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ORIGINAL PAPER

Activity against plant pathogenic fungi of *Lactobacillus plantarum* IMAU10014 isolated from Xinjiang koumiss in China

HaiKuan Wang · Hu Yan · Jing Shin · Lin Huang · HePing Zhang · Wei Qi

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Abstract Thirty-five of the 77 colonies of lactic acid bacteria isolated from koumiss showed antifungal activity against *Botrytis cinerea*, when tested by the Poison Food Technique. Of these, the most promising isolate having a broad spectrum of antifungal activity, including against *Botrytis cinerea*, *Alternaria solani*, *Phytophthora drechsleri* Tucker, *Fusarium oxysporum* and *Glomerella cingulata*, was identified as *Lactobacillus plantarum* IMAU10014. The effect of pH, temperature and protease on the antifungal activity of *L. plantarum* IMAU10014 was determined. The activity substance was heat stable. The maximum antifungal activity was observed at pH 4.0. When the supernatant was adjusted to pH 8.0, the activity was lost irreversibly. However, after enzymatic treatment of supernatant with trypsin, neutral protease and proteinase K, loss of part of the antifungal activity was observed which indicated there are other substances in addition to the proteinaceous substance with the antifungal activity.

Keywords Plant pathogenic fungi · Antifungal activity · Lactic acid bacterium · Fungicides · *Lactobacillus plantarum*

H. Wang · H. Yan · J. Shin · L. Huang · W. Qi (✉)
Key Laboratory of Industrial Microbiology,
Ministry of Education, College of Biotechnology,
Tianjin University of Science and Technology,
Tianjin, People's Republic of China
e-mail: haikuanwangcn@yahoo.com.cn

H. Zhang (✉)
Key Laboratory of Dairy Biotechnology and Engineering,
Ministry of Education, Inner Mongolia Agricultural University,
Huhhot, People's Republic of China
e-mail: hepingdd@vip.sina.com

Introduction

Fungal plant pathogens, which infect all major crops, are a threat to global food security. They cause serious losses both in the field and post-harvest, and some may produce mycotoxins (Strange and Scott 2005). Various artificial chemical fungicides have been successfully developed and applied to fields to control fungal diseases (Sun et al. 2008). However, because of their huge populations and high frequency of mutation, pathogenic fungi may easily acquire resistance to frequently used fungicides. Several important chemical fungicides, for example anilinopyrimidine, benzimidazoles, demethylation inhibitors (DMI), dicarboximide, phenylpyrrole, Qo respiration inhibitors, and strobilurin, have lost high efficacy against pathogenic fungi in the field (Yang et al. 2008). Thus, there is a definite need for safe and efficient ways to prevent fungal growth in raw materials and food products.

Concurrently, biological agents such as fungi and bacteria could also be anticipated to be applied as part of an integrated pest management program to control plant-parasitic fungi, although only a few commercial formulations have been developed, and their use so far is limited. The most significant progress in this area is the discovery of the skeletal structures of new antifungal compounds usually coming from natural sources (Bevan and Ryder 1995). To reduce the risk of crop disease control and enhance the safety of food and the environment, new safe fungicides should be discovered and developed (Yang et al. 2008). This has made people pay more attention to investigating the production of new biological fungicides from microorganisms, while interest in the metabolites of bacterium is increasing.

The metabolites from lactic acid bacterium showed activity against some kinds of important fungi (Jaroszewski

et al. 2009). Sjogren et al. (2003) report the identification and chemical characterization of four antifungal substances produced by *Lactobacillus plantarum* MiLAB 14, 3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid. Racemic mixtures of the saturated 3-hydroxy fatty acids showed antifungal activity against different molds and yeasts with MICs between 10 and 100 μg mL^{-1}. Rouse et al. (2008) characterized the antifungal compounds produced by LAB indicates that their activity is likely to be because of production of antifungal peptides. The trial conducted showed that the antifungal culture has the ability to prevent growth of the mold involved in apple spoilage, using apples as a model.

