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Synergistic antifungal effects of thyme essential oil and *Lactobacillus plantarum* cell-free supernatant against *Penicillium* spp. and in situ effects

Suphamid Sriwattanachai | Muhammad Bilal Sadiq | Anil Kumar Anal

Food Engineering and Bioprocess Technology, Asian Institute of Technology, PO Box 4, Klongluang, Pathumthani 12120, Thailand

Correspondence
Anil Kumar Anal, Food Engineering and Bioprocess Technology, Asian Institute of Technology, PO Box 4, Klong Luang, Pathumthani 12120, Thailand.
Email: anilkumar@ait.asia

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Abstract

This study evaluates the synergistic antifungal effects between thyme essential oils and *Lactobacillus plantarum* cell-free supernatant (LCFS) against *Penicillium* spp. and in situ antifungal activity in rice grains. Thyme essential oil and LCFS showed remarkable antifungal activities against *Penicillium* spp. with the minimum inhibitory concentration (MIC) of 40 and 80 μL/mL, respectively. The analysis of fractional inhibitory concentration (FIC) index showed the antifungal synergism between thyme essential oil and LCFS against *Penicillium* spp. with FIC index of 0.5. This synergism also resulted in fourfold reduction in their MICs when applied in combination. The antifungal modes of action were characterized by observing the changes in cell membrane permeability and degradation of fungal cell wall. The combination of thyme essential oil and LCFS (2 × MIC of each) showed remarkable in situ antifungal effect and completely inhibit the growth of *Penicillium* in rice seeds. The results suggested the possible applications of the observed synergism on actual crops.

Practical applications

Essential oils are used as preservative in food industry and high concentration of essential oil is associated with negative organoleptic characteristics. This study presented a novel approach for synergistic antifungal effects by using the combination of thyme essential oil and *Lactobacillus plantarum* cell-free supernatant (LCFS) against *Penicillium* spp. and systematic evaluation of the antifungal effect by using fractional inhibitory concentration (FIC) index method. This approach will be a role model for future research on synergism and overcome the major drawbacks of using live bacteria and the negative effects arising from antimicrobial activities of essential oils.

1 | INTRODUCTION

Penicillium spp. is soil fungi, commonly found in spoiled foods and feeds, causing a huge Fig. economic loss, especially during post-harvest processing (Oliveira, Zannini, & Arendt, 2014). *Penicillium* is known for its unique characteristics such as, growing in both refrigerated and warmer temperatures (Sanzani, Montemurro, Di Rienzo, Solfrizzo, & Ippolito, 2013). The fungal infections and the contaminations by its mycotoxins such as patulin and ochratoxin-A have also been reported in various agricultural crops such as wheat, rice, maize, barley, apples, pears, and other deciduous fruits (Oliveira et al., 2014).

To control the mold growth and accumulation of mycotoxins in foodstuffs, food preservatives such as acetic, lactic, benzoic, and sorbic acids are primarily employed alone or in combination with physical treatments to ensure the safety and stability of the products during storage (Kocić-Tanackov & Dimić, 2013). The reduced susceptibility of microorganism to synthetic preservatives and the consumers demand for high quality, preservative-free, safe and minimally processed green-label foods urged the food industry to focus more on natural preservation and stabilizing approaches (Reis, Paula, Casarotti, & Penna, 2012). Essential oils and lactic acid bacteria (LAB) have shown great potential as bio-preservative since they have been generally recognized as safe (Sivakumar & Bautista-Baños, 2014).

Thyme (*Thymus vulgaris* L.), a member of *Lamiaceae* family, is well known for its essential oils that exhibit remarkable antifungal activity (Kohiyama et al., 2015). Its major components such as thymol and carvacrol have been shown to exhibit the strong antifungal activity against 13 *Penicillium* spp., including *P. expansum* (Felsociova et al., 2015).

Lactobacillus plantarum, an LAB and probiotic, is also known to have many potential applications against a wide variety of mycotoxigenic molds (da Silva Sabo, Vitolo, González, & Oliveira, 2014). *L. plantarum* produces several antifungal compounds as secondary metabolites such as organic acid mixture and cyclic dipeptides (Muhialdin, Hassan, & Saari, 2013). These antifungal metabolites have shown the antifungal activities against all three genera of mycotoxigenic molds (*Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp.) (Crowley, Mahony, & van Sinderen, 2013).

