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Production and antifungal effect of 3-phenyllactic acid (PLA) by lactic acid bacteria

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Abstract Phenyllactic acid (PLA), which is a known antimicrobial compound, can be synthesized through the reduction of phenylpyruvic acid (PPA) by lactate dehydrogenase of lactic acid bacteria (LAB). PLA-producing LAB was isolated from coffee beans, and the isolated LAB was identified as *Lactobacillus zeae* Y44 by 16S rRNA gene sequence analysis. Cell-free supernatant (CFS) from *L. zeae* Y44 was assessed for both its capability to produce the antimicrobial compound PLA and its antifungal activity against three fungal pathogens (*Rhizoctonia solani*, *Botrytis cinerea,* and *Colletotrichum aculatum*). PLA concentration was found to be 4.21 mM in CFS when *L. zeae Y44* was grown in MRS broth containing 5 mM PPA for 12 h. PLA production could be promoted by the supplementation with PPA and phenylalanine (Phe) in the MRS broth, but not affected by 4-hydroxyphenylpyruvic acid, and inhibited by tyrosine as precursors. Antifungal activity assessment demonstrated that all fungal pathogens were sensitive to 5 % CFS (v/v) of *L. zeae* Y44 with average growth inhibitions ranging from 27.8 to 50.0 % (p <0.005), in which *R. solani* was the most sensitive with an inhibition of 50.0 %, followed by *B. cinerea* and *C. aculatum*. However, pH modification of CFS to pH 6.5 caused an extreme reduction in their antifungal activity. These results may indicate that the antifungal activity of CFS was caused by acidic compounds like PLA or organic acids rather than proteins or peptides molecules.

Keywords 3-Phenyllactic acid · Antifungal effect · *Lactobacillus zeae* Y44 · Supplement

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Introduction

Lactic acid bacteria (LAB) have been known to produce antagonistic compounds which inhibit food spoilage molds and bacteria. A wide variety of active antimicrobial compounds such as lactic acid, benzoic acid, hydroxyl acids, reuterine, and bacteriocins were produced during LAB fermentation (Lavermicocca et al. 2000; Messens and De Vuyst 2002; Kim et al. 2009). Phenyllactic acid (PLA) and 4-hydroxyphenyllactic acid (HPLA), which can be produced by many LAB (Lavermicocca et al. 2000), are also known as antifungal compounds. Especially, PLA was characterized as having a broad inhibitory activity against yeast and bacteria as well as the inhibition of conidial germination of fungi (Dieuleveux et al. 1998; Dieuleveux and Gueguen 1998). Valerio et al. (2004) reported that both the organic acids could be produced by a wide range of LAB species but that their production was strain dependent. It is presumed that the behavior of the antifungal activity is positively related to the PLA and HPLA content in LAB culture filtrates. Although the production of PLA varies greatly based on the strains and species, it is known that PLA can be produced by a wide range of LAB species, such as *Lactobacillus*, *Enterococcus*, *Weissella*, and *Leuconostoc* (Valerio et al. 2004). PLA and HPLA were identified as metabolic by-products of amino acids phenylalanine and tyrosine, respectively, in LAB strains. These amino acids are transaminated to 2-keto-carboxylic acids such as phenylpyruvic acid (PPA) and 4-hydroxyphenypyruvic acid (HPPA) (Yvon et al. 1997), and the ketoacids are further reduced to 2-hydroxy carboxylic acids (PLA and HPLA) (Vermeulen et al. 2006; Li et al. 2007; Mu et al. 2010).

The PPA and Phe were known as the metabolic precursors of PLA, while Tyr and HPPA are the precursors of HPLA in LAB strains (Vermeulen et al. 2006; Zheng et al. 2011).

The supplementation of the above amino acids and ketoacids in the initial culture broth for LAB fermentation could effectively increase the corresponding 2-hydroxy acids (PLA or HPLA) production. The supplementation of ketoacids is more effective

than that of the original amino acids to improve the corresponding 2-hydroxy acids biosynthesis (Li et al. 2007; Mu et al. 2009; Mu et al. 2010).

