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Original Article

Antimicrobial activity optimization of nisin, ascorbic acid and ethylenediamine tetraacetic acid disodium salt (EDTA) against *Salmonella* Enteritidis ATCC 13076 using response surface methodology

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ABSTRACT

Nisin is a commercial bacteriocin produced by *Lactococcus lactis* subsp. *lactis* and widely used as a natural preservative in the food industry. However, while nisin alone cannot inhibit the growth of Gram-negative bacteria, it can in combination with a chelating agent or organic acid. This study combined nisin with some chelating agents, weak organic acids and their salts to inhibit *Salmonella* Enteritidis ATCC 13076. The combinations of nisin (2000 parts per million; ppm) and ascorbic acid (2000 ppm) or ethylenediamine tetraacetic acid disodium salt (EDTA; 7400 ppm) showed significant inhibitory effects on the target strain. Due to regulatory limits, the second part of the study reduced the concentrations of nisin, ascorbic acid and EDTA to 500 ppm, 2000 ppm and 250 ppm, respectively. The mixture of nisin, ascorbic acid and EDTA showed the highest inhibitory effect with a reduction number of 3.41 log colony forming units ($p < 0.05$). To minimize the growth of *S.* Enteritidis ATCC 13076, central composite design and response surface methodology were applied to investigate the combined effect of nisin (0–500 ppm), ascorbic acid (0–2000 ppm), and EDTA (0–250 ppm) on the target strain growth. Among the three factors, nisin had a higher antimicrobial effect than ascorbic acid or EDTA, while an increase in nisin resulted in a decrease in *S.* Enteritidis ATCC 13076 growth. The optimum concentration was 500 ppm nisin with 1515 ppm ascorbic acid and 250 ppm EDTA. Under these conditions, the growth of *S.* Enteritidis ATCC 13076 predicted by the model was 24.99%.

Introduction

It has been well established that fish and shellfish are nutrient-rich foods and consumption of them provides high quality protein, energy and essential nutrients, including the long-chain n-3 poly-unsaturated fatty acids (LCn3PUFAs; Food and Agriculture Organization, 2010). During 2010–2030, the total global consumption of seafood is expected to increase by 30% (World Bank, 2013). This trend potentially leads to an increase in consumer health risk, because of the greater risk of bacterial contamination. Contamination of fish and shellfish with *Salmonella* is a major public health concern as 99% of human infections are caused by *Salmonella enterica* and *Salmonella* Enteritidis, a non-typhoid *Salmonella* serotype, is the most often implicated in human infections (Amagliani et al., 2012). It causes gastrointestinal disease, which presents as nausea, vomiting, diarrhea, cramps and fever (Iwamoto et al., 2010). The U.S. Food and Drug Administration (USFDA) has demonstrated the presence of salmonellae in a variety of fish and shellfish, including ready-to-eat (RTE) products, products requiring minimal cooking and shellfish eaten raw (Iwamoto et al., 2010). The most common factors contributing to *Salmonella* outbreaks are improper cooking, unsuitable storage, cross-contamination and the use of raw ingredients to prepare food (Amagliani et al., 2012). One approach for the inhibition of *Salmonella* is to use natural antimicrobial agents. Peptide or metabolites from bacteria have been used to control pathogenic bacteria in food for a long time (Bagenda and Yamazaki, 2011). Among these, nisin is the only a kind of bacteriocin which is permitted for use as a food preservative (E234) by the European

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Union since 1983 (Phongphakdee and Nitisinprasert, 2015). Nisin is a short-chained antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis* and was classified to be Generally Recognized As Safe (GRAS) for human consumption by the USFDA in 1988 (Phongphakdee and Nitisinprasert, 2015). Nisin shows a broad antimicrobial spectrum against vegetable cells and endospores of Gram-positive bacteria but has no antimicrobial effect on Gram-negative bacteria, including *Salmonella* sp., because of their outer membrane (OM) barriers. The lipopolysaccharides (LPSs) in the OM of Gram-negative bacteria are strongly anionic and are stabilized by Ca^{2+} and Mg^{2+} (Facon and Skura, 1996). It has been reported that the antimicrobial activity of nisin could be enhanced by the use of membrane destabilizing agents, such as chelating agents, polycationic acid and weak organic acids as well as their salts (Facon and Skura, 1996; Alakomi et al., 2000; Helander et al., 2001). Most previous studies focused on combinations of nisin and other substances. These combinations can increase the broad spectrum of antimicrobial activity because nisin and combined substances act on different targets within the cell (Dai et al., 2010). However, nisin and/or combined substances must be used at high concentrations to achieve activity against target microorganisms, and these levels may exceed the flavor threshold acceptable to consumers and exceeds the allowed regulatory level (López-Malo et al., 2005). In theory, the use of combinations of three antimicrobial agents should be more effective than combinations of two antimicrobial agents because it is more difficult for the target organism to adapt to and resist a simultaneous attack on several targets in its cell wall and interior (Dai et al., 2010). However, reports on the use of combinations of three antimicrobial agents are scarce. Thus, the objectives of this study were to identify combinations of nisin with some chelating agents against *S.* Enteritidis ATCC 13076 and to determine the optimum concentrations of nisin and potential combined substances using a central composite design with response surface methodology. The information from this study not only can be used as an effective alternative for controlling target, food-borne, pathogenic bacteria in the food industry but also to reduce consumer risk from an overdose of food additives.