The objective of this research was to develop a novel approach for the synergism between essential oils and LAB against *Penicillium* spp. in vitro and in situ antifungal activity in rice, using LAB metabolites and fractional inhibitory concentration (FIC) index method.

2 | MATERIALS AND METHODS

2.1 | Materials

Lactobacillus plantarum TISTR 858 (isolated from pickle vegetable) and *Penicillium* spp. TISTR 3314 (a soil isolate) were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. Thyme essential oil (*Thymus vulgaris* L.) extracted by hydro-distillation was provided by Botanic-Essence, Thailand. The gas chromatographic (GC) analysis of thyme essential oil showed the major components; p-Cymene (26.32%), Thymol (21.31%), γ-Terpinene (19.50%), Linalool (3.02%), Myrcene (2.56%), Terpinen-4-ol (1.78%), and Carvacrol (1.10%). Rice seeds were obtained from the local farmers in Pathum Thani, Thailand.

2.2 | Preparation of *L. plantarum* cell-free supernatant

The relationship between optical density (OD_{600}) and CFU/mL was determined for the bacterial strain. Afterwards, the OD_{600} of bacterial stock was measured and converted to 20 CFU/mL by dilution with sterile medium.

L. plantarum was cultured in one liter (1,000 mL) of sterile MRS broth (HiMedia, Mumbai, India) with initial cell concentration of 20 CFU/mL and incubated at 30 °C for 60 hr (Sathe, Nawani, Dhakephalkar, & Kapadnis, 2007) without shaking to produce the antifungal metabolites. *Lactobacillus plantarum* cell-free supernatant (LCFS) was collected by centrifugation (Centrikon T-324, Neufahrn, Germany) at 8,000 rpm for 10 min at 25 °C. The supernatant was dried in a freeze dryer (Scanvac Cool Safe 55-4, Lynge, Denmark) at −52 °C for 24 hr. The powdered LCFS was reconstituted in sterile distilled water to obtain concentrated LCFS (17× by volume of the culture obtained after incubation of 60 hr). The obtained stock solution of supernatant was then made sterile by filtering through the 0.22 μm non-pyrogenic filter (Sartorius, Germany) and stored at −40 °C (Sanyo, Osaka, Japan) for further use.

2.3 | In vitro antifungal activity and minimum inhibitory concentration determination of thyme essential oil and LCFS

The antifungal activities and minimum inhibitory concentrations (MICs) of thyme essential oil and LCFS were determined separately by the radial growth inhibition assay adapted from Tian et al. (2011) with minor modifications. Potato dextrose agar (PDA) containing two-fold concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μL/mL) of thyme essential oil or concentrated LCFS were prepared. Tween 80 (2% v/v) was also added as an emulsifier into the media to ensure the formation of homogenous mixture of thyme essential oil. Each concentration was prepared in triplicate, and the media was inoculated at the center with a 5 mm fungal plug, containing actively growing mycelia of *Penicillium* spp. All petri dishes were sealed with parafilm and incubated at 30 °C for 7 days in dark before measuring the radial growth of the fungal colonies with a Vernier caliper (Mitutoyo, Kawasaki, Japan). The control used was PDA, containing 2% Tween 80 without the addition of thyme essential oil or LCFS. The lowest concentration of thyme essential oil or LCFS that showed no visible growth of the fungi after 7 days of incubation was determined as the MIC, while the antifungal activities of other concentrations were expressed in terms of percentage of inhibition using the following formula (Tao, Jia, & Zhou, 2014).

$$\%\ \text{inhibition} = \left[\frac{d_c - d_t}{d_c}\right] \times 100 \quad (1)$$

where d_c is the diameter of control, and d_t is the diameter of the treated samples.

2.4 | In vitro synergism between thyme essential oil and LCFS

The synergistic antifungal effects between essential oil and LCFS were evaluated by using FIC index by following the method of Barros, Conceição, Gomes Neto, Costa, and Souza (2012), with slight modifications. The tested combinations (thyme essential oil + LCFS) were; ½ MIC + ½ MIC, ½ MIC + ¼ MIC, ½ MIC + ⅛ MIC, ¼ MIC + ½ MIC, ¼ MIC + ¼ MIC, ¼ MIC + ⅛ MIC, ⅛ MIC + ½ MIC, ⅛ MIC + ¼ MIC, and ⅛ MIC + ⅛ MIC. The sterile PDA media containing 2% Tween 80 was prepared with the described combinations of thyme essential oil and LCFS at 55 °C, vortexed (Scientific Industries, Inc., Bohemia, NY) for 30 s and allowed to solidify at 25 °C. Each combination was prepared in triplicate, while the control was prepared without essential oil and LCFS. The fungal disk (5 mm), containing actively growing mycelia, was used to inoculate at the center of each plate and incubated at 30 °C for 7 days. The lowest concentration of the combined mixture that showed no visible growth of the fungi after the incubation was then used to determine the FIC index, using the following formula (Valcourt et al., 2016).

