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Stability and Antimicrobial Efficiency of Eugenol Encapsulated in Surfactant Micelles as Affected by Temperature and pH

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ABSTRACT

Growth inhibition of four strains of *Escherichia coli* O157:H7 (H1730, F4546, 932, and E0019) and *Listeria monocytogenes* (Scott A, 101, 108, and 310) by eugenol encapsulated in water soluble micellar nonionic surfactant solutions (Surfynol 485W) adjusted to pH 5, 6, and 7 and incubated at 10, 22, and 32°C was determined. Concentrations of eugenol ranged from 0.2 to 0.9% at a surfactant concentration of 5%. Antimicrobial activity was assessed using a microbroth dilution assay. Eugenol encapsulated in surfactant micelles inhibited both microorganisms at pH 5, 6, and 7. At pH 5, some inhibition occurred in the absence of eugenol, i.e., by the surfactant itself (optical density at 24 h for *L. monocytogenes* = 0.07 and optical density at 24 h for *E. coli* O157:H7 = 0.09), but addition of >0.2% eugenol led to complete inhibition of both microorganisms. Inhibition of *L. monocytogenes* and *E. coli* O157:H7 decreased with increasing pH, that is, the minimum inhibitory concentration was 0.2, 0.5, and 0.5% of micellar encapsulated eugenol solutions at pH 5, 6, and 7, respectively. The encapsulated essential oil component in surfactant micelles was effective at all three temperatures tested (10, 22, and 32°C), indicating that the activity of encapsulated eugenol was not affected by high or low (refrigeration) temperatures. Overall, strains of *E. coli* O157:H7 were more sensitive than strains of *L. monocytogenes.* Improved activity was attributed to increased solubility of eugenol in the aqueous phase due to the presence of surfactants and improved interactions of antimicrobials with microorganisms.

Listeria monocytogenes and *Escherichia coli* O157:H7 are an important cause of foodborne illnesses throughout the world. The Centers for Disease Control and Prevention reported that approximately 76 million illnesses that occur each year in the United States are attributed to foodborne pathogens *(5)*. A reduction of food-related illnesses through control of the growth of foodborne pathogens is of key interest to the food industry to assure consumers of a safe food supply. Consequently, interest in the development of novel methods to reduce or eliminate foodborne pathogens is increasing *(4, 19)*. Because of the need to find more effective food antimicrobials that have a broad-spectrum activity along with difficulties in gaining regulatory approval for synthesized antimicrobials, naturally occurring food antimicrobials, such as spice extracts, have gained increased attention for application in food products *(3, 21)*.

The antimicrobial activity of spices and herbs has been attributed to the presence of active components in the essential oil (EO) extracts of these plants *(7, 31)*. Several researchers have shown that EO components inhibit the growth of pathogenic bacteria *(1, 2, 16, 18–20, 25, 31, 33)*. Many of these EO components are phenolic compounds, such as carvacrol and eugenol *(7, 21)*.

A general mode of action proposed for phenolic compounds involves insertion of the antimicrobial in the cytoplasmic membrane followed by a disruption of membrane integrity and proton motive force *(4, 6, 15, 17, 34)*. Differences in susceptibility to antimicrobial agents have been found between gram-positive and gram-negative bacteria. This can be attributed to the fact that a rigid wall of peptidoglycan surrounds gram-positive bacteria, whereas gram-negative bacteria have an outer membrane in addition to the cell wall *(23, 24)*. Because of the outer membrane, gram-negative bacteria are relatively resistant to hydrophobic antibiotics *(12, 22, 23)*. However, the outer membrane is not completely hydrophobic, and some compounds may pass through the membrane via porins *(12, 22, 26)*.

We previously demonstrated that encapsulation of EO components (eugenol and carvacrol) in surfactant micelles (Surfynol 465 and 485W) increased their inhibitory effect against *E. coli* O157:H7 and *L. monocytogenes* at pH 7 and 32°C *(12)*. The objective of this study was to determine the stability and antimicrobial activity of the most efficient system, eugenol encapsulated in Surfynol 485W, at different pHs and incubation temperatures against strains of two pathogenic bacteria, *L. monocytogenes* and *E. coli* O157:H7.

MATERIALS AND METHODS

Materials. All solutions were prepared with distilled and deionized water. Eugenol (4-allyl-2-methoxyphenol) was purchased from Sigma (St. Louis, Mo.). The nonionic surfactant Surfynol 485W was provided by Air Products and Chemical, Inc. (Allentown, Pa.). The critical micellar concentration is defined as the concentration at which micelle formation begins. The critical mi-

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cellar concentration for Surfynol 485W is 1.65% (wt/wt). The pH was adjusted with 0.1 N HCl or NaOH.

