction of its dimension to the atomic level increasing the atomic surface compared to the bulk equivalents (Divya and Jisha, 2018). The suitable formulation technique using nanoparticles (nanoencapsulation) improve the antimicrobial activity of bacteriocins (Namasivayam et al., 2015). These nanoparticles have the potential to diffuse and cross-biological cellular membrane barrier of different cell type. Several studies have modified chitosan as biocompatible nontoxic polymer with integrated ability to antimicrobial peptides to form chitosan-based nanoparticles vehicle for delivery applications (Tamara et al., 2018; Kahdestani et al., 2021).

The aim of the present study is to evaluate the potential release and stability of bacteriocin encapsulated with CSNps under different values pH and temperatures and as well as its antibacterial activity against common food-borne pathogenic bacteria.
MATERIALS AND METHODS

Bacterial strains

Lyophilized strains of bacteriocin producing bacteria and food-borne pathogenic bacteria were obtained from different cultures collections as shown in (Table, 1).

| Table 1 Source of bacterial strains. |
|-------------------------------------|
| **Bacterial strains** | **Strain number/identification** | **Sources** |
| Lactococcus lactis subsp. lactis | EMCC*11552 | Dairy Department, Minia University |
| Salmonella typhimurium | ATCC*14028 | Cairo MIRCEN* |
| Escherichia coli | ATCC*10536 | Cairo MIRCEN* |
| Bacillus cereus | ATCC*10976 | Cairo MIRCEN* |
| Staphylococcus aureus | ATCC*6538 | Cairo MIRCEN* |
| **Food-borne pathogenic bacteria** | | |
| **Preparation of Chitosan nanoparticles conjugate bacteriocin (CSNps-B)** |

Preparation of different formulations

Under aseptic conditions three-fold tubes (12*73 mm) representing 4 groups include crude chitosan, CSNps, free B or CSNps-B were accurately quantified then added to 0.25% acetic acid to prepare 100 µg/ml concentration according to Abdeltawab et al. (2019).

Assessments and characterizations

In-vitro release of bacteriocin

To determine in-vitro bacteriocin release, dialysis bag method was used according to Bohrey et al. (2016) with slight modifications. 5 ml of release medium (0.1M PBS, pH 7.4) containing 50 mg of CSNps-B or free-B was pipette in dialysis bag at 37°C, then placed in beaker containing 100 ml of PBS. The beaker was stirred at 37±1°C using a magnetic stirrer at 100 rpm. An intermittent bacteriocin release was assessed by withdrawing 2 ml samples at 1, 2, 3, 4, 5, and 6 hours.

Antibacterial activity assay

Agar well diffusion method was applied to evaluate the food-borne pathogenic bacterial response against the formulation groups, by measuring the inhibition zone in millimeters (mm). Sterile cork borer well made wells by removing slug from inoculated Mueller Hinton Agar (Code: 64884, BIO-RAD, USA). The individual bacterial cultures were loaded with 100 µl of the separated treatments and then incubated at 37°C for 24 h. The measurements were carried out in triplicates (Balouiri et al., 2016).

Stability at different pH values

To assess the effect of pH on the prepared formulation groups, tubes containing 5 ml of the concentrates were pH adjusted at different values of pH which ranged from 4 to 11 with sterile lactic acid (1% w/v) or NaOH (1 M) at room temperature (22°C) for 2 hours then readjusted to 7 as pH value. The antibacterial activity of these concentrates was determined using agar well diffusion method (Mostafa et al., 2015).

Statistical analysis

The obtained results representing the data of duplicated experiments were statistically analyzed. Analysis of variance among formulations and treatments were performed by entering data through one-way ANOVA and paired-samples T-test using (IBM-SPSS, 20; USA) with statistical significance declared at p < 0.05 (Rabiei et al., 2015).

RESULTS AND DISCUSSION

In-vitro release of bacteriocin

The efficiency of loaded CSNps with bacteriocin produced by L. lactis subsp. lactis as safe food preservative was in vitro evaluated by comparing formulations, i.e. CSNps-B and its components (chitosan, CSNps and free-B), against Gram positive and Gram negative food-borne pathogenic bacteria.

The release behavior of bacteriocin from CSNps-B and free-B during 24 hours was carried out using dialysis bag method and shown in Figure (1). Gradual increase of bacteriocin release was found to be 79 and 95% from CSNps-B and free-B tubes, respectively. During the initial 4 hours, burst release about 83% of bacteriocin from free-B tubes then slightly increase to release about 94% by the end of 24 hours. On the other hand, cumulative sustained release from tubes containing CSNps-B was observed reaching about 28% within 4 hours and 79% by the end of 24 hours. A significant difference at p < 0.05 was observed when compared with the paired-samples T-test.
Effect of different pH values on the antibacterial activity of CSNps-B. Several studies reported that CSNps-B may cause morphological changes such as formation of pores in Gram-positive and Gram-negative bacterial cell membrane which in turn facilitate the bacteriocin cell penetration (Pan et al. 2011). Several studies reported that bacteriocin conjugated with nanoparticles enhanced and increased the antibacterial activity against food borne pathogenic bacteria by about two to four times more higher than the use of bacteriocin alone (Zohri et al., 2010; Zohri et al., 2013).

Effect of pH on the antibacterial activity

Environmental parameters such as pH and temperature were investigated to define the optimized conditions in order to maximize the antibacterial activity. Figure (3) shows the effect of different pH values on the antibacterial activity of the 4 formulations (chitosan, CSNps, free-B and CSNps-B), on the growth of food borne pathogenic bacteria; B. cereus, Staph. aureus, E. coli, and S. typhimurium. An increase in inhibition zone was observed at lower pH values using the different formulations for all studied strains wherein the highest inhibition zone was recorded at pH = 4. Whereas, a gradual decrease trend in inhibition zone was found with increasing the pH value wherein the lowest inhibition zone was observed at pH = 11. The release of bacteriocin (nisin) is pH dependent; Wu et al. (2016) found higher significant release of bacteriocin (nisin) at pH value of 3 (with 46% cumulative bacteriocin during 72 hrs) than those at 6 as pH value.

