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Effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion

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ABSTRACT

D-Limonene has been considered to be a safer alternative compared to synthetic antimicrobial food additives. However, its hydrophobic and oxidative nature has limited its application in foods. The purpose of this research was to study effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion and develop a novel antimicrobial delivery system by combining the positive effect of these two antibacterial agents at the same time. By the checkerboard method, both the synergistic and additive effects of D-limonene and nisin were found against four selected food-related microorganisms. Then, D-limonene nanoemulsion with or without nisin was prepared by catastrophic phase inversion method, which has shown good droplet size and stability. The positive effects and outstanding antimicrobial activity of D-limonene nanoemulsion with nisin were confirmed by MICs comparison, scanning electron microscopy and determination of cell constituents released. Overall, the research described in the current article would be helpful in developing a more effective antimicrobial system for the production and preservation of foods.

1. Introduction

Microbial contamination of food has for long presented a major public health concern throughout the world (Settanni et al., 2012). It remains a significant source of human foodborne illness and causes severe economic losses for the food industry (Lynch, Painter, Woodruff, & Braden, 2006; Swanger & Rutherford, 2004). Despite the very effective and widespread application of traditional chemical preservatives and synthetic antimicrobials that are used to prevent the growth of food borne and spoilage microorganisms; many have been reported to cause adverse reactions in humans and even produce toxic substances and carcinogens (Fleming-Jones & Smith, 2003; Gutierrez, Barry-Ryan, & Bourke, 2009; Lv, Liang, Yuan, & Li, 2011). Natural antimicrobial substances may be regarded as preferred alternatives and could achieve comparable or improved preservative effects. D-limonene (4-isopropenyl-1-methylcyclohexene), is a major constituent in several citrus-derived essential oils (e.g. orange, lemon, mandarin, lime, grapefruit, etc.) and has been considered as generally regarded as safe (GRAS) for use as a flavouring agent and food preservative (Sun, 2007). Its outstanding antimicrobial activities have already been proven with different species of food-related microorganisms, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella enterica*, *Saccharomyces bayanus* and more (Chikhoune, Hazzit, Kerbouche, Baaliouamer, & Aissat, 2013; Settanni et al., 2012).

However, because of the hydrophobic nature of D-limonene and difficulties in achieving an even dispersion in water, the use of D-limonene requires the application of elevated concentrations in order to achieve equivalent antimicrobial efficiencies in foods. Furthermore, D-limonene is susceptible to oxidative degradation which directly results in its loss of activity (Li & Chiang, 2012; Sun, 2007).

In order to improve these limitations of hydrophobic, oxidation-prone biologically active compounds, a number of approaches have been explored. Many research groups have focused on combining antimicrobial agents. It was reported that combinations of essential oils with synergistic activity (Gutierrez et al., 2009), and the combinations of essential oils with other natural antibacterial compounds (e.g. Nisin) (Govaris, Solomakos, Pexara, & Chatzopoulou, 2010; Moosavy et al., 2008) could achieve effective antimicrobial activity at sufficiently low dosages and remarkably reduce the negative sensory impact on foods. Another efficient method for improving the apparent solubility of D-limonene is the application of nanoemulsion technology (Weiss, Gaysinsky, Davidson, & McClements, 2009; Donsì, Annunziata, Sessa, & Ferrari, 2011; Li, & Chiang, 2012). Nanoemulsions, owing to their subcellular size, provide an effective approach to improve the physical stability of the encapsulated active substances and increase the distribution

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of antimicrobial agents in food matrices where microorganisms are preferably located (Weiss et al., 2009). Nanoemulsions with essential oils and their components have shown to exhibit excellent antimicrobial properties against a range of different microorganisms with lower addition rates and very good stability (Donsì et al., 2011).

As mentioned before, smart combinations of antimicrobial substances can result in lower individual additions. Apart from limonene, nisin, a polycyclic antibacterial peptide produced by certain strains of *Lactococcus lactis*, is readily soluble in water and has good antimicrobial activity (García, Martínez, Rodríguez, & Rodríguez, 2010). To our knowledge, there are no reports about the combination of nisin and D-limonene, or the exploration of the effects of nisin on the antimicrobial activity of D-limonene nanoemulsion for achieving a better preservative. As such, the purpose of this research was to study the effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion and develop a novel antimicrobial delivery system by combining synergistic effect of these two antibacterial agents at the same time.

2. Materials and methods

2.1. Chemicals

D-Limonene was purchased from Florida Worldwide Citrus Products Group Inc. (Bradenton, Florida, USA). Nisin (10^4 IU/ml) was purchased from Lanzhou Weiri Bio-Engineering Co., Ltd., (Lanzhou, China). Sorbitan monooleate (Tween 80), propylene glycol, glutaraldehyde, sodium chloride (purity > 99.5%), kanamycin sulphate were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Double distilled and deionized water was filtered prior to use.

