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An inclusion complex of thymol into β-cyclodextrin and its antifungal activity against *Geotrichum citri-aurantii*

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ABSTRACT

Thymol is a naturally occurring volatile terpenoid in plants that possesses strong antifungal activities against many pathogens. In this research, thymol was included into β-cyclodextrin and the antifungal activity of the resulting complex against *Geotrichum citri-aurantii,* the causal agent of sour rot in citrus fruit, was investigated. The inhibition concentration of the β-cyclodextrin-thymol inclusion complex (β-CTIC) to *G. citri-aurantii* was estimated to be 4.0 g L^{-1}, which was supposed to be a comparable antifungal efficiency of 0.5 g L^{-1} thymol alone. The structure and characteristic of β-CTIC were evaluated by scanning electron microscopy (SEM), X-ray diffraction (XRD), nuclear magnetic resonance (NMR) and entrapment efficiency (EE). The ROESY spectrum of β-CTIC showed appreciable correlation of H-1, H-4 and H-3 protons of thymol with the H-3′, H-5′ and H-6′ protons of β-cyclodextrin, respectively. In addition, β-CTIC was found to reduce the decay of citrus fruit inoculated with *G. citri-aurantii* and maintain the fruit quality. Our present study suggested that β-CTIC might be used as an alternative to chemical fungicides in controlling the postharvest sour rot of citrus fruit.

1. Introduction

Sour rot, caused by *Geotrichum citri-aurantii*, is a devastating postharvest disease that occurs in postharvest citrus (Ferraz et al., 2016; Hao et al., 2010). This disease is difficult to control duo to the lack of effective fungicides. Application of sodium o-phenylphenate (SOPP) is an effective approach for the control of this disease, but its use is limited because of the risk of fruit damage (Feng et al., 2011). In addition, the extensive application of fungicides can easily led to the proliferation of resistant strains, the increase of human health risks and environment problems (McKay et al., 2012). Therefore, there is a need to find alternatives to synthetic fungicides.

Essential oils might be a good alternative to chemical fungicides due to their effectiveness in controlling postharvest diseases (Shao et al., 2015). Thymol is a major component of oregano oil extracted from plants belonging to the *Lamiaceae* family (Marchese et al., 2016; Sharma et al., 2016). It has been approved by the U.S. Food and Drug Administration (FDA) as “generally recognized as safe” (GRAS) and can be used as a food additive (Marchese et al., 2016). The antifungal activities of thymol against *Penicillium digitatum* and *P. italicum* have been reported through *in vitro* and *in vivo* tests and it was found to be able to decrease the fruit decay of citrus in a concentration-dependent manner (Castillo et al., 2014; Pérez-Alfonso et al., 2012). However, the poor water-solubility and high volatility of thymol restricted its application in disease control (Ceborska et al., 2015).

β-Cyclodextrin, a family member of the cyclic oligosaccharides with cone-shaped structure, is commonly used as a kind of nontoxic coating material. Its outer surface is hydrophilic and the central cavity is hydrophobic (Loftsson et al., 2005), therefore it is able to form inclusion complex with many small organic molecules like anethol, carvacrol, eugenol, geranio within its central cavity (Loftsson et al., 2005; dos Passos Menezes et al., 2016; Ren et al., 2016; Waleczek et al., 2003), which could enhance the aqueous solubility (Yang et al., 2016) and the stability of the guest molecules (Yang et al., 2013; Wei et al., 2017). Thus, β-cyclodextrin might be a potential material in the development of controlled-release formulations that aimed at controlling postharvest disease of citrus fruit.

This study aims to (1) prepare an inclusion complex by incorporating thymol into β-cyclodextrin, (2) investigate the formation of inclusion complex of the host molecules β-cyclodextrin and guest molecules thymol through scanning electron microscopy (SEM), nuclear magnetic resonance (NMR), and X-ray diffraction (XRD) and (3) assess the antifungal activity of the inclusion complex against *G. citri-aurantii* by using *in vitro* and *in vivo* tests.