Preparation of eugenol-containing Surfynol 485W micelles. Aqueous surfactant solutions were prepared by dispersing Surfynol 485W in water at room temperature to obtain surfactant solutions with a concentration of 1, 2, 3.5, 5, 7.5, and 10% (wt/wt). Eugenol was added to surfactant solutions at concentrations ranging from 0.025 to 3% (wt/wt). Solutions were stirred until the absorbance remained constant, indicating that solubilization was complete (typically less than 10 min). Solutions were filter sterilized using a 0.22-μm cellulose acetate membrane (Corning, Corning, N.Y.) and stored at 25 ± 2°C until used but not longer than 2 weeks.

Temperature and pH stability of eugenol-containing micelles. The stability of micellar encapsulated eugenol as a function of pH and temperature was determined by measuring the absorbance of the surfactant-antimicrobial combinations (5% Surfynol 485W and 0.1 to 0.9% eugenol) that were adjusted to pH 3 to 9 using HCl and NaOH as a function of temperature (25 to 90°C) at 632 nm using a UV-visible spectrophotometer (8452A Diode Array Spectrophotometer, Hewlett Packard, Palo Alto, Calif.). The wavelength of 632 nm was chosen to avoid interference with the intrinsic absorbance of eugenol.

Bacteria and growth conditions. Growth inhibition of four different *L. monocytogenes* strains (Scott A, 101, 108, and 310) and four strains of *E. coli* O157:H7 (H1730, F4546, 932, and E0019) was investigated. Bacterial cultures were maintained on slants stored at 4°C. A loopful of the culture was transferred to tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) and incubated at 32°C for 24 h. Before exposure to antimicrobials, each strain was subcultured in TSB for 18 h. A microbroth dilution assay *(8)* was used to determine the MIC of eugenol and Surfynol 485W at pH 5, 6, and 7. Microtiter plate wells were filled with 120 μl of double-strength micellar solutions and 120 μl of inoculated double-strength TSB (approximately 5×10^7 CFU/ml) adjusted to pH 5, 6, and 7 using HCl. Plates were incubated at 10, 22, and 32°C. For temperatures of 22 and 32°C, the optical density at 630 nm (OD_{630}) was monitored periodically for 24 h using an El_x800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, Vt.). At 10°C, OD_{630} was read every 24 h for 168 h to account for the slower growth kinetics at the reduced temperature. The MICs were defined as the lowest concentration at which growth was completed inhibited ($OD < 0.07$) after 24 h. All samples were run in duplicate.

Statistical analysis. Tukey's multiple range test was used to determine significant differences ($P < 0.05$) between treatments. Least squares means were analyzed using the general linear model of the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.).

RESULTS

MAC of eugenol in Surfynol 485W. The maximum additive concentration (MAC) is the largest amount of solute that can be incorporated into a micellar solution at a given surfactant concentration. It is a key parameter for systems that contain swollen micelles, because it governs the applicable concentration range. The MAC increased as the surfactant concentration increased. For example, at a surfactant concentration of 2, 3.5, 5, 7.5, and 10% (wt), the MAC was 0.35, 0.87, 0.96, 2 and 2.66% (wt), respectively. Absorbance remained zero, because eugenol was titrated into the solution until the MAC was reached (data not shown). Absorbance increased significantly above the MAC, indicating that micelles were fully saturated and were no longer able to take up eugenol and eventually phase separated. The MAC for Surfynol 485W at 5% surfactant, a typical surfactant concentration used in many emulsion applications, for eugenol was 0.96%. Consequently, all subsequent antimicrobial experiments were conducted at a Surfynol 485W concentration of 5% and eugenol concentrations below the MAC (0.96%) to prevent phase separation and ensure testing of partially and fully filled micelles.

Temperature and pH stability of encapsulated surfactant micelles. All nanoparticles were stable over a wide range of pHs; however, temperature and EO component concentration influenced the stability of the mixed micelles. Figure 1A shows the results of such experiments at pH 7. The absorbance of solutions remained zero until a critical temperature was reached, at which point the absorbance rapidly increased. The critical temperature indicated the so-called cloud point, i.e., the point where micelles phase invert and release the encapsulated compound because of increased dehydration of the polar head group of the surfactant *(27, 30)*. The cloud point decreased with increasing eugenol concentration. For example, at a eugenol concentration of 0.1%, micelles remained stable even if solutions were heated to 90°C. In contrast, at a eugenol concentration of 0.9%, the cloud point decreased to 55°C (Fig. 1B). Results indicate that the thermal stability of micelles decreased with concentration of antimicrobial.

