Innovative Food Science and Emerging Technologies 38 (2016) 98–104

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Electroporation-assisted inactivation of *Escherichia coli* using nisin-loaded pectin nanoparticles

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ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form 19 September 2016
Accepted 20 September 2016
Available online 20 September 2016

Keywords:
Pulsed electric field
Bacteria inactivation
Biopreservation
E. coli

ABSTRACT

Pulsed electric fields (PEF) trigger the electroporation phenomenon in cell membranes, which has found many biotechnological applications including food processing. Nisin is a prominent lantibiotic widely used as a food preservative due to its high potency against many bacteria; its efficacy depends on various environmental factors and nisin resistance is a common problem. In this work we have applied novel nisin-loaded pectin nanoparticles in combination with sub-microsecond and microsecond range electroporation for inactivation of nisin-resistant *Escherichia coli*. The 5–30 $kVcm^{-1}$ square wave 1 kHz pulse bursts of 250 ns, 500 ns, and 100 μs were used. The PEF treatment combined with nisin nanoparticles further improved the inactivation efficacy (up to 3.7 log reduction) compared to separate procedures (up to 1.5 log reduction); this effect is scalable and does not depend on the cell concentration.

Industrial relevance: We have designed experiments and provided data that has direct application in food preservation and processing. Nisin is a prominent lantibiotic that is widely used as a food preservative due to its high potency against many bacteria. Nevertheless, its efficacy depends on various environmental factors and the nisin resistance is a common problem. We have used the pectin encapsulation that allowed addressing the susceptibility of nisin to environmental factors such as pH or food composition, while the PEF technology enabled a non-thermal increase of the membrane permeabilization and overcoming the nisin-resistance of the bacteria. We have also shown that the only reason to use submicrosecond pulses instead of the microsecond ones for eradication of the bacteria is the possible minimization of energy costs for generation of PEF. If the total energy of the pulse burst is the same the inactivation efficacy is also identical.

1. Introduction

Pulsed electric field (PEF) has been shown to be an alternative potent method (electroporation) for pasteurization of liquid foods due to the high efficacy of the treatment (Rivas et al., 2013; Mahnič-Kalamiza, Vorobiev, & Miklavčič, 2014). Electroporation is based on the significant increase in electrical conductivity and permeability of the cell membrane, caused by the externally applied electrical pulses (Kotnik, Kramar, Pucihar, Miklavcic, & Tarek, 2012; Žgalin, Hodžić, Reberšek, & Kandušer, 2012). Depending on the PEF parameters, the formation of transient or permanent pores is possible, resulting in reversible or irreversible electroporation (Korohoda, Grys, & Madeja, 2013). PEF applied at higher intensities triggers irreversible effects (Jiang, Davalos, & Bischof, 2015). In both cases the electroporation phenomenon has found different applications both in biotechnology and food processing (Schrive, Lumia, Pujol, & Boussetta, 2014; Kotnik et al., 2015). For the inactivation of microorganisms with pulsed electric fields, the permanent membrane damage is the baseline for the success of the processing method (Huang, Tian, Gai, & Wang, 2012; Rivas et al., 2013; Pillet, Formosa-Dague, Baaziz, Dague, & Rols, 2016).

Several studies have investigated the inactivation of bacteria in electric fields predominantly in relation to microbial inactivation, while the *Escherichia coli* is the frequent object of the research due to its frequent occurrence and importance in food processing (Wu, Mittal, & Griffiths, 2005; Žgalin et al., 2012; Flisar, Meglic, Morelj, Golob, & Miklavcic, 2014; Rivas et al., 2013; Guionet et al., 2014). However, the research is also performed in the sub-lethal damage range for the molecular