Koumiss is a traditional fermented mare's milk drink of nomads in Central Asia, and is popular among the people of Mongolia, Kazakhstan, Kirgizstan and some regions of Russia (Bouriatie, Kalmoukie and Bashkirie). It is usually made from mare's or camel's milk fermented by its normal microbiota, and has been used in the treatment of tuberculosis and other lung ailments. Some research has been conducted on the isolation and characterization of *Lactobacillus* strains from koumiss (Sun et al. 2009). However, there is no report on the screening and characterization of koumiss-originated *Lactobacillus* which possesses activity against plant pathogenic fungi.

In this contribution, we report for the first time activity against plant pathogenic fungi and the principal antifungal characteristics of *L. plantarum* IMAU10014 isolated from Xinjiang koumiss in China.

Materials and methods

Screening of *Lactobacillus* cultures for antifungal activity

Seventy-seven strains of *Lactobacillus* were isolated from the koumiss collected from Xinjiang, China. One milliliter of the koumiss sample was transferred aseptically into 10 mL sterile litmus milk (DIFCO) incubated at 30°C. After coagulation of the milk, one loop of the milk was streaked onto Man Rogosa Sharpe broth (MRS, Fluka) and Trypticase peptone yeast broth (TPY, DIFCO) agar plate incubated at 37°C for 48–72 h. A representative single colony was selected from the agar plates and examined microscopically. Gram-positive, catalase-negative bacterial isolates were purified and preserved in the same medium containing 15% glycerol at −20°C.

Lactobacillus seed culture was prepared in a 100-mL flask with 70 mL MRS medium incubated at 37°C for 18 h. A 3% volume of seed culture was used as the inoculum for the flask culture. The flask cultures were carried out in a 250-mL flask with 200 mL MRS medium at 37°C for 30 h. The *Lactobacillus* culture was centrifuged at 6,000 *g* for 10 min. Subsequently, the supernatant was used to determine the antifungal activity; plant pathogenic fungi *B. cinerea* was used as the indicator.

Identification of *Lactobacillus*

Identification of *Lactobacillus* was conducted using 16 S ribosomal RNA (rRNA) analysis. Genomic RNA from *Lactobacillus* was isolated using Promega kit according to the manufacturer's instructions. To amplify the full length of 16 S rRNA sequence, PCR was performed using the upstream primer 5 - AGA GTT TGA TCC TGG CTC AG -3 and the downstream primer 5 - CTA CGG CTA CCT TGT TAC GA -3 . The cycling program was 96°C for 90 s, 48°C for 30 s, 72°C for 4 min for the first cycle and 95°C for 30 s, 48°C for 15 s, 72°C for 4 min for the next 35 cycles. The purified PCR products were cloned into the T vector and sequence analysis was performed by Sangni Biosciences (Shanghai, China). A homology search to reference strains registered in DDBJ/EMBL/GenBank was performed using NCBI BLAST.

Antifungal activity assays

The antifungal activity in vitro was determined by a Poison Food Technique (Sridhar et al. 2003). Potato dextrose agar (PDA) was used as the medium for all test fungi. The media incorporating test compounds at a concentration of 10% (v/v) was inoculated with agar discs of the test fungi (5 mm) at the center. Three replicate plates for each fungus were incubated at 27±2°C for all test fungi. Control plates containing media mixed with sterile water (10%, v/v) were included. After incubation for 2–6 days, the mycelial growth of fungi (mm) in both treated (T) and control (C) Petri dishes was measured diametrically in perpendicular directions until the fungi growth in the control dishes was almost complete. The percentage of growth inhibition (I) was calculated using the formula: $I(\%) \times [(C - T)/C] \times 100$. The corrected inhibition (IC) was then calculated as follow: $IC(\%) = [(C - T)/(C - C_0)] \times 100$. C_0 means the diameter of the test fungi agar discs (5 mm).

Production of antifungal substance

Fermentations were carried out in a 30-L fermenter containing MRS medium with stirring (50*g*) at 37°C for 48 h. The production medium was seeded with 5% (v/v) inoculum from an overnight culture of *L. plantarum* IMAU10014. Cell-free supernatant was prepared by centrifugation (8,000*g*, 10 min and 4°C) and sterile filtration (0.22 μm, Millipore). The cell-free supernatant was stored at −20°C and used for further characterizing of the antifungal compounds.