Similarly, the thermal stability of eugenol-containing Surfynol 485W micelles at different pHs was evaluated (Fig. 1B). Variation in pH did not affect significantly the thermal stability of micelles. At pH 3, a small (5°C) decrease in the overall thermal stability of the surfactants was observed, but compared with the decline of thermal stability with increasing EO component concentration, the decrease was not significant ($P < 0.05$).

Influence of pH on growth kinetics of *L. monocytogenes* in the presence of encapsulated eugenol at 22 and 32°C. The growth of the most resistant strain of *L. monocytogenes*, Scott A, at pH 5, 6, and 7 at incubation temperatures of 22 and 32°C in the presence and absence of eugenol-Surfynol combinations is shown in Figure 2. The presence of the surfactant in the absence of eugenol (Surfynol) reduced growth of *Listeria* slightly at pH 5. The OD of both the control cultures and the Surfynol 485W solution increased from 0.02 (base OD of media and antimicrobial preparation) to maximally 0.4 after 24 h. Slightly reduced growth in the presence of surfactant was observed in the case of *L. monocytogenes* at pH 5 at 22 and 32°C (Fig. 2A and 2D). As expected, growth in the absence of eugenol was both temperature and pH dependent. The OD of cultures after 24 h at 22°C increased to 0.19, 0.24, and 0.4 at pH 5, 6, and 7, respectively, indicating that growth was slightly inhibited at lower pH (Fig. 2A through 2C). Growth at 32°C at pH 7 was more rapid than at 22°C (Fig. 2F). No significant difference in growth at pH 5 and 6 be-

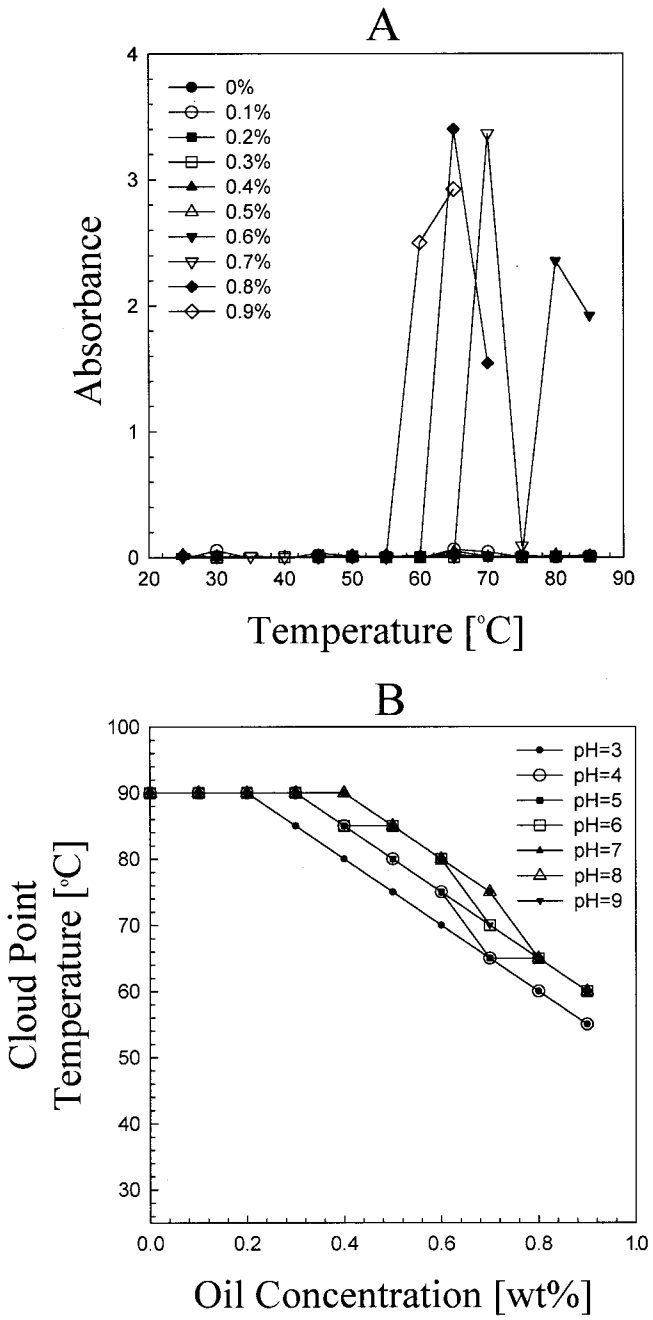

FIGURE 1. *Stability of Surfynol 485W and eugenol nanoparticles at pHs ranging from 3 to 9 and temperatures ranging from 25 to 90°C at eugenol concentrations of 0 to 0.9%. (A) Absorbance of a 5% Surfynol solution titrated with 0 to 0.9% eugenol as a function of temperature at pH 7. (B) Cloud point (temperature where solution turbidity increases above zero) as a function of pH and temperature for Surfynol solutions that contain 0 to 0.9% eugenol.*

tween 22 and 32°C was observed (Fig. 2A, 2B, 2D, and 2E). Similar results were observed for all other strains (data not shown).