2.2. Preparation of nanoemulsions with catastrophic phase inversion (CPI) method

Catastrophic phase inversion (CPI) method is a low-energy method that is an inexpensive and energy efficient method that takes advantage of the chemical energy stored in the system. It has been reported that the nanoemulsions of essential oils prepared by CPI method were used in food preservation (Bilbao-Sainz, Avena-Bustillos, Wood, Willams, & McHugh, 2010; Binks & Lumsdon, 2000). In the tests, a water phase was prepared by mixing distilled water with propylene glycol in a 2:1 mass ratio, including a certain amount of nisin. Nanoemulsions were prepared from a mixture of oil phase (D-limonene) and Tween 80 (6% w/w) by slowly adding water phase with gentle agitation. The addition rate of water was kept constant at approximately 1.0 ml/min. Slowly adding the water phase into a mixture of oil phase and Tween 80, a W/O emulsion with a high oil-to-water ratio was formed, and then increasing amounts of water were added to the system with continuous stirring. The amount of water added to a W/O emulsion was progressively increased, until a phase inversion occurred and an O/W emulsion was formed with continuous stirring for 6 h.

2.3. Droplet size determination

Mean particle diameters (Z-averages) of samples were determined using Dynamic Light Scattering (DLS) at 25 °C (Zetasizer Nano-ZS90, Malvern Instruments, Malvern, Worcestershire, UK). The samples were placed in vertical cylindrical cuvettes (10 mm-diameter). The analysis was performed at a scattering angle of 90°, where each recorded measurement is an average of 10 scans.

2.4. Turbidity measurements

The turbidity was determined in duplicate using an UV-visible spectrophotometer at 600 nm (Ultra-spec 2450 pro, SHIMADZU Ltd., Kyoto, Japan). Distilled water was used as a reference to blank the cells.

2.5. Antimicrobial activity

2.5.1. Microbial strains and growth conditions

Four food-related microorganisms were used to assess the antimicrobial properties of the compounds tested here. These included the Gram-positive bacteria *Staphylococcus aureus* ATCC6538, and *Bacillus subtilis* ATCC 6633, the Gram-negative bacterium *Escherichia coli* ATCC 8739, and the yeast *S. cerevisiae* ATCC 9763. All strains were obtained from China General Microbiological Culture Collection Center (Beijing, China) and maintained on slants of Nutrient Agar (NA, Abxing, Beijing, China) for bacteria and Yeast Peptone Dextrose Agar (YPDA, Abxing, Beijing, China) for the yeast at 4 °C.

Active cultures were prepared by transferring a single colony from the agar slant to a test tube containing 5 ml of Nutrient Broth for bacteria and YPD Broth for the yeast. The bacterial and yeast cultures were incubated overnight at 37 and 30 °C, respectively. Cultures purity was examined by streaking each culture on plates of Nutrient Agar for bacteria and YPD Agar for yeast. The inoculums were prepared from overnight broth cultures and suspensions were adjusted to the required microbial density (1×10^8 CFU/ml) at 600 nm using the UV-visible spectrophotometer (Liang, Yuan, Vriesekoop, & Lv, 2012; Gilles, Zhao, An, & Agboola, 2010).

2.5.2. Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) values of antimicrobial agents or nanoemulsions were determined by a broth dilution method with some modifications as described by Weerakkody, Caffin, Turner and Dyke (2010). After adding appropriate volume of antimicrobial agents or nanoemulsions to the first tube containing 4 ml of broth, serial two-fold dilutions were made in a concentration range in 10 ml sterile test tubes containing Nutrient broth for bacteria and YPD broth for the yeast. A 400 μL suspension (1×10^8 CFU/ml) of tested microorganisms was added to each tube. A negative control tube contained broth and microorganism. Meanwhile, a positive control tube contained 50 μg/ml of kanamycin sulphate in broth and microorganism. MIC was defined as the concentration in the lowest serial dilution of the antimicrobial agents which resulted in the lack of visible microorganism growth in tubes after 24 h (bacteria) and 48 h (yeast) (Al-Reza, Rahman, Lee, & Kang, 2010; Lv et al., 2011).

2.5.3. Synergism testing: checkerboard method

The broth dilution checkerboard method, which has been frequently used to assess interactive inhibition of antimicrobial compounds in vitro (Hemaiswarya, Kruthiventi, & Doble, 2008), was used to determine the antimicrobial effects of D-limonene and nisin obtained in antimicrobial activity testing. The assay was arranged as follows: D-limonene was diluted two-fold in vertical orientation, while nisin was diluted two-fold in horizontal orientation. The concentrations of them were prepared corresponding to 1/2, 1/4 and 1/8 of the MIC values, respectively. Subsequently, 400 μL suspension containing 1×10^8 CFU/ml of the indicator strain was added to each tube. The inoculated tubes were incubated overnight at 37 °C for bacteria and 30 °C for the yeast, and then evaluated for their microbial growth.

The checkerboard method was then combined with calculation of fractional inhibitory concentration indices (FICI) to assess the antimicrobial effects of combinations. The FICI was calculated as