$$\text{FIC index} = \frac{\text{MIC}_{\text{A in mixture}}}{\text{MIC}_A} + \frac{\text{MIC}_{\text{B in mixture}}}{\text{MIC}_B} \quad (2)$$

where MIC is minimum inhibitory concentration and A represents thyme essential oil and B represents LCFS.

The synergistic effect was recognized only, when the FIC index was ≤0.5 and considered as additive when FIC index was >0.5, but ≤4.0. If it exceeded 4.0, it was declared as antagonism.

2.5 | Synergistic effects of combined mixture on *Penicillium* spp.'s hyphae

The combined effect of thyme essential oil and LCFS on hyphae was determined by the method described by Tao et al. (2014) with slight modifications. Potato dextrose broth (PDB) containing 2% Tween 80 (20 mL) was inoculated with *Penicillium* spp. (10^4 spores) and incubated at 30 °C, 200 rpm for 48 hr in a shaking incubator (N-Biotek, South Korea). The hyphae were harvested by centrifugation (Centrikon T-324, Germany) at 8,000 rpm for 5 min. The fungal cells were then washed twice with 5 mL of phosphate buffer saline (PBS, pH 7.4). The cells were harvested by centrifuging at 8,000 rpm for 5 min and re-suspended in 20 mL of PBS containing 2% Tween 80, with (thyme essential oil + LCFS) and without (control) the addition of the combined mixture. Three concentrations: 0 (control), 1/8 MIC + 1/8 MIC (0.5 × MIC of the mixture), and 1/4 MIC + 1/4 MIC (MIC of the combined mixture) of thyme essential oil and LCFS were used respectively, and the samples were incubated at 25 °C for 24 hr. The hyphae of each sample were then stained with lactophenol cotton blue mounting solution before observing the morphology at magnification of 1,000× under a light microscope (Olympus, Tokyo, Japan).

2.6 | Determination of leakage of cellular constituents

The leakage of cellular constituents from *Penicillium* after treatment with the combination of thyme essential oil and LCFS, was determined by the method described by Tao et al. (2014), with minor modifications. PDB containing 2% Tween 80 (20 mL) was inoculated with 10^4 spores of *Penicillium* spp. and incubated at 30 °C, 200 rpm for 48 hr. The fungal pellets were harvested by centrifugation at 8,000 rpm for 5 min and washed twice with 5 mL of PBS (pH 7.0), containing 2% (v/v) Tween 80. The fungal cells were then re-suspended in PBS pH 7.0 (20 mL), containing different concentrations (0, 1/8 MIC + 1/8 MIC, and 1/4 MIC + 1/4 MIC) of the combined mixture (thyme essential oil + LCFS) and kept in a shaking incubator at 30 °C, 200 rpm for 24 hr. Samples (2 mL) were withdrawn after every 4 hr and centrifuged (Centurion K_2S series, UK) at 12,000 rpm for 5 min. The optical density of the resultant supernatant was measured at 260 nm by using UV–visible spectrophotometer (Shimadzu, Kyoto, Japan).

2.7 | In situ antifungal activity of combined mixture (thyme essential oil and LCFS) in rice seeds