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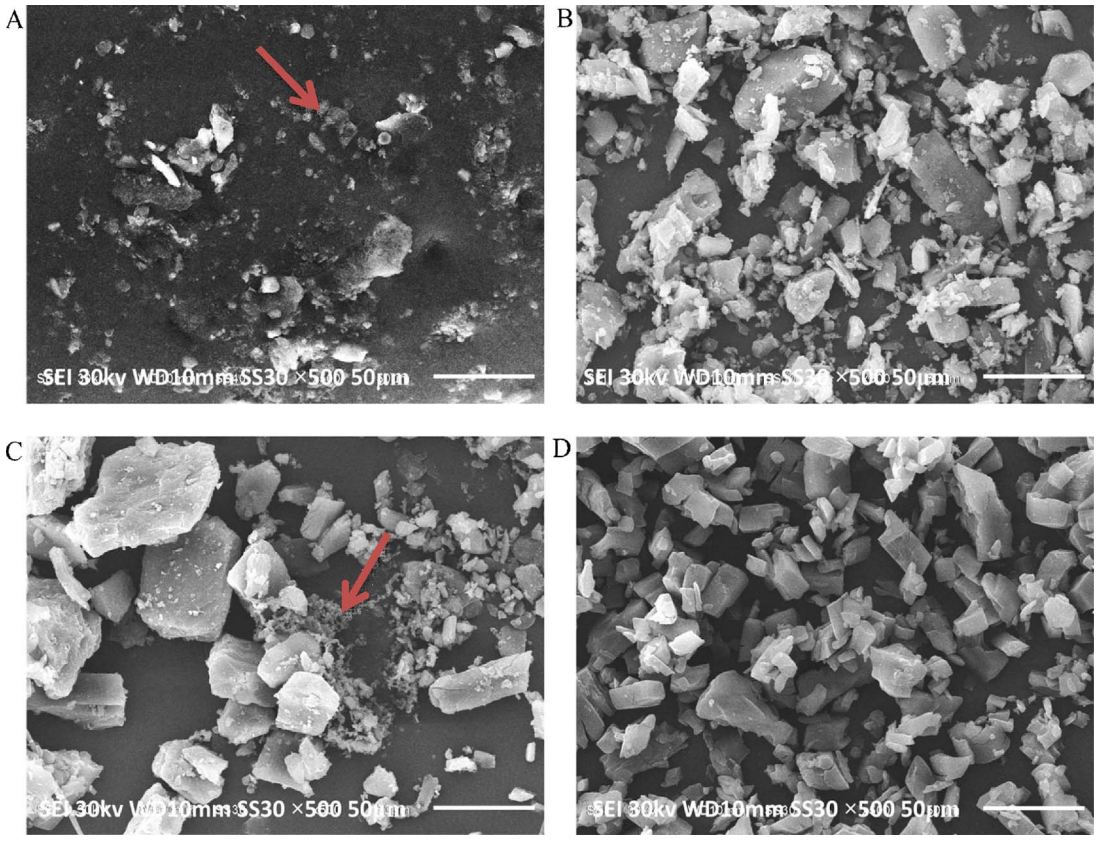

Fig. 1. SEM graphs of thymol (A), β-cyclodextrin (B), the physical mixture (C) and the β-CTIC (D) (Arrows refer to thymol).

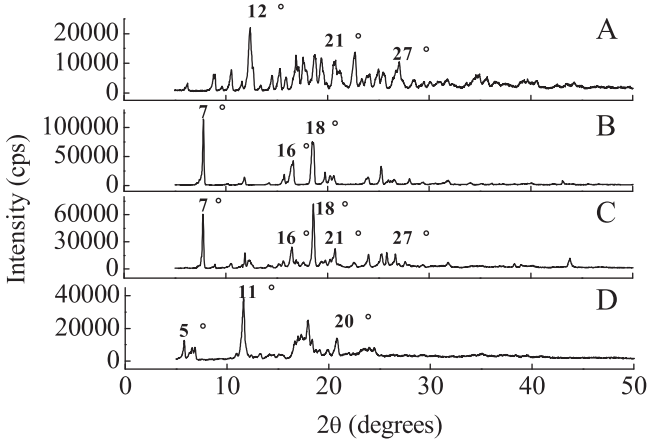

Fig. 2. X-ray diffraction patterns of β-cyclodextrin (A), thymol (B), the physical mixture (C) and the β-CTIC (D).

2. Materials and methods

2.1. Chemicals

Thymol was purchased from Aladdin (Shanghai, China). β-Cyclodextrin was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). D_2O was obtained from Sigma-Aldrich Chemical Company (Shanghai, China).

2.2. Pathogen

The mold of *G. citri-aurantii* used in this study was provided by the Department of Biotechnology and Food Engineering, Xiangtan University, Xiangtan, China. The fungal was preserved on potato dextrose agar (PDA) at 28 ± 2 °C.

2.3. Preparation of the inclusion complex and the physical mixture

The β-CTIC was prepared using the saturated aqueous solution method (Miron et al., 2012) with minor modifications. Firstly, β-cyclodextrin (10 g) was dissolved in 100 mL of distilled water at 60 °C under continuous stirring for completely dissolved. Then, a solution of thymol in ethanol (1:4, w/v) was added slowly to the β-cyclodextrin solution. Subsequently, the mixture was continuous stirring for 4 h and cooled down to the room temperature. The mixture was filtered and washed with double distilled water for three times to wash off the excess β-cyclodextrin and thymol. Finally, the solid powder was dried by a vacuum freeze-drying equipment. The β-cyclodextrin-thymol inclusion complex (β-CTIC) was obtained. The physical mixture was prepared by the reported method (Yang et al., 2016). Two grams of thymol and 2 g β-cyclodextrins were mixed with a mortar until a homogeneous mixture.

2.4. Scanning electron microscopy (SEM)

The morphology of thymol, β-cyclodextrin, the physical mixture, and the β-CTIC were observed using a JSM-6610LV scanning electron microscopy (JEVL, Tokyo, Japan) operated at 25 kV at 500 × level of magnification. The samples were mounted on SEM stubs using double-stick adhesive tabs. To improve conductivity, samples were coated with gold-palladium electroplating (90s, 1.8 mA, 2.4 kV) in a Polaron SEM Coating System sputter coater before determination.

2.5. X-ray diffraction (XRD)

The XRD analysis of the samples thymol, β-cyclodextrin, the physical mixture, and the β-CTIC was performed in a Rigaku D/Max-