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Characterization and antimicrobial activity of a pharmaceutical microemulsion

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ABSTRACT

The characterization of a pharmaceutical microemulsion system with glycerol monolaurate as oil, ethanol as cosurfactant, Tween 40 as surfactant, sodium diacetate and water, and the antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger* and *Penicillium expansum* have been studied. The influence of ethanol and sodium diacetate on oil solubilization capability was clearly reflected in the phase behavior of these systems. One microemulsion formulation was obtained and remained stable by physical stability studies. The antimicrobial assay using solid medium diffusion method showed that the prepared microemulsion was comparable to the commonly used antimicrobials as positive controls. The kinetics of killing experiments demonstrated that the microemulsion caused a complete loss of viability of bacterial cells (*E. coli*, *S. aureus* and *B. subtilis*) in 1 min, killed over 99% *A. niger* and *P. expansum* spores and 99.9% *C. albicans* cells rapidly within 2 min and resulted in a complete loss of fungal viability in 5 min. The fast killing kinetics of the microemulsion was in good agreement with the transmission electron microscopy observations, indicating the antimembrane activity of the microemulsion on bacterial and fungal cells due to the disruption and dysfunction of biological membranes and cell walls.

1. Introduction

Microemulsions are thermodynamically stable, transparent and isotropic dispersions consisting of oil and water stabilized by an interfacial film of surfactant molecules, typically in conjunction with a cosurfactant. There are a limited number of reports in the literature on microemulsion use for antimicrobial purposes. It was reported that an oil-in-water (o/w) microemulsion as pharmaceutical preparations gave a 5 log reduction in the numbers of *Staphylococcus aureus* or *Pseudomonas aeruginosa* in only 45 s (Al-Adham et al., 2000), and effectively reduced the viability of an established biofilm population of *P. aeruginosa* (Al-Adham et al., 2003). A recent report (Teixeira et al., 2007) corroborated some of the previous observations and found that the o/w microemulsion was active against five bacterial pathogens.

Engineering drug itself in nanoparticulate form has emerged as a new strategy for the delivery of hydrophobic drugs due to their unique advantages over colloidal drug carriers (Date and Patravale, 2004). This study was conducted to load glycerol monolaurate (GML), a generally recognized as safe (GRAS) antimicrobial lipid (Shibasaki, 1982), in a pharmaceutical microemulsion system for the control of typical bacteria and fungi. Our previous work (Fu et al., 2006) has confirmed the feasibility of microemulsions which were prepared with GML as oil and Tweens as surfactant for pharmaceutical use.

The microemulsion formulations, by the addition of short-chain alcohols, have been recently discussed (Yaghmur et al., 2002). It was reported that the addition of short-chain alcohol (such as ethanol), which increased the penetration of the surfactant film and decreased the polarity of water, seemed to favor the formation of microemulsions.

The ability of Tweens to form microemulsions for pharmaceutical applications has been studied by few authors (Prichanont et al., 2000; Radomska and Dobrucki, 2000). It was observed, in some investigations, the goal of formulating isotropic water dilution lines was easily achieved in the Tween-based system (Garti et al., 2001).

There have been many investigations about the effects of added salts on the phase behavior (Kabalnov et al., 1995; Iwanaga et al., 1998). Sodium diacetate (SD), the sodium salt of the acetic acid, is an economical GRAS ingredient by the US FDA (Shelef and Addala, 1994). Our previous work (Zhang et al., 2008a) showed that the solubilization of water-soluble salts with antimicrobial properties in microemulsions could enhance the antimicrobial effects.

A preliminary study on the effects of possible compositions, including short-chain alcohols and several nonionic surfactants, on the formulation of GML-loaded microemulsions has been conducted (Zhang et al., 2008b). In the present work, attempts were made to establish a pharmaceutical microemulsion system with GML as oil, ethanol as cosurfactant, Tween 40 as surfactant, SD and water, and evaluate the antimicrobial effects against typical

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bacteria (*Escherichia coli*, *S. aureus*, *Bacillus subtilis*), yeast (*Candida albicans*) and mycelial fungi (*Aspergillus niger*, *Penicillium italicum*).