In situ antifungal activity in rice grains was evaluated by following the procedure described by Naseer, Sultana, Khan, Naseer, and Nigam (2014) with slight modifications. Rice seeds were sterilized by autoclaving at 121 °C for 15 min and inoculated with the spores of *Penicillium* spp. (2.5×10^6 spores/100 seeds) by spraying and mixing thoroughly inside a sterile plastic bag to obtain the homogenous distribution of spores. The rice seeds were then placed separately into the petri dishes (50 seeds per plate), containing a moistened filter paper (prepared by adding 1 mL sterile distilled water per plate). The samples were treated with different concentrations of the combined mixture of thyme essential oil and LCFS (0, 1/4 MIC + 1/4 MIC, 1/2 MIC + 1/2 MIC, 1 MIC + 1 MIC, and 2 MIC + 2 MIC) by spraying 1 mL of each solution on the samples. Sterile distilled water was used as the control. The wide range of mixture concentrations was selected to observe the dose-dependent response as in situ responses are observed at relatively higher concentration than the in vitro. All samples were incubated at 30 °C for 7 days in an incubator (Memmert, Büchenbach, Germany), while the humidity was maintained (70%) throughout the incubation period. After the incubation, 10 subsamples of each treatment were used to inoculate on the PDA in triplicate and further incubated at 30 °C for 24 hr to visualize the contaminations. Seed contamination index (SCI) was determined using the following formula (Sumalan, Alexa, & Poiana, 2013).

$$\mathrm{SCI}(\%) = \frac{\text{Number of contaminated seeds}}{\text{Total number of seeds}} \times 100 \quad (3)$$

2.8 | Statistical analysis

The analysis of variant (one-way ANOVA) and Tukey analysis were used to analyze the obtained data to identify the significant differences between the treated conditions and the control. The significant difference was considered when the p value was less than 0.05. The data were then presented in the form of mean ± *SD* (standard deviation). All statistical analyses were carried out using statistical software package (SPSS, version 17.0, SPSS Inc., Chicago, IL).

3 | RESULTS AND DISCUSSIONS

3.1 | In vitro antifungal activity and MIC of thyme essential oil and LCFS

Antifungal activities of thyme essential oil and LCFS and their MICs were evaluated by the radial growth inhibition assay. The results showed that thyme essential oil and LCFS possessed a strong antifungal activity against *Penicillium* spp. Moreover, the radial growth of the fungi was significantly inhibited ($p < .05$) in a dose-dependent manner as illustrated in Table 1. The percentage of inhibition increased as the concentration of essential oil or LCFS increased, whereas, the control (PDA + 2% Tween 80) showed the normal growth of *Penicillium* spp. with green spores production after the incubation. The MICs of thyme essential oil and LCFS against *Penicillium* spp. (incubation at 30 °C for 7 days) were found to be 40 and 80 μL/mL respectively with complete inhibition of the visible growth of the fungi.

The observed antifungal activities of thyme essential oil were in accordance with the report as described by Felsociova et al. (2015). The antifungal effects of thyme essential are likely due to the overall inhibitory effects exerted by its major components; p-Cymene, thymol, γ-Terpinene, Linalool, and Carvacrol (Tao et al., 2014). However, the determined MIC value of thyme essential oil (40 μL/mL) was higher than the previous report (19.17 μg/mL) (Šegvić Klarić, Kosalec, Mastelić, Piecková, & Pepeljnak, 2007). These differences are caused by variations in major components of Thyme essential oil and strains of fungi and their cultivation conditions (Calo, Crandall, O'Bryan, & Ricke, 2015).

TABLE 1 Antifungal activities and MICs of thyme EOs and LCFS and evaluation of seed contamination index after the in situ application of combined mixture of EOs and LCFS

Sample	Concentration (μL/mL)	Percent inhibition (%)
Thyme essential oils (EO)	Control (0.00)	0.00 ± 2.23^{a}
	1.25	24.15 ± 3.53^{b}
	2.5	41.88 ± 1.45^{c}
	5.0	78.57 ± 3.20^{d}
	10	92.45 ± 4.76^{e}
	20	96.80 ± 4.00^{ef}
	40	100 ± 0.00^{f}
Lactobacillus cell-free supernatant (LCFS)	Control (0.00)	0.00 ± 1.17^{a}
	10	1.33 ± 2.84^{a}
	20	6.42 ± 2.69^{b}
	40	24.40 ± 1.59^{c}
	80	100.00 ± 0.00^{d}
	160	100.00 ± 0.00^{d}
Concentrations of mixture (EO + LCFS)		Seed contamination index (%)
	Control (0.00)	100.00 ± 0.00^{a}
	1/4 MIC + 1/4 MIC	100.00 ± 0.00^{a}
	1/2 MIC + 1/2 MIC	96.67 ± 5.77^{a}
	1 MIC + 1 MIC	10.00 ± 0.00^{b}
	2 MIC + 2 MIC	0.00 ± 0.00^{c}

Note. The different superscript letters within a column indicate the significant difference between means ($p < .05$). The sterile PDA containing 2% Tween 80 was used as control for antifungal assay whereas, sterile distilled water was used as control for seed contamination index assay.