Furthermore, PLA is an antimicrobial compound with a wide activity spectrum against some yeast such as *Candida pulcherrima* and *Rhodotorula mucilaginosa* (Schwenninger et al. 2008); and molds including some mycotoxigenic species such as *Aspergillus ochraceus* and *Penicillium citrinum* (Valerio et al. 2004). In addition, PLA has been found to inhibit a range of Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus, Listeria monocytogenes, Enterococcus faecalis,* and *Klebsiella oxycota* (Lavermicocca et al. 2000; Valerio et al. 2004). Recently, several LAB have been screened for their antifungal potential and their ability to produce PLA (Gerez et al. 2010; Ryan et al. 2011; Gerez et al. 2013), but additional studies are required in this field due to the wide diversity of both LAB and food spoilage molds. Therefore, the aim of this work was to isolate novel LAB strains and assess their antifungal activity against three fungal pathogens (*Rhizoctonia solani*, *Botrytis cinerea,* and *Colletotrichum aculatum);* and subsequently to study their capability to produce the antimicrobial compound 3-phenyllactic acid as well as its relationship with the antifungal activity of the LAB.

Materials and Methods

Microorganism and culture media

LAB were obtained from coffee beans and cultivated in deMan-Rogosa-Sharpe (MRS) medium at 30°C without shaking. MRS medium contained 10 g L^{-1} peptone, 10 g L^{-1} beef extract, 5 g L^{-1} yeast extract, 20 g L^{-1} glucose, 2 g L^{-1}, K_2HPO_4, 2 g L^{-1} triammonium citrate, 5 g L^{-1} CH3COONa, 1 mL L^{-1} Tween-80, 0.58 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.25 g L^{-1} $MnSO_4 \cdot 4H_2O$, and pH was adjusted to 6.2 with 1 M HCl or NaOH. All the microbial fermentation experiments were developed in 10 mL test tubes containing 5 mL MRS broth. LABs producing PLA (Sigma Co., St. Louis, MO, USA) were isolated with 5 mL MRS broth containing 5 mM PPA (Sigma Co.). To prepare the inoculum for fermentation experiments, the isolated LABs were inoculated into a 10 mL test tube containing 5 mL MRS broth and grown for the seed culture at 30°C for 24 h. A 2 % (v/v) inoculum of the seed was aseptically added to 5 mL of MRS for fermentation experiments. In order to evaluate the effect of amino acids and ketoacids precursors on PLA production, different substrates, PPA, HPPA, phenylalanine, and tyrosine purchased from Sigma were added as supplements to the initial MRS broth at various concentrations. The fermentation was carried out in a 10 mL test tube at 30°C for 36 h without shaking. Control strains for fungal pathogens *Rhizoctonia solani*, *Botrytis cinerea, Colletotrichum aculatum*, and *Aspergillus oryzae* were provided by the Chungcheongnam-Do Agricultural Research & Extension Services.

Identification of LAB

The partial sequencing of 16S rRNA for the LAB strain was done with the help of a DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The online program BLAST was used in identifying the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using the CLUSTAL X program (Thompson et al. 1997), which involved sequence alignment by neighbor joining method (Saitou and Nei 1987) and maximum parsimony using the MEGA4 program (Kumar et al. 2001). Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura's two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Preparation of cell-free supernatants (CFS)

LAB colonies were precultured for 18 h at 30°C in 10 mL of MRS broth. An aliquot (200 μL) of the preculture was then inoculated into fresh sterile MRS broth (20 mL) and allowed to grow at 30°C without shaking for 72 h. CFS were recovered by centrifugation (7200×g, 10 min) of the culture, and filtered through a 0.45-μm Millipore membrane to remove the subsistant cells. The resulting CFS were used for identifying PLA and screening their antifungal activity.

Determination of antifungal activity

Antifungal activity was tested with cell-free supernatants of LAB by the modification method of Wang et al. (2012). Briefly, CFS was mixed with sterilized PDA (pH 4) to achieve a final concentration of 5–10 % for *Rhizoctonia solani*, *Botrytis cinerea, Colletotrichum aculatum* (v/v), *and Aspergillus oryzae* as a control strain. The mixture was then poured into Petri dishes. Resulting media was centrally inoculated with 5 μL of the previously prepared fungal pathogen spore suspensions and incubated at 25°C. Sterile MRS broth was used for control plates instead of CFS in the same proportions. During the 8-day incubation period to grow the pathogens onto the plate, the areas of mycelial growth in both treated and control plates were determined from the mean perpendicular diameter measurements assuming a circular growth. The percentage of growth inhibition (I) was calculated as $I = 100(A_C - A_T)/A_C$, where A_T are the area of mycelial growth in both treated and control plates, respectively. CFS were also subjected to heating (121°C, 15 min) treatment to investigate the possible presence of antifungal compounds other than PLA in each CFS, and the remaining antifungal activity of treated CFS was further assessed as described above.