Incorporation of eugenol in surfactant micelles strongly inhibited growth of *L. monocytogenes* (Fig. 2), confirming the potent antimicrobial activity of the eugenol-Surfynol 485W combination. Above 0.5% eugenol, growth of *L. monocytogenes* was completely inhibited. Growth inhibition at 22°C with 0.2% eugenol was pH dependent, i.e., the OD after 24 h increased from 0.02 to 0.03, 0.11, and 0.32 at pH 5, 6, and 7, respectively (Fig. 2A through 2C), and similar results were observed at 32°C (Fig. 2D through 2F). Using an MIC definition based on a minimum OD increase of 0.07 between 0 and 24 h, the MIC decreased from 0.5 to 0.2 on a reduction of pH to 5 regardless of incubation temperature (Fig. 2A and 2D). Less resistant strains were inhibited by 0.2% of eugenol at pH 6 and 7 as well (data not shown), i.e., no growth was observed in the presence of any of the tested eugenol-Surfynol combinations.

Influence of pH on growth kinetics of *E. coli* O157:H7 in the presence of encapsulated eugenol at 22 and 32°C. The growth of the most resistant strain of *E. coli* O157:H7, F4546, at pH 5, 6, and 7 at incubation temperatures of 22 and 32°C in the presence and absence of eugenol-Surfynol combinations is shown in Figure 3. Presence of Surfynol micelles without eugenol did not inhibit growth of *E. coli* compared with the control. A slight inhibition by the surfactant is shown at pH 7.0 at both temperatures (Fig. 3C and 3F). Growth rate decreased in the controls and in the presence of Surfynol 485W–only micelles at 22°C at lower pHs, i.e., the OD decreased from 0.6 to 0.52 and 0.21 as the pH was reduced from 7 to 5, respectively (Fig. 3A through 3C). Growth of controls was slightly greater at 32 than 22°C. The OD increased at pH 7 to a maximum of 1.1 at 32°C compared with 0.6 at 22°C. Addition of eugenol-containing Surfynol micelles completely suppressed growth of *E. coli* O157:H7 at all tested concentrations (Fig. 3). There was no significant increase in OD of culture suspensions that contained 0.2% eugenol and 5% Surfynol during the 48-h incubation period.

Growth kinetics of four strains of *L. monocytogenes* in the presence of encapsulated eugenol at 10°C. *L. monocytogenes* is a psychrotrophic microorganism and can thus grow at refrigeration temperatures. The change in OD as a function of time for all four tested strains of *L. monocytogenes* incubated at 10°C at pH 7 is shown in Figure 4. Presence of Surfynol micelles without eugenol inhibited growth of three strains of *L. monocytogenes* (Scott A, 101, and 310) compared with the control. *L. monocytogenes* 108 showed a slight inhibition in the presence of the surfactant micelles. Results indicated that maximal growth of the controls was reached after 60 h. Addition of ≥0.2% eugenol inhibited growth of all strains for the entire incubation period (168 h), indicating that the micellar encapsulated eugenol was effective even at 10°C. Similar results were found at pH 5 and 6 (data not shown).

DISCUSSION

Surfactant micelles incorporate relatively large amounts of nonpolar compounds in their interior. The formation of micelles in an aqueous phase and the inclusion of hydrophobic compounds are thermodynamically driven. Surfactant micelles are nanometer-sized aggregates of surfactants, amphiphilic molecules that are surface active. The self-assembly process enables the hydrophobic tails of surfactant monomers to minimize undesirable interactions with water molecules by forming spherical particles in which the