In terms of LCFS, the observed antifungal activity corroborates with the previous report (Rouse, Harnett, Vaughan, & Sinderen, 2008). The concentrated cell-free supernatant of *L. plantarum* showed potential antifungal activities due to the presence of various antifungal metabolites including phenyllactic, hydroxy fatty acids, benzoic acid, organic acid mixture, methylhydantoin, mevalonolactone, and cyclic dipeptides (Muhialdin et al., 2013; Yang & Chang, 2010). Moreover, Ojha, Kerry, Alvarez, Walsh, and Tiwari (2016) reported that cell-free extracts obtained after fermentation of ultrasound treated samples showed remarkable antimicrobial activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella typhimurium* at lower concentrations than control.

3.2 | Synergistic antifungal effects of thyme essential oil and LCFS

The synergistic antifungal effects of thyme essential oil and LCFS were evaluated by nine combinations and results were elaborated by FIC index method. The lowest concentration of the combined mixture that fully inhibited the growth of *Penicillium* spp. after 7 days of incubation was 1/4 MIC thyme + 1/4 MIC LCFS (Figure 1). This concentration corresponded to the FIC index of 0.5, suggesting the synergistic antifungal effects of essential oil and LCFS against *Penicillium* spp. (Zamani-Zadeh, Soleimanian-Zad, Sheikh-Zeinoddin, & Goli, 2014) reported synergistic effects of thyme essential oil and *L. plantarum* against *Botrytis cinerea* in strawberry. Furthermore, the observed synergism also suggested that there was a fourfold reduction in MICs of essential oil and LCFS when applied together in the mixture.

The synergistic antifungal effects of thyme essential oil and LCFS are explained though the interactions between essential oil and its phenolic compounds with lactic acids as proposed earlier by Barros et al. (2012). Lactic acid in LCFS provides the acidic environment that increases the hydrophobicity of essential oils. This increased hydrophobicity of essential oils allows the better penetration and solubility of essential oil into the lipid layers of cell membrane, resulting in the cytoplasmic membrane disruption and cell lysis. Furthermore, the phenolic compounds present in the essential oil are known to cause sub-lethal injury to the cell membranes through the disruption of proton motive force (H^{+}) and the interference of cellular energy production. This injury makes the cells more vulnerable to the acidic environment caused by lactic acids and organic acids present in LCFS (Barros et al., 2012).

3.3 | Synergistic effects of combined mixture on the *Penicillium* spp.'s hyphae

To evaluate synergistic effects of combined mixture on *Penicillium*'s hyphae, the fungi's hyphae were treated with PBS containing three different concentrations of the combined mixture (0, 1/8 MIC + 1/8 MIC, and 1/4 MIC + 1/4 MIC) and stained with lactophenol cotton blue mounting solution. The visualization of hyphae under a light microscope (Figure 2) presented a shrinkage and distortion of hyphae in the treated samples, whereas the control showed a smooth surfaced hyphae. These alternations are the characteristics of hyphal death caused by the antifungal effects of treatments (Miguélez, Hardisson, & Manzanal, 2000).

The treatments of *Penicillium* spp. hyphae with the combined mixture of thyme essential oil and LCFS significantly resulted in chitin degradation on the fungal cell wall. This was confirmed by the absence of blue color in treated samples after staining with lactophenol cotton blue mounting solution, a dye that stains chitin on the fungal cell wall and appears blue under a light microscope (Goldman & Green, 2009). The degree of degradation increased with increase in the concentration of the mixture applied, suggesting that the effect was derived from the addition of the mixture. In contrast, the control showed homogenous blue color even after 24 hr.

Thyme essential oil and the supernatant of *L. plantarum* lack chitinase and chitinolytic enzymes that degrade chitin. Therefore, the degradation of chitin in the cell wall of *Penicillium* spp.'s hyphae is mediated by the hydrolytic activity of endogenous chitinases present in the cell wall of hyphae (Brzezinska, Jankiewicz, Burkowska, & Walczak, 2014; Nguyen et al., 2012). These endogenous chitinases are triggered and released during the induced autolysis caused by the antifungal metabolites of Thyme essential oil and the supernatant of *L. plantarum* (Hartl, Zach, & Seidl-Seiboth, 2012). The weakened hyphae and damaged fungal cells resulted from the effects of antifungal metabolites that increased the accessibility of the released chitinases into the structural chitin (Gruber & Seidl-Seiboth, 2012).