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Glycerol monolaurate (GML) (>99%) was supplied by Hangzhou Kangyuan Food Science & Technology Co., Ltd., China. Ethoxylated sorbitan esters Tween 40 (ethoxylated-20 EO-sorbitan monopalmitate) (Wenzhou Qingming Chemical Engineering Company, China) was of commercial grade. Ethanol, sodium diacetate (SD), sodium lactate and potassium sorbate were of analytical grade. Natamycin (50%) was purchased from DSM Food Specialties, Netherlands. All these components were used without further purification. The water was double-distilled.

2.1.2. Microorganism

The strains *E. coli* CMCC(B) 44102, *S. aureus* CMCC(B) 26003, *B. subtilis* CMCC(B) 63501, *C. albicans* CMCC(F) 98001, *A. niger* CMCC(F) 98003 and *Penicillium expansum* ATCC 1117 were provided by Institute of Microbiology, Chinese Academy of Sciences and preserved at the Department of Food Science and Nutrition, Zhejiang University. The bacteria (*E. coli*, *S. aureus* and *B. subtilis*) were maintained in Nutrient Agar medium (NA, Hangzhou Microbiological Agents Co., Ltd., China) at 37 °C, the yeast (*C. albicans*) was maintained in Sabouraud Dextrose Agar medium (SDA, Hangzhou Base Biotech Co., Ltd., China) at 28 °C, and the mycelial fungi (*A. niger* and *P. expansum*) were maintained on Potato Dextrose Agar medium (PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g; and deionized water, 800 ml) at 28 °C.

2.2. Phase diagrams

The behavior of multi-component microemulsions was described on a pseudo-ternary phase diagram. The phase diagrams were constructed as reported recently (Zhang et al., 2008b) at 25 °C. Briefly, mixtures of surfactant–oil phase (alcohol and oil) were prepared in glass test tubes sealed with caps at predetermined weight ratios of oil phase (alcohol plus oil) to surfactant and kept in a 25 °C water bath. Microemulsion areas were determined in phase diagrams by titrating the oil–surfactant phase with the aqueous phase. All samples were vigorously stirred. The samples were allowed to equilibrate at 25 °C for at least 24 h before they were examined. Every sample, which remained transparent and homogeneous after vigorous vortexing, was considered as belonging to a monophasic area in the phase diagram. The accuracy in the location of the phase boundaries is within 4 wt.%.

Two suitable solubilization parameters were used in this work to describe the solubilization capacities of GML-loaded microemulsions: maximal amount of aqueous phase, denoted as W_m, and total monophasic region, denoted as A_T. These solubilization parameters have been used by many other researchers (Garti et al., 2001; Yaghmur et al., 2002).

2.3. Antimicrobial activity assay

2.3.1. Solid medium diffusion method

Solid medium diffusion procedure using wells in plates was used to determine the antimicrobial activity of the prepared microemulsion. Petri plates (85 mm × 15 mm) were prepared by pouring 15 ml of NA for bacteria, SDA for yeast and PDA for mycelial fungi, and allowed to solidify. Plates were dried and 0.1 ml of an inoculum suspension (bacteria, 1.5×10^4 cfu/ml; yeast, 1.5×10^4 cfu/ml; mycelial fungi, 2.5×10^3 spores/ml) was poured and uniformly spread. After inoculum absorption by agar, wells were made using sterile tubes (diameter 5 mm) which were filled with 100 μl of the microemulsion. The plates were incubated in incubators at 37 °C for 24 h (bacteria), 28 °C for 48 h (yeast) and 28 °C for 72–96 h (mycelial fungi). Positive controls used in this assay were sodium lactate (for bacteria), natamycin (for yeast) and potassium sorbate (for mycelial fungi). The inhibition zones were measured in millimetres using vernier calipers. Each assay in this experiment was carried out in triplicate.

2.3.2. Kinetics of killing

This experiment was designed according to the method of Al-Adham et al. (2000) with modifications. The bacterial or fungal cultures in fresh medium with a known inoculum size (*E. coli*, 2.0×10^8 cfu/ml; *S. aureus*, 2.0×10^8 cfu/ml; *B. subtilis*, 2.0×10^8 cfu/ml; *C. albicans*, 9.0×10^6 cfu/ml; *A. niger*, 2.4×10^6 spores/ml; *P. expansum*, 1.6×10^6 spores/ml) were added at 1% (v/v) to the microemulsion and incubated on a tube rotator at 200 rpm for 10 min at 37 °C (for bacteria) and 28 °C (for fungi). 0.1 ml samples were taken from each tube at 1, 2, 5, 10 min, and prepared by dilution series for viable counts on sterile NA plates (bacteria) at 37 °C for 24 h, SDA plates (yeast) and PDA plates at 28 °C for 48 h (mycelial fungi). The experiments were performed in duplicate for each set of conditions.