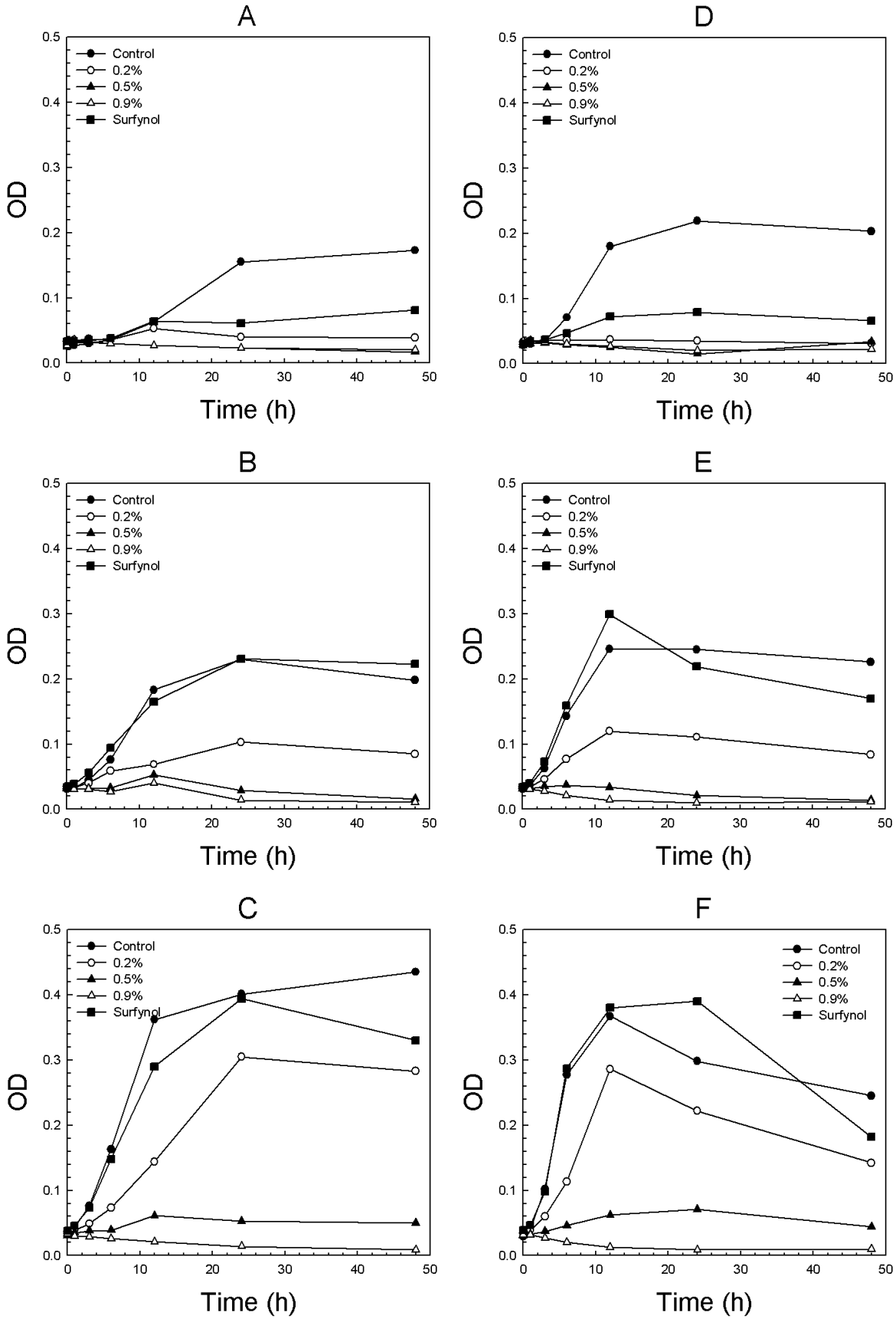

FIGURE 2. *Growth of* Listeria monocytogenes *Scott A as measured by* OD_{632} *in the presence of 5% Surfynol 485W and 0, 0.2, 0.5, and 0.9% eugenol as a function of incubation time at (A) pH 5, 22°C; (B) pH 6, 22°C; (C) pH 7, 22°C; (D) pH 5, 32°C; (E) pH 6, 32°C; and (F) pH 7, 32°C.*