Chitin accounted for approximately 10–42% of the fungal cell wall in filamentous fungi, its hydrolysis causes the significant deformation

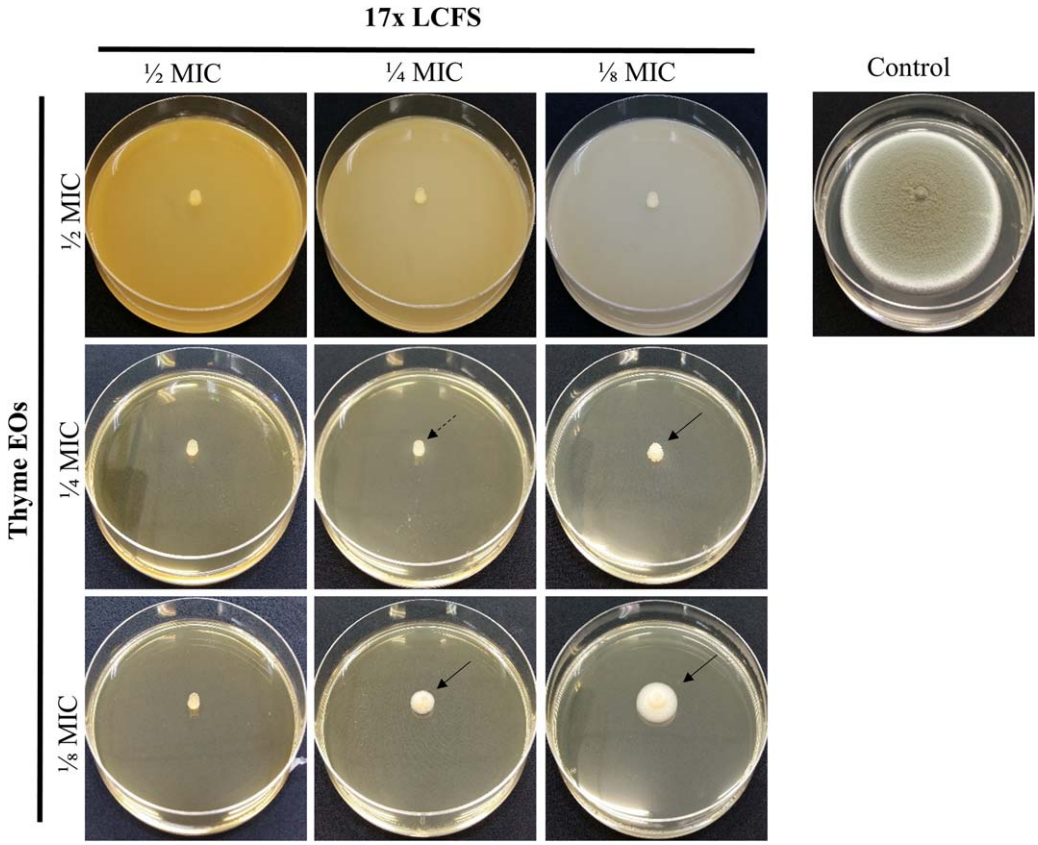

FIGURE 1 Radial growth inhibition of various twofold combinations between Thyme EOs and LCFS after 7 days of incubation at 30 °C against *Penicillium* spp. The matrix shows nine combinations of the combined mixture generated by FIC index method. The control was PDA, containing 2% Tween 80. The arrows indicated the visible growth and the doted arrow indicated the lowest concentration of the combined mixture of EO and LCFS that did not show any visible growth of *Penicillium* spp

of the fungal cells and makes it vulnerable to osmotic stresses, eventually resulting in the death of fungal cells (Hamed, Özogul, & Regenstein, 2016). The degradation of chitin in the fungal cell wall could be a possible mode of action resulting from synergistic effects of Thyme essential oil and LCFS.