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http://dx.doi.org/10.1016/j.ifset.2016.09.019
1466-8564/

transfer, transformation and proteomic assays (Piggot, Holdbrook, & Khalid, 2011; Rivas et al., 2013). To improve the efficacy of the antimicrobial treatment, the alternative methods are also introduced such as photodynamic, high pressure, chemical procedures, which are studied both separately and in synergy with each other (de Alba, Bravo, & Medina, 2013; de Melo, A., Lee, Perussi, & Hamblin, 2013; Liu et al., 2015). Subsequently, a number of biopreservatives have been developed such as nisin, pediocin and many others to improve the quality of the food processing, where the inactivation of different microbes is of high importance (de Alba et al., 2013). At the same time nisin, which is a natural polypeptide from *Lactococcus lactis* subsp. *lactis* has been the only approved bacteriocin by World Health Organisation (de Alba et al., 2013). It was also approved as a food additive in European Union (EU) and was assigned the number E234 (Balciunas et al., 2013). The high efficacy and unique effects of the prominent lantibiotic versus Gram-positive bacteria makes nisin an effective agent for low cost, contamination-free and high efficacy biopreservation (Cheigh & Pyun, 2005; Hui, Liu, Feng, Li, & Gao, 2016). Nevertheless, the development of nisin resistance has been reported among Gram-negative bacteria and the treatment efficacy is highly affected by the environmental factors such as pH, temperature or food composition (Zhou, Fang, Tian, & Lu, 2014). In order to improve the quality and enhance the stability of antimicrobial peptides against environmental factors, the encapsulation using food-grade biopolymers such as chitosan/alginate (Zohri et al., 2013), alginate/pectin (Khaksar et al., 2014) or chitosan/carrageenan (Chopra, Kaur, Bernela, & Thakur, 2014) is applicable. The encapsulation partly solves the challenges of the treatment resulting in a simple and low cost method; however, the resistance of the bacteria (including *E. coli*) to nisin still possess a problem that requires investigation (Kramer et al., 2004; Zhou et al., 2014). One of the possible solutions is the application of the synergistic methods for the enhancement of the treatment efficacy (Pol, Mastwijk, & Smid, 2000; Wu et al., 2005; Liu et al., 2015; Mate, Periago, & Palop, 2016).

Previously in our work the novel nisin-loaded pectin nanoparticles have been developed, that have shown a good potential as a biopreservative and exhibited antimicrobial activity dependent on the type of the biopolymer applied for particles preparation (Krivorotova et al., 2016). The highest antimicrobial activity against Gram-positive bacteria *Arthrobacter* sp. was observed; however, the inhibition effect toward the Gram-negative *E. coli* was at least two-fold weaker based on agar-diffusion assay (Krivorotova et al., 2016). As a result, in this work we apply a synergistic methodology using PEF and nisin-loaded pectin nanoparticles and investigate the inactivation kinetics of the nisin-resistant Gram-negative *E. coli.* The resultant increased cell permeabilization rate due to PEF will depend on the PEF pulse parameters (Pucihar, Krmelj, Reberšek, Napotnik, & Miklavčič, 2011).

Even though the 100 μs × 8 electroporation protocol is common, several studies have focused the inactivation of *E. coli* in relation to the pulse duration (Žgalin et al., 2012; Guionet et al., 2014; Guionet et al., 2015; Haberl Meglic, Marolt, & Miklavcic, 2015). It was reported that the increase of the pulse duration above 100 μs in the 10–30 $kVcm^{-1}$ PEF range does not result in any improvement of inactivation, while the short 10-ns pulses alone do not induce any detectable effect regardless of the treatment time (Žgalin et al., 2012). At the same time the use of combination of nanosecond and microsecond pulses allowed inducing a synergistic effect – therefore decrease the energy cost of the treatment, which is considerable in industry (Žgalin et al., 2012; Guionet et al., 2015). The empirical and modeling results suggest that the exposure to high fields with durations less than the charging time of the cell's plasma membrane results in poration of both intracellular and plasma membranes and the various degrees of selective electroporation could be achieved (Tekle et al., 2005; Kotnik & Miklavčič, 2006; Weaver, Smith, Esser, Son, & Gowrishankar, 2012). In case of bacteria inactivation, the use of nanosecond protocols is applicable; however, the number of pulses should be increased dramatically to the range of several thousands (Guionet et al., 2015), thus application of synergistic protocols looks more promising. However, the major limiting factor could be the capacitive charging time of the cell plasma membrane, which also depends on the external solution conductivity (Marszalek, Liu, & Tsong, 1990). In order to acquire significant permeabilization the electric field must be high enough to induce critical transmembrane potential (TMP), while if the steady state of TMP is not reached, the field effect on the membrane will remain limited (Guionet et al., 2015; Blanckaert, Salles, Thomas, & Teissie, 2016).