2.3.3. Transmission electron microscopy

The microorganism cells, either untreated or treated with the microemulsion, were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.0), washed three times in phosphate buffer, then post-fixed with 1% osmium tetroxide in phosphate buffer (pH 7.0) for 1 h and washed three times in the phosphate buffer. The specimen were dehydrated by a graded series of ethanol (50%, 70%, 80%, 90%, 95% and 100%) for about 15–20 min at each step, and transferred to absolute acetone for 20 min. After placed in 1:1 mixture of absolute acetone and the final Spurr resin mixture for 1 h at room temperature, the specimen were transferred to 1:3 mixture of absolute acetone and the final resin mixture for 3 h and to final Spurr resin mixture for overnight. After placed in capsules contained embedding medium and heated at 70 °C for about 9 h, the specimen sections were stained by uranyl acetate and alkaline lead citrate for 15 min, and observed in the transmission electron microscopy (TEM) of Model JEM-1230 (JEOL, Japan).

3. Results

3.1. Characterization of microemulsion system

Ethanol with different concentrations in oil phase was studied in the present work (see Fig. 1a–d). The phase diagram of a three-component system of GML/Tween 40/water is presented in Fig. 1a, it could be clearly seen that a small microemulsion region [A_T = 16.5%] was formed. The changes of the oil solubilization in the presence of ethanol are dramatic. When ethanol was used at a 1:3 weight ratio to GML (Fig. 1b), the one-phase region increased [A_T = 21.7%]. When the weight ratio of GML to ethanol was 1:1 (Fig. 1c), the area of the two-phase region shrank significantly [A_T = 81.4%; W_m (50) = 52.5%]. The solubilization parameters increased further [A_T = 92.0%; W_m (70) = 50.0%] when GML/ethanol = 1:3 was used (Fig. 1d).

The phase diagrams of the system GML/ethanol/Tween 40/SD/water are presented in Fig. 1e–g. When the weight ratio of Tween 40/SD was 3:1 (Fig. 1e), a larger one-phase region dominated most of the phase diagram area [A_T = 77.2%]. However, when Tween 40/SD = 1:1 (Fig. 1f) or 1:3 (Fig. 1g) was used, the microemulsion area diminished greatly [A_T < 40%].