Table 1 The antifungal activity of 35 LAB against *Botrytis cinerea*

LAB	*I* (%)[a]	LAB	*I* (%)[a]	LAB	*I* (%)[a]	LAB	*I* (%)[a]
IMAU10014[b]	86.0±2.0	IMAU10010[c]	63.7±5.3	IMAU50032[e]	30.9±0.5	IMAU20003[b]	21.1±8.3
IMAU40010[b]	84.6±1.9	IMAU10013[b]	56.3±4.6	IMAU20087[d]	29.2±4.2	IMAU20026[b]	16.9±2.9
IMAU10004[c]	81.4±2.7	IMAU60092[c]	56.3±1.3	IMAU20009[b]	25.6±1.3	IMAU10011[b]	15.6±5.2
IMAU40007[b]	78.1±2.2	IMAUB0014[d]	51.4±4.1	IMAU10116[b]	25.2±2.2	IMAU10124[b]	13.8±2.7
IMAU30001[b]	76.9±1.4	IMAUB0011[d]	48.4±1.9	IMAU40105[b]	25.1±2.7	IMAU10016[b]	20.3±1.3
IMAU10043[c]	69.4±5.8	IMAU10128[b]	43.0±0.8	IMAU10123[f]	24.8±4.1	IMAU10012[b]	20.1±3.1
IMAU10032[c]	65.3±6.4	IMAU50085[c]	41.9±1.5	IMAU10126[c]	23.8±6.4	IMAU20029[b]	19.2±2.2
IMAU10024[b]	64.1±4.1	IMAU30163[e]	36.0±2.2	IMAU10015[b]	21.4±1.2	IMAU20091[b]	18.9±4.0
IMAU30160[c]	63.9±2.2	IMAU10129[b]	33.7±8.3	IMAU20089[d]	21.4±5.3		

[a] The percentage of growth inhibition. Data are means of triplicate determinations±standard deviation

[b] *Lactobacillus plantarum*

[c] *Lactobacillus casei*

[d] *Lactobacillus fermentum*

[e] *Lactobacillus. helveticus*

[f] *Enterococcus faecalis*

Characteristics of the active antifungal principles from cultures in the fermenter

The filter sterilized cell-free supernatant from the test culture were subjected to different pH values namely 3.5, 4.0, 5.0, 6.0 and 7.0 before carrying out the antifungal activity assay. The pH of the cell-free supernatant was adjusted with HCl (2 M) and NaOH (2 M). The antifungal activity after each treatment was determined with the Poison Food Technique assay with *Alternaria solani* as the indicator strain. The antifungal activity test of culture filtrate was repeated three times.

The filter sterilized cell-free supernatant was treated with different temperature 80°C and 100°C for 10 and 60 min, separately. The antifungal activity after each treatment was determined as described above.

The filter sterilized cell-free supernatant was separately treated with trypsin (NuoAo, TianJin), proteinase K (Amresco, American) and neutral protease (NuoAo, Tian-Jin) in 100 mM sodium phosphate buffer, pH 7.0. The samples and the controls were incubated at 37°C for 120 min before testing them for the antifungal activity assay as described above.

Determination of antifungal spectra

Seventy-seven strains of *Lactobacillus* were screened against the indicator mold *B. cinerea*, and among these, 45 isolates against the *B. cinerea* were futher selected for

