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Antimicrobial effect of emulsion-encapsulated isoeugenol against biofilms of food pathogens and spoilage bacteria

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ABSTRACT

Food-related biofilms can cause food-borne illnesses and spoilage, both of which are problems on a global level. Essential oils are compounds derived from plant material that have a potential to be used in natural food preservation in the future since they are natural antimicrobials. Bacterial biofilms are particularly resilient towards biocides, and preservatives that effectively eradicate biofilms are therefore needed. In this study, we test the antibacterial properties of emulsion-encapsulated and unencapsulated isoeugenol against biofilms of *Lis. monocytogenes*, *S. aureus*, *P. fluorescens* and *Leu. mesenteroides* in tryptic soy broth and carrot juice. We show that emulsion encapsulation enhances the antimicrobial properties of isoeugenol against biofilms in media but not in carrot juice. Some of the isoeugenol emulsions were coated with chitosan, and treatment of biofilms with these emulsions disrupted the biofilm structure. Furthermore, we show that addition of the surfactant Tween 80, which is commonly used to disperse oils in food, hampers the antibacterial properties of isoeugenol. This finding highlights that common food additives, such as surfactants, may have an adverse effect on the antibacterial activity of preservatives. Isoeugenol is a promising candidate as a future food preservative because it works almost equally well against planktonic bacteria and biofilms. Emulsion encapsulation has potential benefits for the efficacy of isoeugenol, but the effect of encapsulation depends on the properties of food matrix in which isoeugenol is to be applied.

1. Introduction

Biofilms are communities of microorganisms living at an interphase (solid-liquid or liquid-air), where they attach to each other through the extracellular polymeric substance (EPS) also known as the biofilm matrix. The EPS, which consists of extracellular DNA, proteins, lipids and polysaccharides, offers structural stability and protection (Flemming and Wingender, 2010). Bacteria in a biofilm show up to 1000 times greater tolerance to antibiotics and biocides than their planktonic counterparts (Ceri et al., 1999). Due to this resilience, biofilms are difficult to eliminate once they are formed, and they are therefore a persistent source of contamination in the food industry. Biofilms can develop directly on food or on food-contact surfaces whereby they cause contamination of the food (Shi and Zhu, 2009), leading to food spoilage or to the spread of foodborne illnesses. Nontyphoidal *Salmonella* serotypes, *Clostridium perfringens*, *Campylobacter* spp. and *Staphylococcus aureus* are amongst the top five of pathogens causing domestically acquired foodborne illnesses in the USA, and non-typhoidal *Salmonella*, *Listeria monocytogenes* and *Campylobacter* spp. are amongst the top five of pathogens causing death from foodborne illnesses in the USA (Scallan et al., 2011). All of these bacteria readily form biofilms. *Pseudomonas* and *Lactic Acid Bacteria* species are common causes of food spoilage (Gram et al., 2002). Relevant to this study, both *Pseudomonas fluorescens* and *Leuconostoc mesenteroides* are able to form biofilms. The Centers for Disease Control and Prevention have estimated that 48 million citizens in the U.S.A. become ill from ingesting foodborne pathogens every year (Scallan et al., 2011) and roughly one third of foods globally go to waste due to spoilage (Food and Agricultural Organization United Nations, 2011). These numbers underline that much can still be done to improve food preservation.

Essential oils have attracted attention in recent years as possible new food preservatives. They are volatile compounds extracted from plants and many possess antimicrobial properties (Burt, 2004). There is an increasing consumer demand for more natural food products and preservatives. Essential oils could potentially help in meeting this demand. We recently developed an emulsion encapsulation system for the essential

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oil isoeugenol using β-lactoglobulin or n-OSA starch, and found that emulsion encapsulation enhanced the antibacterial efficacy of isoeugenol against planktonic bacteria (Krogsgård Nielsen et al., 2016). Emulsion encapsulation enhanced the antibacterial efficacy against *Lis. monocytogenes* and *E. coli* in TSB and against *E. coli* in carrot juice, but not in milk. It was demonstrated through release profiles that isoeugenol was fully released from emulsions after 12 h. In this study, we investigate if emulsion encapsulation enhances the antibacterial efficacy of isoeugenol against biofilms of common food pathogens and spoilage bacteria. Emulsions were negatively charged and we therefore also investigated if coating emulsions with the positively charged biopolymer chitosan would further enhance the antibacterial efficacy by mediating contact with the surface of bacteria through electrostatic interactions.