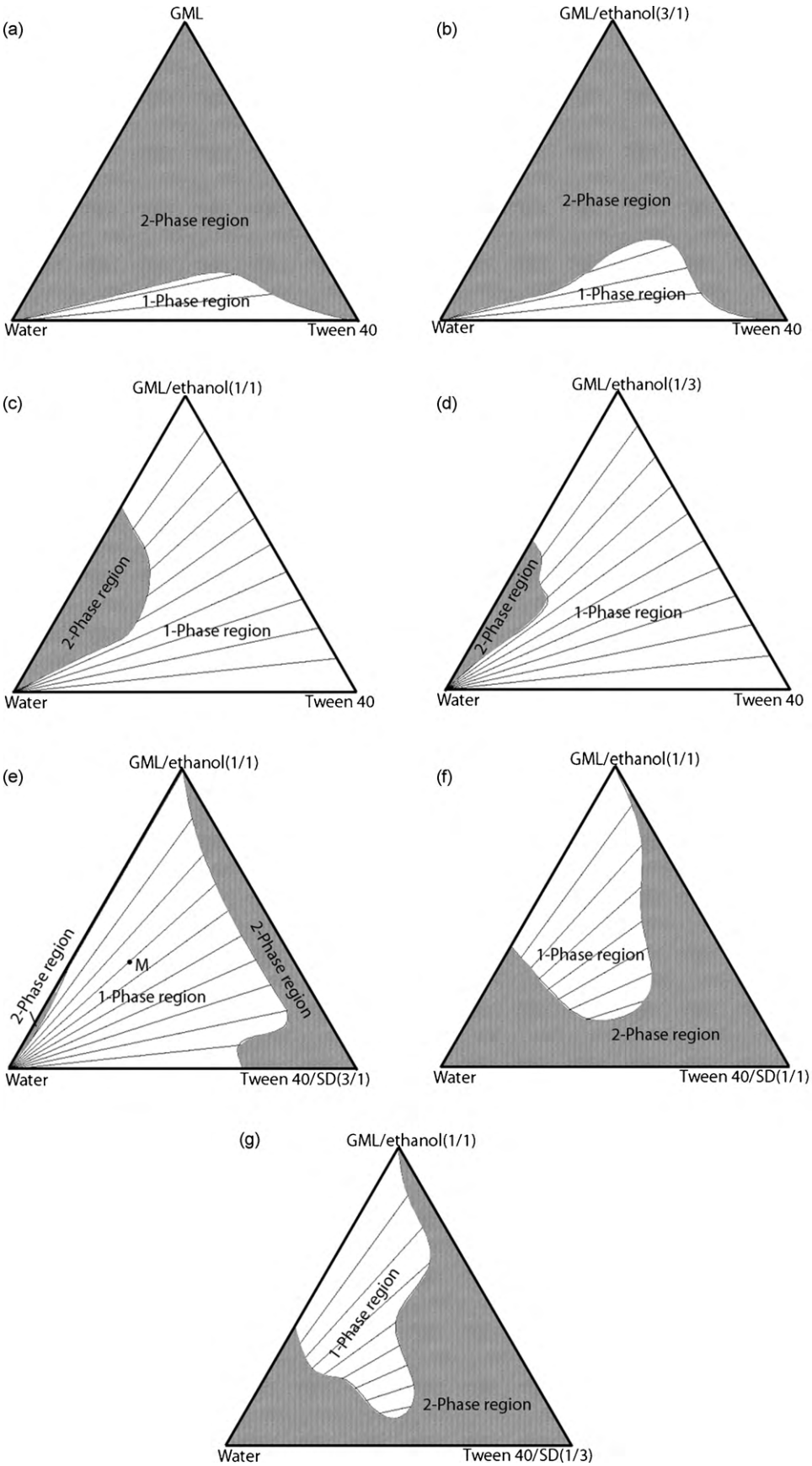

Fig. 1. Phase diagrams of the system water/GML/ethanol/Tween 40 at 25 °C with different weight ratios of GML/ethanol: (a) GML, (b) GML/ethanol (3:1), (c) GML/ethanol (1:1), (d) GML/ethanol (1:3), and the system water/GML/ethanol/Tween 40/SD at 25 °C with different weight ratios of Tween 40/SD: (e) 3:1, (f) 1:1, (g) 1:3. The composition of the oil phase in these phase diagrams is given in the upper corner. Point M denotes a formulation composed of an oil phase of GML (10% w/w), ethanol (10%), and a surfactant phase of Tween 40 (35%) and SD (12%).

Table 1
The inhibition zones (mm) of microemulsion against bacteria, yeast and mycelial fungi (mean ± SD).

	Bacteria			Yeast	Mycelial fungi	
	Escherichia coli	*Staphylococcus aureus*	*Bacillus subtilis*	*Candida albicans*	*Aspergillus niger*	*Penicillium expansum*
Microemulsion	15.5 ± 2.5	19.2 ± 4.2	22.9 ± 2.1	13.2 ± 5.8	18.0 ± 3.0	25.0 ± 2.0
Positive control[a]	5.5 ± 1.8	6.0 ± 0.6	6.0 ± 0.8	17.3 ± 1.7	6.0 ± 0.5	12.2 ± 0.8

[a] Positive controls used in this assay were 0.1% (w/v) sodium lactate (for bacteria), 0.01% natamycin (for yeast) and 0.5% potassium sorbate (for mycelial fungi).

3.2. Determination of microemulsion formulation

One microemulsion formulation of GML/ethanol/Tween 40/SD/water microemulsion system was selected (see Point M in Fig. 1e). The microemulsion had an oil phase of GML (10% w/w), ethanol (10%), and a surfactant phase of Tween 40 (35%) and SD (12%). Further examination of the physical stability of the microemulsion was performed according to the method of Al-Adham et al. (2000). No phase separation was observed in the sample after placing it in a centrifuge at 4000 g for 15 min at room temperature. The same sample was then stored for 1 month at 28 °C and 37 °C, no phase separation was observed.