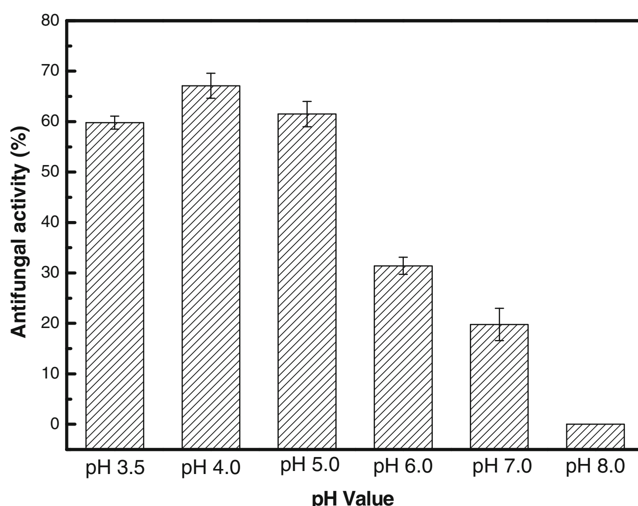

Fig. 1 Effect of pH on the antifungal activity of *L. plantarum* IMAU10014

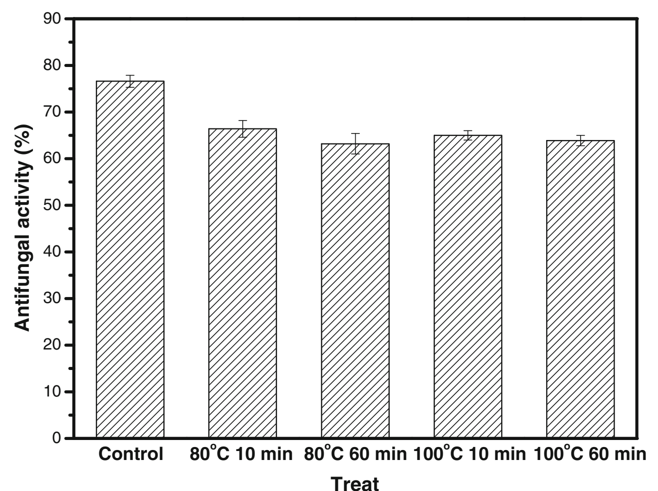

Fig. 2 Effect of temperature on the antifungal activity of *L. plantarum* IMAU10014

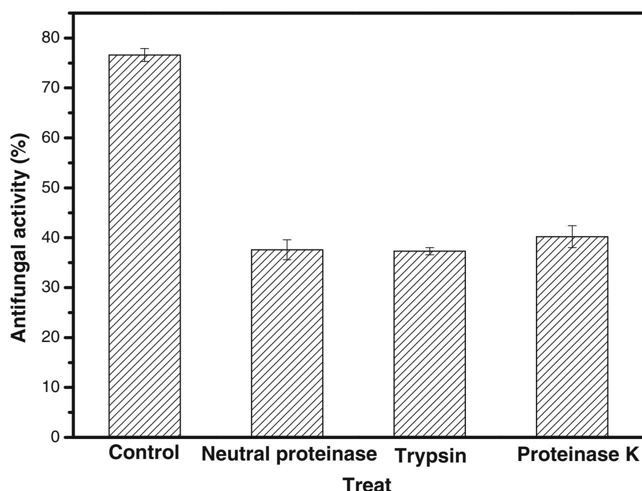

Fig. 3 Effect of protease on the antifungal activity of *L. plantarum* IMAU10014

Table 3 Growth inhibition (%) of *B. cinerea*, *P. drechsleri Tucker*, *A. solani*, *G. cingulata* and *F. oxysporum* by lactic acid* compared with cell-free supernatant fluids of *L. plantarum* IMAU10014

strain	Growth inhibition (%±SD.)	
	Lactic acid[a]	*L. plantarum* IMAU10014
B. cinerea	13.4±2.1	86.0±2.0
P. drechsleri Tucker	21.1±5.0	100.0±0.0
F. oxysporum	16.7±4.3	79.7±3.7
A. solani	9.7±3.5	79.3±6.4
G. cingulata	8.2±4.0	92.4±2.9

[a] The concentration of lactic acid in the well was 67.6 mM, corresponding to the amount of lactic acid added along with the cell-free supernatant fluid of *L. plantarum* IMAU10014. In the control plates, lactic acid was replaced by an equal volume of sterile water. Data are means of triplicate determinations±standard deviation

determining antifungal activity against 5 plant pathogenic fungi: *B. cinerea*, *A. solani*, *P. drechsleri* Tucker, *F. oxysporum* and *G. cingulata*.