Materials and methods

Bacterial strains

S. Enteritidis ATCC 13076 and *Micrococcus luteus* IFO 12708 were used as the target indicator strains. Stock cultures of each strain were kept in tryptic soy broth (TSB; Difco; Sparks, MD, USA) containing 20% (v/v) glycerol (Merck, Darmstadt, Germany) and 0.6% (w/v) yeast extract (Difco; Sparks, MD, US) at −18 °C. Before use, both target strains were cultured in TSB and incubated at 35 °C for 18 h. After incubation, each strain was serially diluted with TSB to a final population of approximately 7.0 log colony forming units (CFU)/mL.

Chemicals and reagents

Commercial nisin (Nisaplin®) containing 10^6 international units (IU) of pure nisin per gram was purchased from Danisco (Grindsted, Denmark). Stock solution of nisin and all chelating agents including ascorbic acid (Riedel-Graham; Barrington, IL, USA), citric acid (Carlo Erba; Rodano, MI, Italy), ethylenediamine tetraacetic acid disodium salt (EDTA; Ajax; Taren Point, NSW, Australia), sodium acetate (Carlo Erba; Rodano, MI, Italy), sodium citrate (M&B; Dagenham, UK), sodium lactate (Wako; Osaka, Japan) and potassium sorbate (Fluka; St. Louis, MO, USA) were prepared by dissolving each chemical in distilled water to the designed final concentration before sterilization using filtration through a syringe filter membrane pore size 0.22 μm (Minisart; Göttingen, Germany).

Screening of the potential chelating agents for enhancing nisin antimicrobial activity

A stock solution of nisin, ascorbic acid, citric acid, sodium acetate, sodium citrate, sodium lactate, potassium sorbate and a mixture of nisin with each chelating agent were separately added into 10 mL of Mueller Hinton broth (MHB; Difco; Sparks, MD, USA) to get a final concentration of 2000 parts per million (ppm) except for the EDTA which was added to a final concentration of 7400 ppm. Thereafter, 100 μL of each target strain was separately inoculated into each tube of treated MHB to a final population of approximately 5.0 log colony forming units (CFU)/mL and then incubated at 35 °C for 24 h. After incubation, the growth of the target strain under different treatments was determined using optical density measurement at 600 nm (OD_{600}; Suksathit and Tangwatcharin, 2013) with a microplate reader (Power wave; Biotek; Winooski, VT, USA).

Antimicrobial activity of nisin in combination with ascorbic acid and ethylenediamine tetraacetic acid disodium salt at legal limit concentration

Stock solutions of nisin in combination with ascorbic acid or EDTA or both were added into 10 mL of MHB to a final concentration of 500 ppm; 2000 ppm or 250 ppm. Then, 100 μL of each target strain was separately inoculated into treated MHB to a final population of approximately 5.0 logCFU/mL and then incubated at 35 °C. After 24 h, the viable cell number in each treatment were enumerated using the spread plate technique with tryptic soy agar (TSA; Difco; Sparks, MD, USA) and incubated at 35 °C for 24 h (Suksathit and Tangwatcharin, 2013).

Determination of the optimal mixing concentration of nisin, ascorbic acid and ethylenediamine tetraacetic acid disodium salt

In this study, central composite design (CCD) and response surface methodology (RSM) were applied to determine the optimal nisin, ascorbic acid and EDTA concentrations in simulated mixed solutions that exhibited the highest antimicrobial activity against *S.* Enteritidis ATCC 13076 (Bezerra et al., 2008). Then nisin, ascorbic acid and EDTA concentrations were assigned as independent variables, whereas the percentage of target strain growth was assigned as the response variable in the proposed regression equation (Table 1). Moreover, the upper-limit concentrations for nisin, ascorbic acid and EDTA were designed to comply with the legal limit concentrations of European Food Safety Authority, 2006 and Food and Agriculture Organization, 2015. Using this approach, 20 experiments consisting of 2^3 factorial central composite experimental designs with six axial points and six replicates at the central point were conducted. MHB without any antimicrobial agent was used as a control experiment.

A stock solution of nisin, ascorbic acid, and EDTA was added into 5 mL of MHB to the designed final concentrations. *S.* Enteritidis ATCC 13076 was inoculated into prepared MHB to a final population

Table 1
Levels of independent variables—nisin, ascorbic acid and ethylenediamine tetraacetic acid disodium salt (EDTA)—in a 2^3 full factorial experimental design.

Independent variable	Unit[a]	Level				
		−1.682 (-α)	−1	0	+1	1.682 (+α)
nisin	ppm	0.00	101.37	250.00	398.63	500.00
ascorbic acid	ppm	0.00	405.47	1000.00	1594.53	2000.00
EDTA	Ppm	0.00	50.68	125.00	199.32	250.00

[a] ppm = parts per million.