2. Materials and methods

2.1. Emulsion encapsulation of isoeugenol

Emulsion encapsulation of isoeugenol was described and characterized in previous work (Krogsgård Nielsen et al., 2016). Briefly, the emulsifier n-OSA starch (3% (w/w), Cargill, Germany, C*Emcap 12635) and glucose syrup (24% (w/w), Cargill, Hamburg, Germany, C*Dry GL 01934) were dissolved in water before addition of isoeugenol (18% (w/w), Sigma-Aldrich, Copenhagen, Denmark, W246808). Pre-emulsions were prepared with a high-sheer homogenizer (Ystral, Germany) at 24,000 rpm for 1 min. The pre-emulsions were run through a high-pressure homogenizer (Niro Soavi) with a stage 1 pressure at 250 bar and a stage 2 pressure at 50 bar. The material was passed through twice. For emulsions coated with chitosan (Sigma-Aldrich), a 0.6% (w/v) chitosan solution was prepared in 0.172 M/0.028 M NaOAc/AcOH (pH 5.4) buffer and filtered before adding glucose syrup. The chitosan solution was mixed with the emulsion solution in a 1:1 ratio and passed through the high-pressure homogenizer with stage 1 pressure at 50 bar. The emulsions were spray-dried at 180/70 °C inlet/outlet temperature and 4 bar with rotary atomization (Niro A/S, Denmark).

2.2. Antibacterial efficacy

The antibacterial efficacy of isoeugenol emulsions with and without chitosan coating was measured as the Minimum Biofilm Eradication Concentration (MBEC) for biofilms of *Lis. monocytogenes* (NCTC 12426), *P. fluorescens* (DSMZ 4358), *Leu. mesenteroides*, (DuPont Collection Safety, DCS512) and *S. aureus* (DSMZ 2569) grown in TSB (30 g/L), pH adjusted to 6 with HCl (Merck 1054590500) at 6 °C, 12 °C, and 25 °C. In order to compare this with the antibacterial efficacy against planktonic bacteria we measured the Minimum Bactericidal Concentration (MBC) for the same organisms in TSB at 25 °C. Lastly, to test the effects in real foods, we measured MBECs at 6 °C and 25 °C in carrot juice (Biotta, lactofermented, pasteurized, organic).

2.2.1. Culture preparations

Cultures were kept in glycerol stocks at −80 °C and streaked once or twice on agar plates that were kept at 4 °C before use. Overnight cultures were prepared by inoculating from one colony of *S. aureus*, *P. fluorescens*, *Leu. mesenteroides* or *Lis. monocytogenes* into a 100 mL Erlenmeyer flask containing 20 mL TSB and incubating overnight at 25 °C with shaking at 180 rpm. All experiments were carried out with 3 biological replicates inoculated from individual colonies.

2.2.2. Minimum Bactericidal Concentration

Stock solutions of spray-dried emulsions were prepared by dissolution in TSB to a stock concentration of 8000 mg/L isoeugenol. Two controls were included: pure isoeugenol and isoeugenol with 0.5% (v/v) Tween 80 (Fluka analytical, Sigma-Aldrich, 59924). All concentrations of pure and encapsulated isoeugenol are given in mg/L isoeugenol. Pure isoeugenol was dissolved in TSB and for the control with Tween 80, isoeugenol was dissolved in 0.5% (v/v) Tween 80 in TSB. Both were shaken at 180 rpm for ca. 1 h.