Analysis of lactic acid

Lactic acid was determined by Agilent 1100 HPLC system with a u.v. detector at 210 nm (Laitila et al. 2002). Agilent ZORBAX SB-AQ column 4.6×150 mm, 5 μm was used at 35°C with 1% ACN/99% 20 mM $NaHPO_4$ (pH 2) as the mobile phase, at a flow rate of 1 mL min^{-1}.

Tomato leaves test

To determine if the antifungal-producing LAB had a potential application in agriculture, a trial was undertaken using tomato leaves as model, and *B. cinerea* was used as the indicator. *L. plantarum* IMAU10014 fermentation broth was uniformly sprayed on fresh tomato leaves, while the control were sprayed with sterile water. And then the tomato leaves were put in a petri dish with two layers of filter paper. The filter paper was used to maintain the required humidity by adding appropriate amounts of sterile water. The agar discs of the test fungi (5 mm diameter) were put on the center of the leaves with mycelia facing the leaf surfaces. The treated and control dishes were kept in the laboratory at 25±2°C incubator for 5–7 days.

Results

Screening of antifungal activity of 77 LAB strains

A total of 77 LAB isolates from Xinjiang koumiss in China were screened for antifungal activity using the Poison Food Technique with *B. cinerea* as the indicator mold. Screening results revealed that antifungal activities of 13 strains were higher than 50%, and among them three strains were higher than 80%. The result of the antifungal activity of the 35 strains is shown in Table 1. Of all strains, 35 were obtained based on antifungal activity, while the others were discarded based on their comparatively poor antifungal activity. Since *L. plantarum* IMAU10014 had the maximum

Table 2 Antifungal activity of 7 LAB strains against 5 fungi

I (%)[a]	IMAU10014[b]	IMAU40010[b]	IMAU10004[c]	IMAU40007[b]	IMAU30001[b]	IMAU10043[c]	IMAU10032[c]
Botrytis cinerea	86.0±2.0	84.6±1.9	81.4±2.7	78.1±2.2	76.9±1.4	69.4±5.8	65.3±6.4
Alternaria solani	79.3±6.4	80.2±1.0	79.8±2.7	82.0±0.5	83.5±1.0	64.3±1.4	83.5±1.2
Phytophthora drechsleri Tucker	100±0.0	22.5±0.4	100±0.0	100±0.0	100±0.0	97.8±1.5	100±0.0
Fusarium oxysporm	79.7±3.7	87.8±1.8	88.0±1.3	89.4±4.0	91.1±5.2	80.2±4.0	85.3±4.6
Glomerella cingulata	92.4±2.9	55.9±3.4	40.4±1.2	61.1±0.5	67.4±4.6	63.7±3.2	50.4±1.7

[a] The percentage of growth inhibition. Data are means of triplicate determinations±standard deviation

[b] *L. plantarum*

[c] *L. casei*

antifungal activity, *L. plantarum* IMAU10014 was chosen from the 35 isolates for further analysis.

The taxonomic identification of the *L. plantarum* IMAU10014 strain was conducted,and the sequence of 16 S rRNA of the IMAU10014 strain showed 100% homology to *L. plantarum* compared with the GenBank database. The 16 S rRNA gene sequences of *L. plantarum* IMAU10014 have been uploaded to the National Center for Biotechnology Information (NCBI), serial number FJ984043.1.

Characteristics of the active antifungal principles

The culture broth pH was 3.8 after 48 h fermentation. *L. plantarum* IMAU10014 started to produce antifungal activity at 12 h during the early logarithmic growth phase. Maximum antifungal activity production was reached after 36 h of incubation (the end of the exponential growth) and remained constant during the stationary phase. The *L. plantarum* IMAU10014 strain was tested to determine whether the antifungal activity was stable after different treatments, the results of which are presented in Figs. 1, 2 and 3. The maximum antifungal activity was observed at pH 4.0. When the supernatant was adjusted to pH 8.0, the activity disappeared irreversibly (Fig. 1). The antifungal activity of the filter sterilized supernatant was stable by heating at 100°C for 1 h (Fig. 2). After enzymatic treatment of supernatant with trypsin, neutral protease and proteinase K, loss of part of the antifungal activity was observed which indicated there are other substances in addition to the proteinaceous substance with the antifungal activity (Fig. 3).