3.4 | Determination of cellular constituents leakage

The leakage of cellular constituents from mycelia of *Penicillium* spp. treated with various concentrations of the mixtures (essential oil and LCFS) was determined by measuring the absorbance at 260 nm of the samples collected at different incubation times. The leakage of cellular constituents from *Penicillium* spp. mycelia resulting from the treatments of combined mixture (⅛ MIC + ⅛ MIC and ¼ MIC + ¼ MIC) is presented in Figure 3. The absorbance at 260 nm increased sharply within 4 hr of incubation and continued to increase until the 8th hr followed by the gradual decline within 24 hr of incubation. Ifesan, Joycharat, and Voravuthikunchai (2009) also observed leakage in bacteria and the absorbance at 260 nm of cellular constituents increased in response to the treatments applied and ceased within 24 hr of incubation. The decline in absorbance at 260 nm, observed after the highest peak is likely due to the interactions between the released constituents and combined mixture, hindering its sensitivity to the absorbance measurement.

The higher concentration of the combined mixture (¼ MIC + ¼ MIC) showed the higher absorbance at 260 nm, indicating the higher extent of cellular leakage and the cytoplasmic membrane-damaging activity of the combined mixture. Conversely, the control (PBS, pH 7.0, containing 2% Tween 80) showed lesser extent and rate of release of cellular constituents.

Macromolecules such as proteins, lipids, polysaccharides, and pigments are secreted from the fungal cells through the vesicle-based mechanisms (Rodrigues et al., 2011). As the diffusion and secretion of macromolecules proceeds, the absorbance at 260 nm of control increases with the variation of incubation period. In contrast, the leakage of cellular constituents in treated samples might be mediated by the antifungal effects of various components present in the combined mixtures. The organic acids are also known to increase the permeability of the cytoplasmic membrane which further supports the leakage of intracellular components (Dalié, Deschamps, & Richard-Forget, 2010).

3.5 | In situ antifungal activity of combined mixture in rice seeds

Sterile rice seeds contaminated with spores of *Penicillium* spp. were treated with various concentrations of the combined mixture (thyme essential oil and LCFS) and evaluated for the seeds