Identification of PLA

The presence of PLA in CFS was identified by HPLC as described by Valerio et al. (2004) and Gerez et al. (2010) with minor modifications. CFS were adjusted to pH 2.0 using 10 M formic acid and extracted three times with 20 mL of ethyl acetate. The extracts were dried using Na_2SO_4 and concentrated in a rotary evaporator (Büchi model R210/215, Flawil, Switzerland). The dried residues were reconstituted with 5 mL of 2.5 mM H_3PO_4. The PLA component was filtered with a 0.45 µm pore-size membrane and analyzed in an HPLC system (Agilent, 1260 infinity, USA) fitted with an CAPCELL PAK C18 column (4.6× 250 mm, 5 µm, Shiseido Co., Tokyo, Japan) at 30°C, using (A) 0.5 % H_3PO_4 (v/v) and (B) 0.5 % H_3PO_4-CH_3CN (V/V) as the mobile phases. AUV-visible detector set to 210 nm was used. Linear gradient elution was used with solvent A and solvent B at 1 mL min^{-1} and A/B ratios of 80:20, 80:20, 0:100 and 0:100 with run times of 0, 12, 13, and 15 min, respectively (Li et al. 2007; Mu et al. 2010). Commercial PLA was used as reference (retention time=11.66 min).

Statistical analysis

Three independent replicates of each experiment were performed, and their results were expressed as mean values ± standard deviation. The data were subjected to analysis of variance (ANOVA) using SAS package (SAS 1999). The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at $p \leq 0.05$.

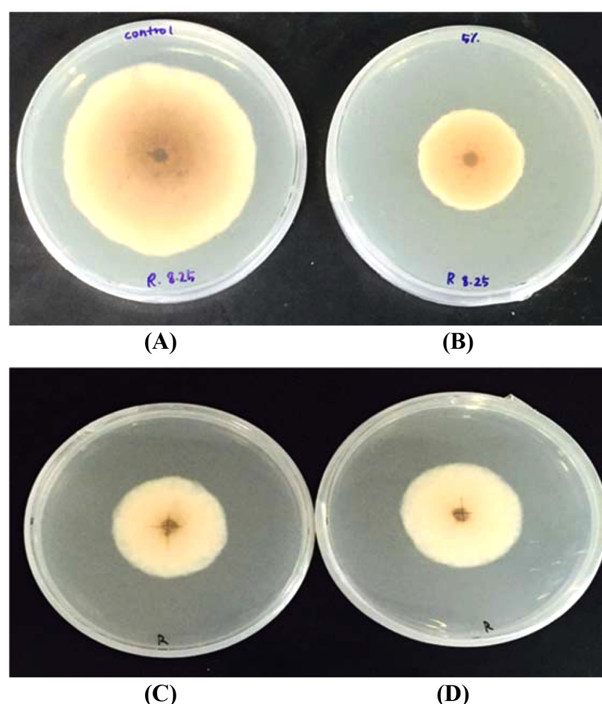

Fig. 1 Growth of *Rhizotonia solani* in PDA after 10 days at 25°C in presence of CFS of *Lactobacillus zeae* Y44 fermentation in MRS broth: (A) PDA+5 % MRS broth (v/v), (B) PDA+5 % CFS (PLA 702 mg L^{-1}, v/v), (C) PDA+PLA (Sigma, 500 mg L^{-1}), (D) PDA+PLA (Sigma, 550 mg L^{-1})

Results and Discussion

Isolation and identification of PLA producing LAB

A PLA producing strain was selected by assessing the mycelial growth inhibition of *Rhizoctonia solani* on MRS media with culture filtrates of LAB isolated from coffee beans and finally by confirming the presence of PLA in the culture filtrate through HPLC analysis. The selected strain produced a marked inhibition of mycelial growth of fungal pathogens. The perpendicular diameter of a circular growth after 7 days of incubation was similar to that produced at the same concentration of commercial PLA (500 mg L^{-1}) used as a control (Fig. 1). According to 16S rRNA sequence analysis, the strain was identified as *Lactobacillus zeae.* Comparison of the 16S rRNA sequence among available strains of *Lactobacillus* species showed high homology (99.4 %) to *Lactobacillus zeae* ATCC 15820T/D86516. The Neighbor-joining method was employed to construct the phylogenetic tree which illustrates the relationships between 16S rRNA gene sequences of a strain to other *Lactobacillus* species (Fig. 2).