3.3. Antimicrobial activity assay

3.3.1. Solid medium diffusion method

Results from Table 1 showed that the formulated microemulsion possessed antibacterial and antifungal activities against the typical bacteria and fungi. When the microemulsion was assayed against the test microorganisms by solid medium diffusion assay, the mean zones of inhibition obtained were between 13 mm and 25 mm, compared to those of the positive controls in the range of 5–17 mm.

3.3.2. Kinetics of killing

No viable bacterial cells including *E. coli*, *S. aureus* and *B. subtilis* were observed after 1 min treatment with the microemulsion (figure not shown). Fig. 2 showed the rate of killing observed for the cultures of fungal cells and gave clear evidence of a true biocidal dynamic treated by the microemulsion. For *C. albicans*, an over 2 log reduction in fungal titre was obtained when treated by the microemulsion in 1 min and a nearly complete loss of viability was achieved in 5 min. For *A. niger* and *P. expansum*, more than 99.9% viable spores were killed within 2 min and the viability decreased rapidly over a period of less than 5 min until no viable spores were observed.

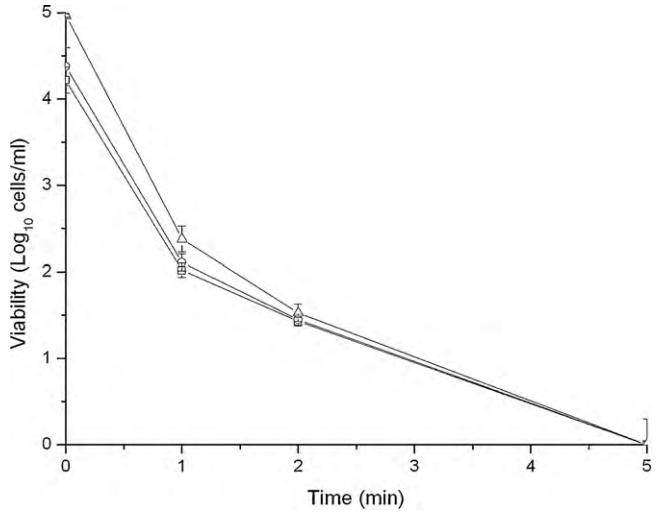

Fig. 2. Time exposure viability curves for cultures of 9.0×10^6 cfu/ml *C. albicans* (△), 2.4×10^6 spores/ml *A. niger* (○) and 1.6×10^6 spores/ml *P. expansum* (□) at 1% (v/v) addition to microemulsion at 28 °C for 5 min. Error bars are calculated from the standard error of the dataset ($n = 2$).

3.3.3. Transmission electron microscopy

A comparison of untreated cells and microemulsion-treated cells using TEM was shown in Figs. 3–5. The bacterial cells (*E. coli*, *S. aureus* and *B. subtilis*) with no treatment showed a normal rod or spherical shape with intact intracellular components (Fig. 3a–c), while the microemulsion-treated cells exhibited abnormal contour, membrane disturbance and cell content disorganization (Fig. 3d–f). The TEM photomicrographs of the untreated cells of the yeast (*C. albicans*) showed a typically structured nucleus and vacuoles. The cytoplasm contained several element of endomembrane system and enveloped by a typical structure of cell wall (Fig. 4a). As for the microemulsion-treated yeast cells, the cytoplasmic volume decreased and the plasma membrane invaginated with notable structural disorganization within the cell cytoplasm (Fig. 4b). The untreated fungal conidia (*A. niger* and *P. expansum*) were shown in Fig. 5a and b, plasma membrane was pressed to the cell wall and the structured cytoplasm. However, the ultrastructural damage in conidia was significant with cell wall collapse, plasma membrane retraction, cytoplasm aggregation and cell content disorganization (Fig. 5c and d).