Inhibitory spectrum

The antifungal spectrum of cell-free culture supernatant of *L. plantarum* IMAU10014 against 5 plant pathogenic fungi are shown in Table 2. The antifungal activity was directed against several plant fungi, including *B. cinerea*, *A. solani*, *P. drechsleri* Tucker, *F. oxysporum* and *G. cingulata*. All the fungi selected represent economically important spoilage organisms in the production of agriculture. Among the 35 isolated LAB strains, *L. plantarum* IMAU10014 strains showed strong antifungal activity against all the plant fungi.

Rule out the possibility of lactic acid

Table 3 shows the effects of cell-free culture filtrates compared with the effect of lactic acid, corresponding to the amount of lactic acid produced in MRS broth, against selected *B. cinerea*, *A. solani*, *P. drechsleri* Tucker, *F. oxysporum* and *G. cingulata* strains. After 48 h fermentation, the pH of the *L. plantarum* MRS culture supernatant was 3.8 and the concentration was 67.6 mM of lactic acid. The results indicated that lactic acid alone could not explain the antifungal action of the *L. plantarum* IMAU10014 against fungi (Table 3). The maximum inhibitory effect of lactic acid was 21% against *P. drechsleri* Tucker, whereas the cell-free supernatant of *L. plantarum* IMAU10014 inhibited the growth of fungi by 100%.

Tomato leaves test

The results of the tomato leaves test shows that *B. cinerea* colonies were smaller on tomato leaves that were sprayed with *L. plantarum* IMAU10014 culture thanthose sprayed with sterile water (Fig. 4). This result indicates that *L. plantarum* IMAU10014 has some prospect for application in agriculture.

Discussion

The bacterium as a source of biologically active metabolites represents an enormous source for natural products with diverse chemical structures and activities. The culture broths of the some bacterium screened for plant pathogenic fungi-antagonistic activity have been reported. LAB occurs naturally in foods or is added as pure cultures to various food products. They are considered to be harmless or even to improve human and animal health (probiotics). LAB has a GRAS status (generally recognized as safe) and it has been estimated that 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods.

A series of studies on lactic acid bacteria indicated that some of them have antifungal activity. The antifungal activity of 322 lactobacilli strains isolated from Edam

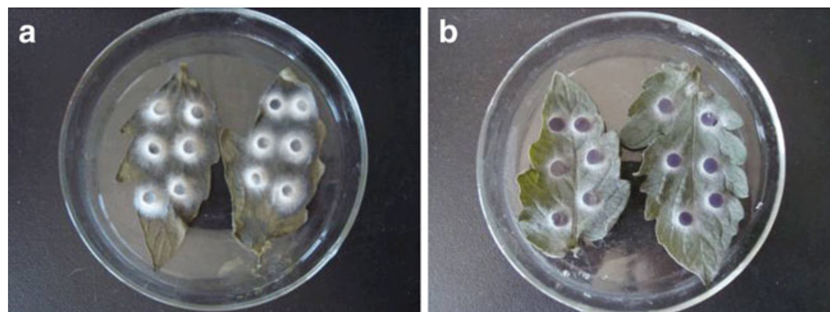

Fig. 4 Effect of cell-free supernatant of *L. plantarum* IMAU10014 on tomato leaves. **a** Control: tomato leaves sprayed with sterilized water. **b** Tomato leaves sprayed with cell-free supernatant of *L. plantarum* IMAU10014