Cell growth

L. zeae fermentation was performed at 30°C with MRS broth without PPA to assess PLA production and pH change. Growth and PLA production curves for *L.* zeae are shown in Fig. 3. Cell growth reached the stationary phase after 12 h, and PLA production progressively increased thereafter until the end of this phase. The highest production reached 0.80 mM (132.93 mg L^{-1}) after 36 h, during which the initial pH of 6.5 was reduced to pH 4.0, indicating that the acidification of the media was directly related to the production of PLA by LAB. A similar result was described by Vermeulen et al. (2006) who reported the continued production of PLA by *L. sanfranciscensis* DSM20451 cells in stationary phase. Valerio et al. (2004) also reported that the production of PLA by *L. plantarum* ITM21B grown in synthetic media reached a maximum concentration of 0.165 mM after 72 h. Recently, the investigation of optimal growth conditions of LAB during fermentation has become a topic of study in order to achieve the maximum production of a given metabolite with antifungal properties, such as PLA (Mu et al. 2012; Rodríguez et al. 2012; Song et al. 2015).

Effect of precursor supplements on PLA production

The effect of ketoacids (PPA and HPPA) and amino acids (Phe and Tyr) supplementation on PLA during *L. zeae* Y44 fermentation process was investigated. As shown in Fig. 4, PLA production was effectively improved by the supplementation to a concentration of 5 mM PPA and Phenyalanine (Phe). Although

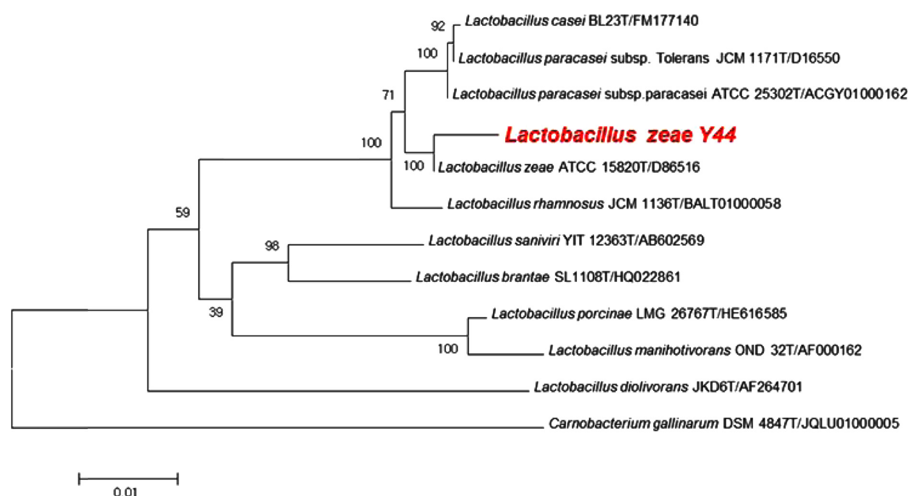

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Lactobacillus zeae* Y44 strain with respect to related species. The scale bar indicates 0.01 substitutions per nucleotide position and accession numbers are given in parenthesis

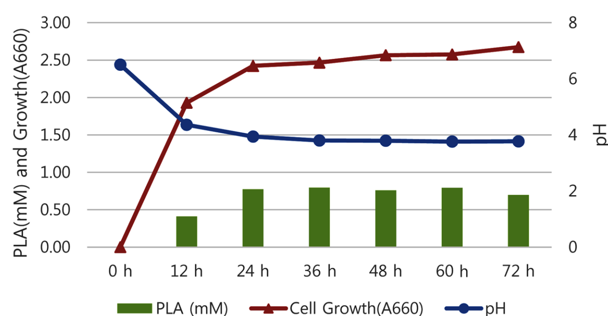

Fig. 3 Cell growth, pH variation, and PLA production by *Lactobacillus zeae* Y44 in MRS broth. All the fermentations were developed at 30°C for 72 h in MRS broth without the precursors