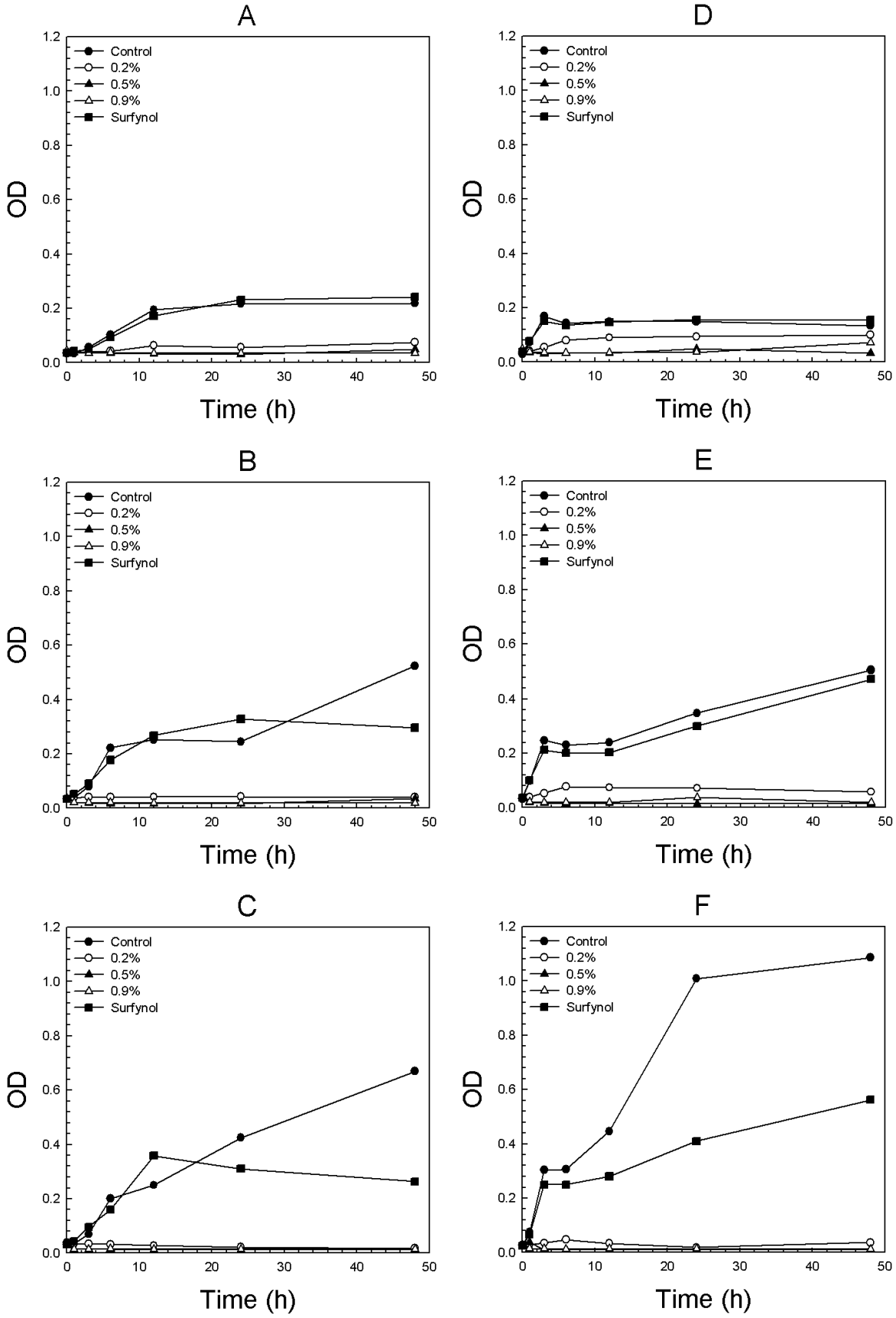

FIGURE 3. *Growth of* Escherichia coli *O157:H7 F4546 as measured by* OD_{632} *in the presence of 5% Surfynol 485W and 0, 0.2, 0.5, and 0.9% eugenol as a function of incubation time at (A) pH 5, 22°C; (B) pH 6, 22°C; (C) pH 7, 22°C; (D) pH 5, 32°C; (E) pH 6, 32°C; and (F) pH 7, 32°C.*