4. Discussion

Ethanol was thought to be able to change the polarity of the polar and apolar phases and increase the flexibility of the interface (Garti et al., 2001). Compared to GML/Tween 40/water system in Fig. 1a, the addition of ethanol induced larger microemulsion area (see Fig. 1b). One possible explanation is that addition of ethanol enhances the oil incorporation into the interface in favor of microemulsions, due to the hydroxyl group in GML molecular structure, which makes it well-dissolved in ethanol. Gradzielski (1998) found that the addition of short-chain alcohols to nonionic o/w microemulsions lowered the bending moduli significantly. Moreover, the changes of the solubilization parameters were dramatic when the concentration of ethanol in oil phase was increased. When the weight ratio of GML to ethanol was 1:1 (Fig. 1c) or 1:3 (Fig. 1d), it is obvious that the addition of ethanol induced the formation of a U-type microemulsion system (Clausse et al., 1987) (see Fig. 1c and d), which is actually an improved self-microemulsifying drug delivery system (SMEDDS), capable of being infinitely and progressively aqueous phase diluted (Spernath and Aserin, 2006). It was reported (Thevenin et al., 1996) that with shorter-chain alcohols U-type microemulsions based on sucrose esters were identified. The results are comparable with similar studies by Garti et al. (2001) who suggested that the increase in the isotropic phase area is an increased incorporation of ethanol into the surfactant film, which would lead to a decrease in the bending moduli of this film.

The phase behavior in Fig. 1e–g indicated the difficulty to solubilize SD in the GML-loaded microemulsions, especially at high salt concentration. It seemed that in a phenomenological sense, SD produced a salting-out effect in correlation with the microemulsion phase behavior. This may be explained that the addition of SD to the surfactant monolayer dehydrates its hydrophilic part through