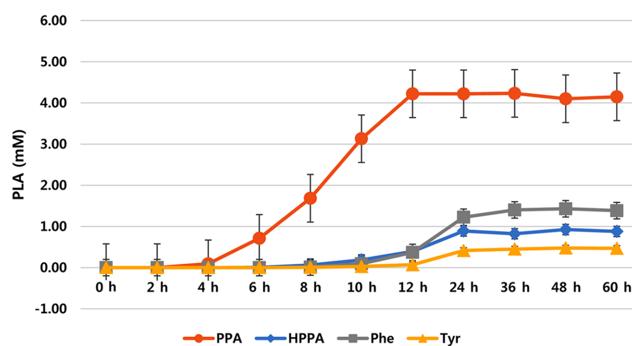

Fig. 4 The effects on supplementation of ketoacids and amino acids on PLA production during *L. zeae* Y44 fermentation process. All the fermentations were developed at 30°C for 60 h in MRS broth with each 5 mM of PPA, HPPA, Phe and Tyr. Values are means of three replications ± standard deviation

the PPA precursors had more promotional effect on the corresponding 2-hydroxy acids (PLA) production than Phe did, maximum PLA production was reached at 4.23 mM (702.89 mg L^{-1}) and 1.43 mM, respectively, after 24 h fermentation, in comparison to the result of 0.80 mM PLA obtained by the fermentation with MRS broth only without PPA supplementation (Fig. 1). However, when the LAB was fermented in MRS broth supplemented with 5 mM HPPA and Tyr, PLA production reached 0.76 mM and 0.47 mM, respectively, indicating that PLA production by *L. zeae* Y44 was somewhat inhibited by Tyr. Several reports suggest that PPA and Phe are the metabolic precursors of PLA, while Tyr and HPPA are the precursors of HPLA in LAB strains (Vermeulen et al. 2006; Zheng et al. 2011). Furthermore, PLA production could be improved by supplementing with the corresponding precursors (PPA and Phe) (Valerio et al. 2004). A similar result was obtained in the present study in which the ketoacid (PPA) and amino acid (Phe) supplements, employed as substrates, must have affected the yield of the metabolic precursors such as PLA during fermentation process. In addition, the influence of various supplement amounts of amino acid and ketoacid precursors on PLA production during *L. zeae* Y44 fermentation process was investigated. Both the promotional effect of the corresponding precursors (PPA and Phe) and the inhibitory effect of the non-corresponding precursors (HPPA and Tyr) on PLA production were strengthened with increasing the supplement amounts (Table 1). Yvon et al. (1997) reported that aminotransferase initiated the degradation of Phe and Tyr to produce the corresponding ketoacids, PPA and HPPA. Subsequent reports identified that PLA was the hydrogenation-reduction product of PPA (Mu et al. 2010; Zheng et al. 2011). Furthermore, in the PLA metabolic pathway, the supplement of non-corresponding amino acid precursor (Tyr) might inhibit the aminotransferation of the corresponding amino acid precursor (Phe). A similar inhibitory effect on PLA production was identified with Tyr supplementation, in which Tyr showed more inhibitory effect on non-corresponding PLA production than HPPA. In addition, when the

Table 1 Effect of various supplement amounts (HPPA, PPA, PHE, Tyr) for PLA production by *Lactobacillus zeae* Y44

PLA production (mM)				
Supplement amount (mM)	PPA	HPPA	PHE	Try
0	0.81±0.02	0.81±0.01	0.8±0.05	0.81±0.02
2	2.34±0.03	1.32±0.04	1.25±0.02	0.61±0.01
4	3.79±0.04	1.76±0.03	1.49±0.04	0.80±0.04
6	6.45±0.08	1.48±0.02	1.91±0.03	0.69±0.03
8	7.55±0.131	1.39±0.06	2.30±0.04	0.66±0.02
10	10.65±0.21	1.32±0.05	2.43±0.06	0.70±0.03

Values are means of three replications ± standard deviation

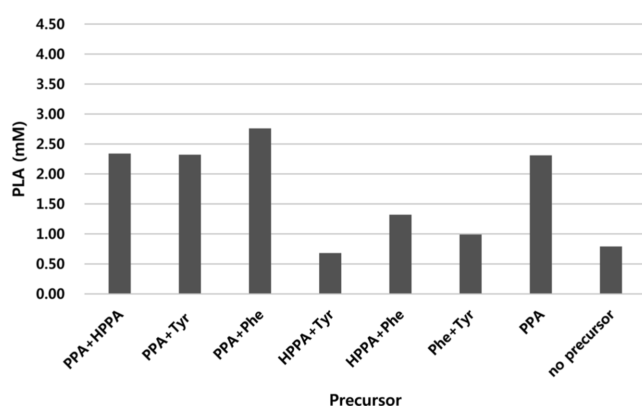

Fig. 5 The effects on supplementation with the mixture of ketoacids and amino acids on PLA production during *L. zeae* Y44 fermentation process. All the fermentations were developed at 30°C for 24 h in MRS broth with the mixture of each 2.5 mM PPA, HPPA, Phe and Tyr. Values are means of three replications ± standard deviation

mixture effect on PLA production was investigate with the mixture of either 2.5 mM of the corresponding precursors (PPA and Phe) or the non-corresponding ones (HPPA and Tyr), even though the highest inhibitory precursor, Tyr was supplied to one of the mixture, the mixtures combined with the corresponding ketoacid, PPA have been appeared to promote PLA synthesis during LAB fermentation (Fig. 5). This results suggested that PPA was the highest promoting precursor in PLA metabolite pathway by *L. zeae* and other precursors besides PPA have not a decisive effect on PLA production.