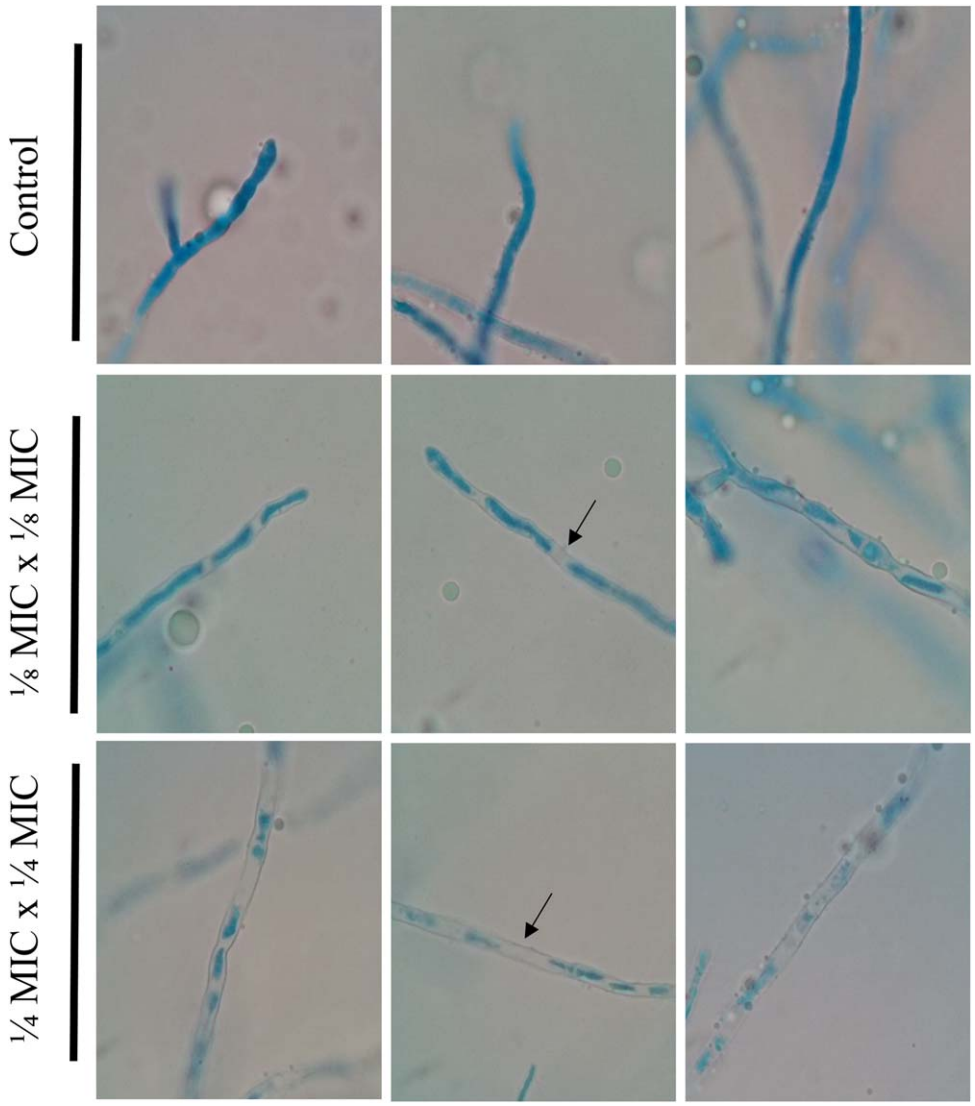

FIGURE 2 Microscopic evaluation of *Penicillium* spp.'s hyphae demonstrating the morphological changes after the treatments with three different concentrations of the combined mixture of Thyme EOs and LCFS (Control, 1/8 MIC + 1/8 MIC, and 1/4 MIC + 1/4 MIC). The hyphae were stained with lactophenol cotton blue mounting solution and visualized at 1,000× magnification under a light microscope. The pointed arrows indicate the absence of blue color and the degradation of chitin in the fungal cell wall

contamination index (SCI, %) after 7 days of incubation. The combined mixtures exhibit the in situ antifungal activity against *Penicillium* spp. in rice seeds, as the growth of fungi is inhibited in a dose-dependent manner. There is a sharp decrease in SCI with increase in the concentration of the mixture (Table 1). Furthermore, the in situ MIC of combined mixture is estimated to be 2 MIC + 2 MIC as there was no visible growth of the fungi at this treatment after 7 days of incubation.

In comparison with the in vitro conditions, the increased MIC of combined mixtures (from 1/4 MIC + 1/4 MIC in vitro to 2 MIC + 2 MIC in situ) might be due to the interactions between antifungal metabolites in the combined mixture and the compositions of rice grains (Calo et al., 2015).

The combined mixture of thyme essential oil and LCFS exhibited significant antifungal activities against *Penicillium* spp. in situ with rice seeds, this observation indicated its potential applications for the industrial uses.

4 | CONCLUSIONS

This study demonstrates a novel approach for synergistic antifungal effects of thyme essential oil and LCFSs against *Penicillium* spp. which resulted in fourfold reduction of individual MICs when applied together in the mixture. The in situ antifungal effects of the mixture observed in rice grains also demonstrate the possible applications of this synergism on actual crops. This approach will overcome the current barrier in the synergism study which are based on the utilization of lived bacteria that are negatively affected by the high antimicrobial activity of essential oil. Moreover, the reduction in MICs of essential oils when applied

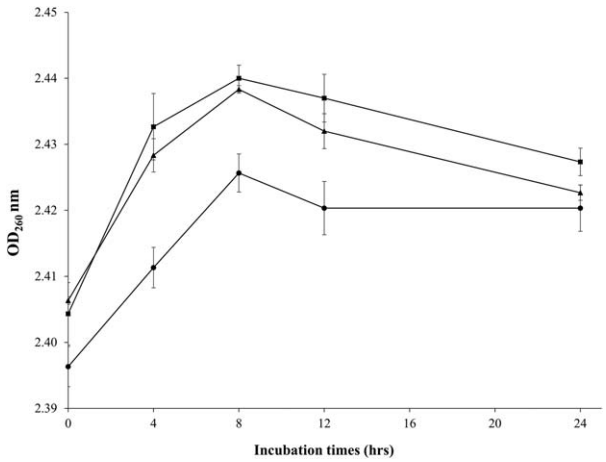

FIGURE 3 The leakage of cellular constituents after treatment with the combined mixture of Thyme EOs and LCFS. The leakage was evaluated by the measuring the release of 260 nm-absorbing materials from *Penicillium* spp.'s mycelia at various time intervals, during 24 hr of incubation period. (•) Control (▲) 1/8 MIC + 1/8 MIC. (■) 1/4 MIC + 1/4 MIC

in combination will help to provide a solution for organoleptic characteristics of the food and feed at industrial scale, that are effected when treated with higher concentrations of essential oils.

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