We prepared a dilution series with 2/3 step sizes of stock solutions and 195 μL of each dilution was transferred to four wells in a microtiter plate (Nunclon™ surface, Nunc, cat. no.: 161093) – one well was used as a negative control (blank), whereas five μL inoculation culture was added to each of the other wells (one well per replicate culture). Inoculation cultures were prepared by diluting overnight cultures in fresh TSB to the following densities (OD_{600}) corresponding to ca 1.1×10^8 CFU/mL. These were for *Lis. monocytogenes*: 0.18, *P. fluorescens*: 0.18, *S. aureus*: 0.12, *Leu. mesenteroides*: 0.5.

The microtiter plates were then incubated at 25 °C for 24 h, and the presence of viable cells at the end of the incubation was determined by transferring 5 μL from each well to a new microtiter plate with 195 μL fresh TSB per well. Assuming that any surviving cells would give rise to planktonic growth, OD_{620} of this plate was measured before and after incubation at 25 °C for 72 h. The MBC was defined as the lowest concentration of isoeugenol resulting in no detection of viable cells by this measurement, corresponding to a >log 5 decrease in CFU during incubation with isoeugenol.

2.2.3. Minimum Biofilm Eradication Concentration

Biofilms were grown on peg lids (Thermo Scientific, Nunc-TSP, cat. no.: 445497) and the MBEC was determined as follows: Overnight cultures of *Lis. monocytogenes*, *S. aureus*, *P. fluorescens* and *Leu. mesenteroides* were diluted in fresh TSB to $OD_{620} = 0.5$, and 160 μL was transferred to wells in a microtiter plate. The peg lid was inoculated by inserting it into this plate and incubating at room temperature for 10 min. The peg lid was then transferred to a new microtiter plate containing 160 μL/well of TSB, and incubated in a zip-lock bag at 25 °C with orbital shaking at 50 rpm for 48 h to allow the adhered bacteria to grow and form a biofilm. After biofilms were formed, the biofilms were washed by inserting the peg lid twice in a microtiter plate containing 180 μL/well of Ringer's solution (RINGER tablets, Merck 1.15525.0001). All biofilms were prepared the same way, regardless of the subsequent incubation conditions during exposure to isoeugenol. Exposure to pure or encapsulated isoeugenol was obtained by transferring the peg lid with biofilms to a new microtiter plate (the “treatment plate”) containing the dilution series of isoeugenol emulsions, pure isoeugenol or isoeugenol with 0.5% (v/v) Tween 80 in TSB or carrot juice as described below. Peg lids with biofilms were incubated in the treatment plates for 24 h at 6 °C, 12 °C or 25 °C with orbital shaking at 50 rpm. After incubation, viability of the biofilms was investigated. The peg lids were washed once by insertion in microtiter plates with 180 μL/well of Ringer's solution and transferred to a new plate (the “recovery plate”) with 180 μL fresh TSB per well. Assuming that any viable cells in the biofilms would recover and lead to planktonic growth in these wells, the OD_{620} of this plate was measured before and after incubation with the peg lid at 25 °C for 72 h. The MBEC was defined as the lowest concentration of isoeugenol that resulted in no increase in OD_{620} of the recovery plate.

Dilution series of pure or encapsulated isoeugenol were prepared as follows: Stock solutions of spray-dried emulsions were prepared by dissolution in TSB or carrot juice to a stock concentration of 8000 mg/L isoeugenol. We prepared dilution series with step sizes of 2/3 and added 180 μL to each well. Growth controls did not contain isoeugenol.

2.3. Confocal laser scanning microscopy

To visualize the antibacterial efficacies of isoeugenol emulsions, we imaged biofilms of *P. fluorescens* and *S. aureus* via confocal laser scanning microscopy with live/dead staining with SYTO 60 (ThermoFisher Scientific, Waltman, MA USA), and TOTO-1 (ThermoFisher Scientific, Waltman, MA USA)