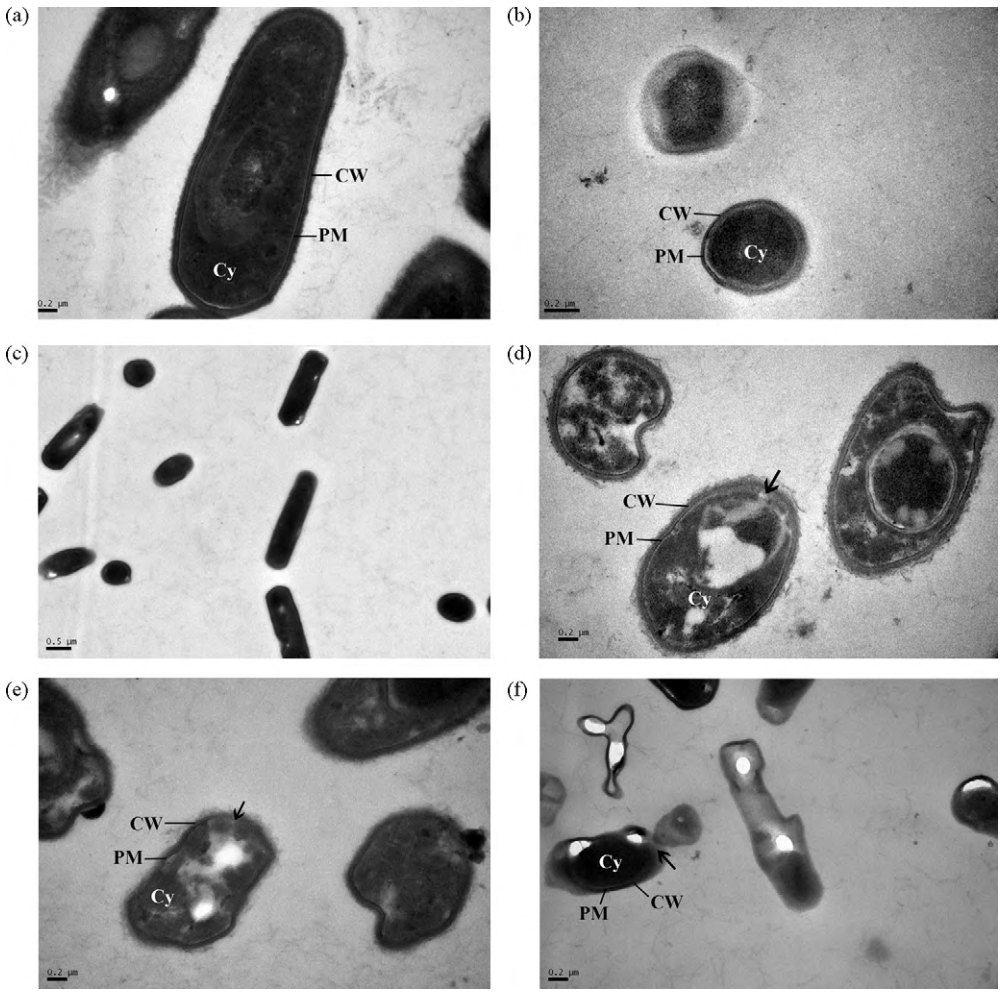

Fig. 3. Transmission electron microscopic photomicrographs of the untreated cells of *E. coli* (a), *S. aureus* (b), *B. subtilis* (c), and the microemulsion-treated cells of *E. coli* (d), *S. aureus* (e), *B. subtilis* (f) by the kinetics of killing method for 1 min. CW = cell wall; PM = plasma membrane; Cy = cytoplasm. The arrows show a low density region, which is indicative of membrane disturbance in the microemulsion-treated cells.

an osmotic equilibrium and bends the oil–water interface toward water, according to the theory of Kabalnov et al. (1995), which proves that the salting-out phenomena do not only correlate with, but also are driven by, the depletion at the surfactant monolayer.

Recent work (Al-Navajeh, 2003) has analysed the relationship between antimicrobial activity and the position of the microemulsion within its existence area (or stability region), and suggested that the maximal activity is to be found at the centre of the stability region. Therefore, one microemulsion formulation at the centre of the stability region in Fig. 1e was selected. The physical stability studies indicated that the prepared microemulsion was stable under conditions of centrifugation and incubation for extended periods.

The antimicrobial assay using solid medium diffusion method showed that the microemulsion was comparable to the commonly used antimicrobials as positive controls (Table 1). Our previous GML-loaded microemulsion with solubilization of potassium sorbate showed 80% *A. niger* growth inhibition at 0.2% and 72% *P. italicum* growth inhibition at 0.1%, and a delay of conidiation of 2 days compared with the control (Zhang et al., 2008a). The GML-

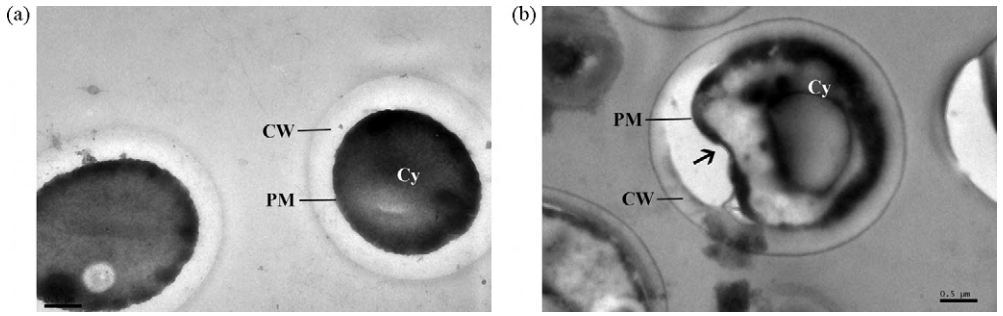

Fig. 4. Transmission electron microscopic photomicrographs of the untreated cells of *C. albicans* (a) and the microemulsion-treated cells of *C. albicans* (b) by the kinetics of killing method for 5 min. CW = cell wall; PM = plasma membrane; Cy = cytoplasm. The arrows show the obviously invaginated plasma membrane in the microemulsion-treated cells.