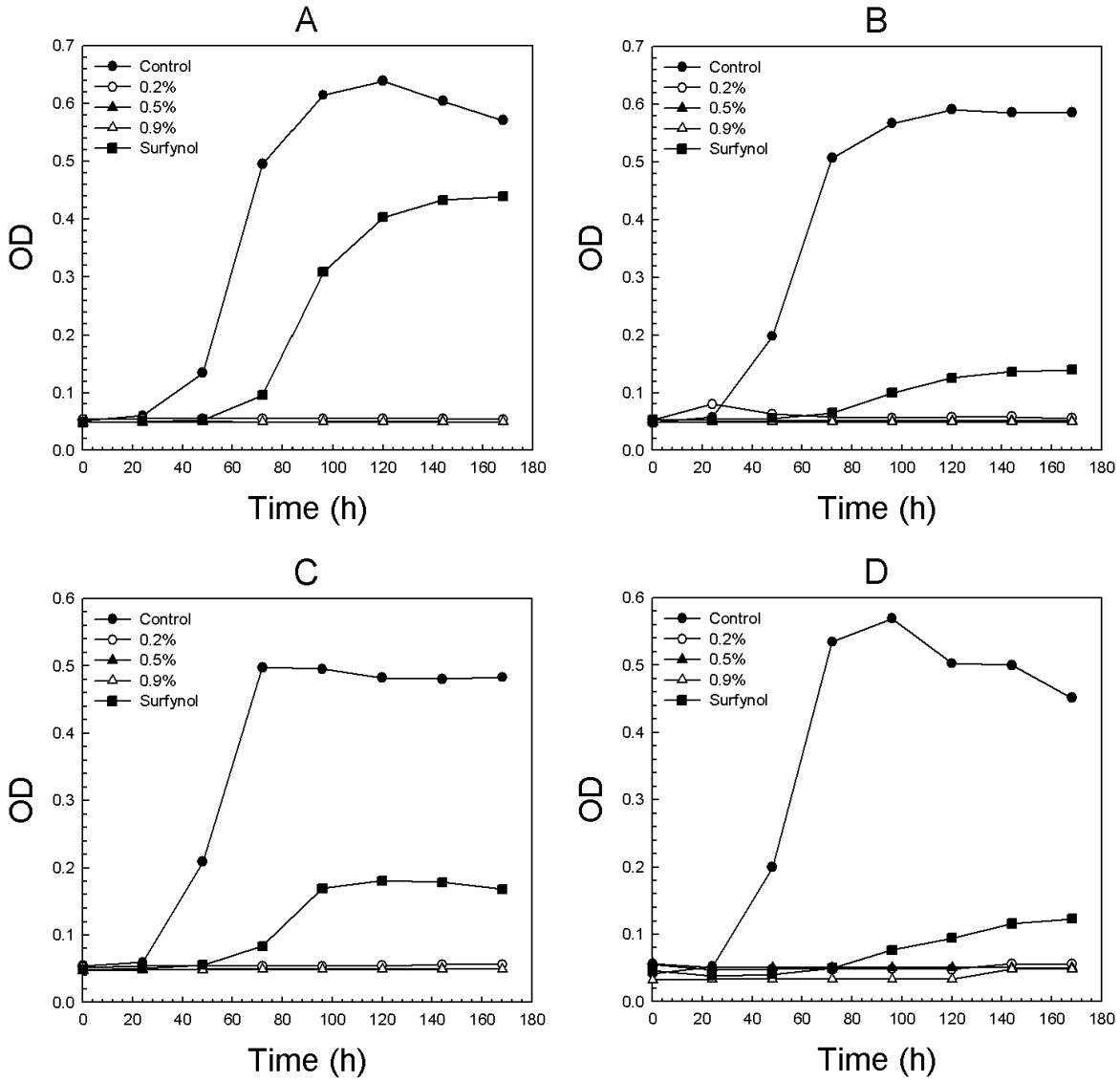

FIGURE 4. *Growth of four strains of* Listeria monocytogenes *at 10°C as represented by increase in* OD_{632} *in the presence of 5% Surfynol 485W and 0, 0.2, 0.5, and 0.9% eugenol as a function of incubation time at pH 7: (A) 108, (B) Scott A, (C) 310, and (D) 101.*

hydrophobic tails are located in the interior of the aggregate and the hydrophilic head groups are located at the exterior of the aggregate, where they can interact with water *(9)*. Consequently, the interior of micelles is hydrophobic in nature and is said to have properties similar to that of an organic solvent. Micelles are therefore capable of encapsulating lipophilic antimicrobials, a class to which phenolics such as eugenol and carvacrol belong. The solubilization capacity is a function of the nature of the surfactant monomer, which determines the size of the micelles through surfactant-surfactant interactions and the chemical nature of the antimicrobial, which determines the surfactant-antimicrobial interactions *(14, 17)*

Surfynol 485W, a surfactant used in the food industry, has been demonstrated to have a very high solubilization capacity. MAC values are generally very large. Because of this, the surfactant was chosen as a model system for our experiments. Surfynol 485W can incorporate up to 2.66% of EO component at 10% and 0.96% at 5%. Other surfactants have typical MACs that are significantly lower than that of the Surfynol. In a solubilization study with a series of short chain alkanes and alkenes, Weiss *(35)* demonstrated that the MAC of Tween 20 for hexadecane at a surfactant concentration of 5% was less than 0.045% or approximately 1/20th that of Surfynol 485W and eugenol. It has been suggested that this extraordinary capacity of Surfynol to include phenolics such as eugenol and carvacrol is due to the formation of a mixed micelle, a micelle that consists of a shell composed of both the solute and the surfactant monomers *(11)*. This is, for example, the case for compounds that possess polar groups that may interact with water molecules. Eugenol, a 2-methoxy-4-(2-propenyl)-phenol, has a methoxyl and phenol group that may well show some interaction with water, despite the fact that the overall solubility of the molecule in water is virtually zero. The mixed micellar structure may contribute to the enhanced antimicrobial activity of the micelle, since eugenol molecules can directly interact with cell membranes on interaction of the micelle with bacterial surfaces. As such, mixed micelles can be thought of as transport vehicles that allow the antimi-

crobial to better interact with foodborne pathogens. They act as virtual ''solubilizers'' by increasing the amount of antimicrobial that can be dissolved in the aqueous phase. Since the mass transport process is driven by the concentration difference between the aqueous phase and the interior of the bacterial cell and cell membrane, an increase in the rate of antimicrobial uptake followed by accelerated destabilization and loss of biological activity of bacterial membranes is observed. Clearly, this differentiates the high antimicrobial activity achieved using the nanostructured systems with traditional applications of eugenol, including ethanol or emulsion-based systems, that have significantly reduced and selective antimicrobial activity.