It was found that CSNps-B exhibited the highest antibacterial activity for all bacterial strains followed by free-B, CSNps, and chitosan at different pH values. Furthermore, CSNps and chitosan did not exhibit any antibacterial activity at pH values of 10 and 11 against both B. cereus and Staph. aureus. It is worth to mention here that at pH value of 10; only the CSNps-B exhibited an antibacterial activity on the growth of E. coli and S. typhimurium while, no effect was observed at pH value of 11. Moreover, the Gram-negative bacteria; E. coli and S. typhimurium, were more resistant to all formulations compared to Gram-positive bacteria; B. cereus and Staph. aureus. The obtained results are in agreement with those reported by Aidesina and Enerijiofi (2016) and Kumari et al. (2018) who reported that the most active bacteriocin against bacterial indicators was in pH values ranged from 6 to 7 with slight sensitivity at pH = 7, while a decrease in antibacterial activity was observed at pH values varied from 2 to 5.
In contrast, Zacharof and Lovitt (2012) showed that bacteriocins produced from LAB had high antibacterial activity at pH values below 5. Similar results concerning the antibacterial activity of chitosan were obtained by Qi et al. (2004) who reported a high antibacterial activity of chitosan only in acidic medium, as it loses its solubility at pH value higher than 6.5. A complete loss of bacteriocin activity especially nisin was also observed by Benkerroum et al. (2002) at neutral pH as a result of decreasing its solubility. Furthermore, the free amino groups in the d-glucosamine units may result in its protonation (Kahdestani et al., 2020). The obtained results are in disagreement with those found by Alishahi, (2014) who reported that the release of bacteriocin was faster at higher pH than that at the lower pH wherein the diffusion process controlled the release of bacteriocin at acidic environment.

The high antibacterial effect of CSNPs compared to chitosan may be due to the interfacial interaction between CSNPs small particle size and the bacterial cell membrane throughout the endocytosis (Divya and Jisha, 2018; Lee et al., 2018). The high bacteriocin release at lower pH values may be due to the dissolution and swelling of CSNPs (Khan et al., 2020). Bacteriocins exhibited high activity at pH 2-5 however, a loss by about 5.9-10% of its activity was observed at alkaline levels (Abanorz and Kanduhoglu, 2018; Kaktcham et al., 2019). While, the antibacterial activity of chitosan may be due to the interactions between the positive charged amino groups and the negative charged bacterial membrane (Kravanja et al., 2019).

The obtained results could be explained by 1) changes in the molecular interactions which take place between biopolymers as a result of the variation in pH values (Wu et al., 2016); 2) increasing the positive charges of chitosan as a result of lowering pH value which in turn increases its affinity towards the bacterial cell wall; increased protonated amino groups such as –NH3 groups with positive charges can bind to bacterial membrane components with negative charges. Moreover, the antibacterial activity at pH values below 6 may be due to the consequent of positively charged protonation which interacts with teichoic acid in Gram-positive bacteria and anionic lipopolysaccharides in Gram-negative bacteria (Malinowska-Pańczyk et al., 2015), and 3) alteration of mRNA functions and limiting the interactions of DNA through their binding with the low molecular weight which can pass through the cell (Rizeq et al., 2019).

Effect of temperature on the antibacterial activity

In order to investigate the heating effect on the antibacterial activity of the 4 formulations expressed by the inhibition zone using different temperatures which varied from 0 to 100°C for 30 min. Figure (4) shows that the 4 formulations exhibited different heat stability representing different antimicrobial activity against Gram-positive and Gram-negative bacteria. The highest stability was recorded for temperatures ranged from 0 to 70°C. Beyond these temperatures, a gradual decrease trend was observed until the absence of inhibition zone except CSNPs-B at 100°C. The highest inhibition zone was observed for CSNPs-B (p < 0.05) followed by free-B, CSNPs, and chitosan. CSNPs-B showed higher antibacterial activity against Gram-positive bacteria more than Gram-negative bacteria. The obtained results are in agreement with those reported by Azhar et al. (2017), Le et al. (2019) and Mostafa et al. (2019). The combined use of CSNPs-B with high temperature may provide synergistic effect which includes the effect of high

Figure 3 Stability of chitosan, CSNPs, Free-B and CSNPs-B at different pH against food-borne pathogenic bacteria: A) B. cereus, B) Staph. aureus, C) E. coli and D) S. typhimurium. [* Significant difference (p < 0.05) between CSNPs-B and the other formulations was observed.]
temperature and the antibacterial activity of CSNps-B increasing the inhibition zone which reflects the growth inhibition of food-borne bacteria (Prudêncio et al., 2015). Therefore, the heat stability of CSNps-B, Free-bacteriocin, CSNps and chitosan allows their applications as food preservative under the mentioned conditions of temperatures.

CONCLUSION

The combination of bacteriocins with CSNps (CSNps-B) increased the antibacterial activity of bacteriocin as food preservative extending the shelf life of food without altering its quality attributes. CSNps-B showed high antibacterial activity at wide range of temperature and pH. It exhibited higher antibacterial activity against Gram-positive pathogenic bacteria which was observed at lower values of pH than Gram-negative pathogenic bacteria. So, CSNps-B could be used as food bio-preservative inhibiting the growth of food-borne bacteria.

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