Inhibitory effect of CFS against fungal pathogens

The observation of antifungal activity by CFS of *L. zeae* Y44 demonstrated that all fungal pathogens were sensitive to 5 % CFS (v/v) with average growth inhibitions ranging from 27.8 to 50.0 % ($p<0.005$). *Rhizoctonia solani* was most sensitive with 50.0 % inhibition, followed by *B. cinerea*, *C. aculatum*, *and A. oryzae* (Table 2). The apparent results were also confirmed in further minimum inhibitory concentration (MIC) experiments using commercial PLA and chemical antimicrobial agents. Moreover, CFS of *L. zeae* Y44 was subjected to heating (121°C, 20 min) and pH modification (from 4.0 to 6.5) in order to assess the characteristics of other potentially present antifungal compounds besides PLA. The inhibition characteristics of CFS against pathogens were not affected by the heating or protease treatments. However, pH modification to 6.5 caused an extreme reduction in their antifungal activity (Table 3). These results indicate that antifungal activities of CFS are very likely to be lost by acidic compounds neutralizing the pH of the media rather than by thermal denaturation of proteins or peptide molecules. It is known that the antimicrobial activity of organic acids on molds and bacteria is pH-dependent and that a maximum inhibitory activity can be maintained at low pH values favoring the undissociated state of the acid molecule (Gerez et al. 2010; Schillinger and Villareal, 2010). Similar results were obtained by Wang et al. (2012) and Zhang et al. (2014) who reported the antifungal activity of CFS derived from *L. plantarum* IMAU10014 against

Table 2 Minimal inhibitory concentrations of commercial phenyllactic acid (PLA) in the studied fungal pathogens*

Antimicrobial	Fungal pathogens			
	Aspergillus oryzae	*Botrytis cinerea*	*Colletotrichum aculatum*	*Rhizoctonia solani*
Sodium benzonate (mg mL^{-1})	0.58±0.03	0.51±0.01	0.42±0.03	0.35±0.02
Polyoxin (mg mL^{-1})	0.19±0.01	0.12±0.02	0.14±0.01	0.13±0.01
PLA (mg mL^{-1})	0.67±0.02	0.37±0.04	0.43±0.02	0.29±0.01

*Minimal inhibitory concentrations were determined at pH 4.0 and 25 °C in PDA medium. Values are means of three replications ± standard deviation

Table 3 Percentage of growth inhibition (I) of selected fungi by CFS obtained from by *Lactobacillus zeae* Y44 fermentation with some modifications

Fungal pathogens	CFS (a)	pH 6.5 (b)	121 °C (c)	Trypsin (d)
Aspergillus oryzae	40.6	0	37.5	40.6
Botrytis cinerea	32.9	0	26.8	31.7
Colletotri chumaculatum	27.8	0	29.1	26.6
Rhizoctonia solani	50	0	63.7	41.9

(a) PDA treated with CFS, (b) PDA treated with pH-modified CFS (pH=6.5), (c) PDA with heat-treated CFS (121 °C, 20 min), and (d) PDA treated with CFS + Trypsin. All plates were prepared with a 5 % CFS (v/v). The percentage of growth inhibition (I) was calculated as $I=100(A_C-A_T)/A_C$, where A_T are the area of mycelial growth in both treated and control plates

B. cinerea, *Glomerella cingulate*, *Phytophthora drechsleri*, *Penicillium citrinum*, *P. digitatum*, and *Fusarium oxysporum*. Furthermore, growth inhibition properties of CFS can also be caused by synergism with other acidic compounds from LAB metabolism such as 4-hydroxyphenyllactic acid (HPLA) and organic acids (Schillinger and Villareal 2010; Hladíková et al. 2012). In this study even though authors have not identified the existence of HPLA in CFS, conclusively, the inhibitory effect was due to acidic metabolites in fermentation broth, and PLA present in CFS was involved in the inhibitory activity against fungal pathogens.

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