Because of the low solubility of EO components, they are generally incorporated into agar media, forming an emulsion by adding emulsifiers or solvents such as ethanol to disperse the EO component *(14, 15, 28)*. It is hypothesized that the type of emulsifiers and the interaction with other solvents may actually interfere with the activity of EO components. It has also been suggested that solubilization of eugenol into micellar systems may improve the subsequent interaction between the cell membrane and phytophenols. Balzyk and Holley *(2)* showed that at 1,000 μg/ml of eugenol, growth of *E. coli* O157:H7 and *L. monocytogenes* was inhibited, demonstrating the antimicrobial potency of this EO against these two pathogenic bacteria. In this study, Tween 20 was used at ≥0.25% to disperse the compound in the aqueous phase. Some eugenol may have been incorporated into the surfactant micelles that were spontaneously formed by this surfactant, but since the MAC of Tween 20 is very low, the eugenol concentrations were most likely <0.05% *(35)*. Clearly, there is a distinct difference between adding antimicrobials in the form of emulsion droplets and adding antimicrobials in the form of swollen micelles. Remmal et al. *(28)* showed that when 10% of the EO was added to 2.5% of the emulsifying agent or 2% (wt/wt) to ethanol, the antimicrobial activity varied. Ethanol had higher interference than those systems prepared with Tween 20, Tween 80, and Triton X-100. For example, oregano EO was found to be more efficient than thyme EO against *Bacillus megaterium* when ethanol or Tween 80 were used, whereas it was less active when the emulsion was prepared with Tween 20. However, Juven et al. *(15)* showed that Tween 80 may cause an increase in hydrophilicity of thymol and cellular membrane proteins, causing an increased interaction between compounds in the aqueous phase and phytophenols.

These data showed that the type of emulsifying agent or solubilizing agent used may interfere or enhance the activity of EO or EO components. Also, the type of antimicrobial test, the type and strain of microorganism, and conditions such as temperature and pH may affect how the surfactant and the EO or EO component interact.

Nonionic surfactant micelles have been shown to be thermodynamically stable systems that are able to withstand elevated temperatures and extreme pHs *(27, 32)*. Nevertheless, temperature has a significant effect on the organization and stability of surfactant micelles. The cloud point of a nonionic surfactant is the temperature at which a surfactant solution becomes turbid. This point varies, depending on the structure of the nonionic surfactant (i.e., length of the polyoxyethylene groups) and the nonpolar compound solubilized. The development of a cloud point indicates a phase separation that is due to the increase in aggregation number of the micelles and the low intermicellar repulsion that is a direct consequence of the dehydration of the oxyethylene groups in the polyoxyethylene chain with increasing temperature *(32)*. Addition of cosurfactants has been shown to generally lower the temperature stability of micelles *(28, 30)*. Similar results were found in this study, where the temperature stability of Surfynol 485W micelles on inclusion of eugenol decreased.

A change in system pH had little or no effect on stability of the encapsulated eugenol system. This may be attributed to the fact that the surfactant used is a nonionic and carries a zero net charge regardless of system pH. Unlike anionic or cationic surfactants, Surfynols do not dissociate into positively charged cations or negatively charged anions. The results are therefore not unexpected and confirm that nonionic surfactants are not only highly functional emulsifiers for food systems, where pHs may vary depending on application, but are also best suited to serve as carrier systems for lipophilic antimicrobials. It should be noted that results may be significantly different if ionic surfactants are used, an investigation that is currently under way.

A comparison of *E. coli* O157:H7 and *L. monocytogenes* susceptibility to these systems indicates that *E. coli* O157:H7, a gram-negative bacterium, is more strongly inhibited by the Surfynol-eugenol combinations. These results are interesting, because other studies have shown that gram-negative bacteria are, in general, more resistant to hydrophobic antimicrobial compounds due to the presence of the outer membrane *(13)*. The precise reason behind this observation is not clear, but other authors have suggested that biological membranes (such as the outer membrane) may be solubilized by surfactants above 1% *(10, 13)*. For example, it has been reported that Tween 20 improved the diffusion and enhanced the antimicrobial activity of nisin *(13)*. The inclusion of the phenolic antimicrobial (eugenol) in a surfactant micelle resulted in a 10-nm particle that has a hydrophilic surface *(11)*. The change in the molecular nature of the antimicrobial may allow passage through the previously impermeable membrane; however, more detailed studies on the interaction of a mixed micellar system with model membranes will be required to verify this hypothesis.

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