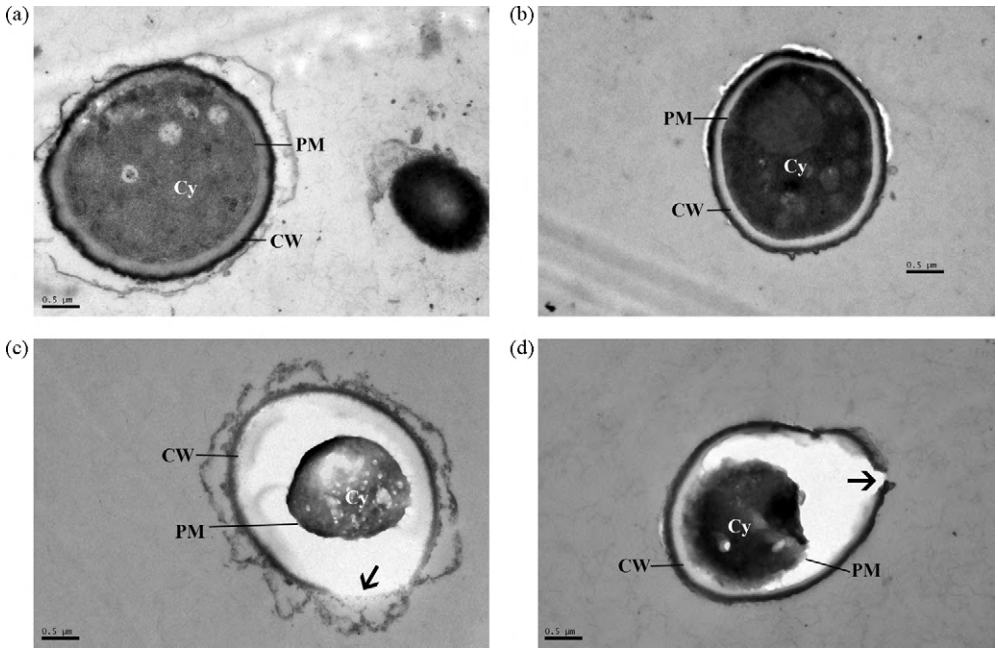

Fig. 5. Transmission electron microscopic photomicrographs of the untreated spores of *A. niger* (a), *P. expansum* (b), and the microemulsion-treated spores of *A. niger* (c), *P. expansum* (d) by the kinetics of killing method for 5 min. CW = cell wall; PM = plasma membrane; Cy = cytoplasm. The arrows show the significant cell wall collapse.

loaded microemulsion prepared by Fu et al. (2006) inhibited 78% *A. niger* growth at 0.05% after 5 days of incubation.

The kinetics of killing results give clear evidence of a good biocidal activity for the microemulsion against the bacteria, but a lesser effect against the fungi. The bacterial cells had a complete loss of viability immediately after their addition to the microemulsion within 1 min. These results are in agreement with those previous reports. Al-Adham et al. (2000) reported that an o/w microemulsion of ethyl oleate emulsified by Tween 80 and pentanol gave a 5 log reduction in the numbers of *S. aureus* or *P. aeruginosa* in only 45 s. Teixeira et al. (2007) extended the previous investigations and found that the o/w microemulsion was active against five bacterial pathogens including *S. aureus*, *E. coli*, *P. aeruginosa*, *Salmonella typhimurium* and *Listeria monocytogenes*. On the other hand, the microemulsion killed over 99% *A. niger* and *P. expansum* spores and 99.9% *C. albicans* cells rapidly within 2 min and a complete loss of viability was obtained in 5 min. This is comparable to our previous work (Zhang et al., 2008c), which has proved that the GML-loaded microemulsions are effective antifungal systems with sporicidal activities against *A. niger*.

The antibacterial and antifungal activities of the prepared microemulsion were confirmed by the TEM observations. The cytoplasmic cell membrane undoubtedly is the target for many antimicrobial agents. Interactions of microbial membranes with antimicrobials frequently cause fundamental changes in both membrane structure and function. TEM observations of relevant work (Al-Adham et al., 2000) exhibited many signs of membrane dysfunction of the microemulsion-exposed *P. aeruginosa* cells, and were confirmed by the subsequent report (Al-Adham et al., 2003) on the microemulsion-treated biofilms of *P. aeruginosa*. Teixeira et al. (2007) reported that the microemulsion was active against biofilms of *P. aeruginosa*, *E. coli*, *S. typhimurium* and *S. aureus*, with the exception of the biofilm formed by *L. monocytogenes*. However, there are very few literatures concerning the antifungal mechanism of microemulsions. Our previous work (Zhang et al., 2008c) has evaluated the antifungal activities of a prepared GML-loaded microemulsion against *A. niger*. The excellent growth inhibition and sporicidal activities of the microemulsion were confirmed by the observation of scanning electron microscopy, light microscopy and increased Ca^{2+}, K^{+} and Mg^{2+} leakages, indicating the disruption and dysfunction of fungal cell walls and biological membranes. Besides, the antimicrobial activity of microemulsions against yeasts, such as *C. albicans*, has never been documented to the best of our knowledge.

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