cheese was tested by Tuma et al. (2007). Approximately 21% of the isolates showed a certain level of inhibitory activity. The *L. plantarum* strain can produce a broad spectrum of some compounds against fungi and bacteria in agar plate assay (Prema et al. 2010; Todorov and Dicks 2004). Lactobacilli strains can not only act against the growth of fungi, but can also act against the production of mycotoxins (Hudacek et al. 2007). A series of studies on LAB indicated that some of the antifungal activity is caused by organic acids, low pH, proteinaceous compounds and other end products (Park et al. 2009; Schnurer and Magnusson 2005; Hassan and Bullerman 2008). The antifungal protein produced by *Enterococcus faecalis* CHD 28.3 was partially purified by Roy et al. (2009), the molecular mass of the antifungal protein from the high resolution gel filtration was estimated to be around 11 kDa. Lavermicocca et al. (2000) reported that phenyl lactic acid and 4-hydroxy-phenyllactic acids were the antifungal compounds in the culture of *L. plantarum* 21B. Antifungal compounds against several fungi include *Eurotium*, *Penicillium* and *Aspergillus* sp. Broberg et al. (2007) investigated the antifungal metabolites of LAB on silage. The following metabolites were found to be present at elevated concentrations in silos inoculated with LAB strains: 3-hydroxydecanoic acid, 2-hydroxy-4-methylpentanoic acid, benzoic acid, catechol, hydrocinnamic acid, salicylic acid, 3-phenyllactic acid, 4-hydroxybenzoic acid, (trans, trans)-3,4-dihydroxycyclohexane-1-carboxylic acid, p-hydrocoumaric acid, vanillic acid, azelaic acid, hydroferulic acid, β-coumaric acid, hydrocaffeic acid, ferulic acid, and caffeic acid. Niku-Paavola et al. (1999) identified benzoic acid, 5-methyl-2,4-imidazolidinedione, tetrahydro-4-hydroxy-4-methyl-2 H-pyran-2-one and 3-(2-methylpropyl)-2,5-piperazinedione from *L. plantarum* VTT E-78076 against Gram-negative test organisms, *Pantoea agglomerans* VTT E-90396 and *Fusarium avenaceum* VTT D-80147. Strom et al. (2002) have isolated a *L. plantarum* strain (MiLAB 393) from grass silage that produces broad-spectrum antifungal compounds: cyclo(L-Phe-L-Pro), cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Yang and Chang (2010) purified an antifungal compound 3,6-bis(2-methylpropyl)-2,5-piperazinedion from *L. plantarum* AF1, which was isolated from kimchi.

Some authors have reported that the antifungal activity of LAB is lost after treatment with proteolytic enzymes. Roy et al. (1996) isolated a *Lactococcus lactis* ssp. *lactis* CHD-28.3 with antifungal activity against *Aspergillus flavus*. However, after thtreatment of the supernatant with chymotrypsin, trypsin and pronase E, the antifungal activity disappeared which indicated the proteinaceous nature of the antifungal substance. We have observed that the antifungal activity of *L. plantarum* IMAU10014 was lost after treatment of the supernatant with trypsin, proteinase K and neutral protease.

The inhibitory activity of lactobacilli against molds can be realized at different times and is caused by different factors. Some antifungal compounds such as phenyl-lactic acid were produced in the early phase during the fermentation; it is displayed by some strains but not by others. In the late phase, at the end of cell growth, the antifungal activity arises from the release of peptidic compounds. It is a common characteristic of all the strains as the physiological consequence of cellular autolysis (Fabio et al. 2007).

LAB are important producers of antifungal substances (Lavermicocca et al. 2000; Cabo et al. 2002; Magnusson et al. 2003; Rouse et al. 2008), but there is no report on the activity against plant pathogenic fungi and the antifungal principles characteristics of *L. plantarum* so far as we know. This study showed that the metabolites of *L. plantarum* IMAU10014 have a pretty high activity against plant pathogenic fungi. In any case, this is a really exciting result due to the many unprecedented advantages compared with the applied fungicides on the market, such as environmentally safe, cost-effective and high thermal stability, meaning it has a great potential for practical application for plant pathogenic fungi control. The results concerning the principal active antifungal characteristics indicate that there are other substances in addition to the proteinaceous substance with antifungal activity in the metabolites of *L. plantarum* IMAU10014, which means that more research is needed to identify the kinds of antifungal compounds and the antifungal activity of these compounds for other types of plant pathogenic fungi.

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