In our work the aim was to determine if PEF could be used to improve the efficacy of nisin-loaded nanoparticles for the inactivation of the nisin-resistant *E. coli* strain. We have studied the inactivation dynamics in the submicrosecond (250, 500 ns) and microsecond (100 μs) PEF pulse duration space to provide new data and assess any synergism between submicrosecond and microsecond range protocols for our defined application.

2. Materials and methods

2.1. Preparation of nisin-loaded pectic acid (PecA) nanoparticles

Nisin (NisinZ™ P) was purchased from Handary S.A. (Brussels, Belgium). Pectic acid (PecA, M_w 30,000) was purchased from Serva (Heidelberg, Germany). Nisin-loaded PecA nanoparticles were prepared as described previously (Krivorotova et al., 2016). Briefly, prior to use, PecA was dissolved in distilled water at the concentration of 1 mg/mL by adjusting the pH of solution to the value of 8.0 with 0.1 M NaOH. The solution was dialysed against distilled water containing 0.03% of sodium azide using 3000 molecular weight cut-off dialysis membrane to remove Na^+ ions. The stock solution of nisin in water at the concentration of 2 mg/mL was filtered through 0.2-μm pore size filters and the pH of solution was adjusted to the value of 6.0. For the formation of nanoparticles, 10 mL of nisin solution was added dropwise to the PecA solution under constant stirring at room temperature. Prior to the addition of nisin, PecA solution was diluted with water to obtain 50 mL of nisin-pectin final mixture at the PecA and nisin concentration of 0.4 mg/mL. Finally, a pH of the solution was adjusted to the value of 6.0. Determination of nisin loading efficiency and physicochemical characteristics of nanoparticles was performed according to Krivorotova et al., 2016.

2.2. Bacterial cultures and growth conditions

Escherichia coli BL21 (F-dcm ompT hsdS(rB-mB-) galλ (DE3)) cells (ThermoFisher Scientific, Vilnius, Lithuania) were propagated in Luria-Bertani (LB) medium (2% tryptone, 2% yeast extract, 1% NaCl) for 16–18 h with continuous shaking at 37 °C until reaching stationary stage. Aliquot of overnight growing culture was transferred to fresh LB medium and incubated at 37 °C for 3 h (exponential phase culture). The growth phase of *E. coli* strain was based on standard growth curve. Stationary and exponential *E. coli* cells were collected by centrifugation at 3000 × *g* for 5 min (1×10^9 cells/sample or 1×10^7 cells/sample) and used for PEF and/or nisin treatment.

2.3. Analysis of antimicrobial activity of nisin-loaded pectin nanoparticles

Bacterial cells (1×10^9 or 1×10^7 CFU/mL) were mixed with nisin-pectin nanoparticle solution or pectic acid only (control). Samples with nisin-PecA nanoparticles were incubated at room temperature (20 °C) for 2 h, serial dilutions were performed in 0.9% NaCl and 50 μL of each solution was spread onto LB-agar plates with following overnight incubation at 37 °C. Colonies were counted and the CFU (colony forming units) in the samples after the nisin treatment (CFU_T) were compared with the CFU in the control samples (no treatment, CFU_C).