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ORIGINAL ARTICLE

In vitro antifungal activity of lactic acid bacteria low molecular peptides against spoilage fungi of bakery products

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Abstract

Bio-preservation, a promising preservation method that involves the use of "friendly" microorganisms such as lactic acid bacteria, has recently become a topic of considerable interest. In the present study, 16 lactic acid bacteria isolates were evaluated for antifungal activity against six fungi commonly associated with bread spoilage. The antifungal compounds were heat stable at 121 °C, and only four isolates, DU15, IT10, TE10, and IS10, showed partial loss of activity when supernatants were treated with proteolytic enzymes. The four isolates showed high inhibition activity at pH 3 and were identified using 16S rDNA sequencing as belonging to *Leuconostoc mesenteroides* DU15, *Lactobacillus plantarum* TE10, *Lactobacillus plantarum* IT10, and *Lactobacillus plantarum* IS10. The minimum germination inhibitions were 30 mg, 50 mg, 40 mg, and 50 mg for TE10, IT10, DU15, and IS10 respectively. The optimum conditions for the strains to produce antifungal compounds were 37 °C for 48 h for IT10, IS10, and TE10, and 30 °C for 24 h for DU15. Antifungal activity was increased threefold when supernatants were filtered using 10 KDa membranes. These findings demonstrate the potential of using lactic acid bacteria antifungal peptides as natural preservatives in bakery products to control the growth of spoilage fungi.

Keywords Antifungal peptide · LAB · Shelf life · Food bio-preservation · Antimicrobial proteins

Introduction

Fermented foods have a long history in human nutrition and food processing systems and have numerous advantages including boosting of the immune system and enhancement of general health. Fermentation microorganisms such as lactic acid bacteria (LAB) convert raw substrates, mainly carbohydrates and proteins, to organic acids, low molecular bioactive peptides, and a significant number of aroma and flavor compounds via their proteolytic activity (Ghaffar et al. 2014). Over centuries, fermented foods have become a traditional part of the diet in many countries, especially in Asia, because of their pleasant taste, texture, and color. Among such foods are *Budu*, *maman*, *tempoyak*, and *tempeh*, fermented dishes prepared from fish, fruit flesh, and soybeans (Moreno et al. 2002). Of note, recent studies have reported that health benefits associated with the consumption of these fermented foods is attributable to the presence of LAB compounds with antagonistic activity towards a significant number of pathogenic microorganisms such as organic acids, hydrogen peroxide, bacteriocins, and bioactive peptides (Nuraida 2015).

LABs have been isolated from different sources including vegetables and fruits (Gerez et al. 2010), sourdough (Sáez et al. 2018), raw milk (Wassie and Wassie 2016), dairy products (Lynch et al. 2014), meat products (Lengkey et al. 2017), and fermented beans (Moreno et al. 2002). Specific LAB strains have demonstrated antimicrobial activity arising from the production of antimicrobial compounds that can be used as natural preservatives in a broad range of food products. With respect to the types of antimicrobial compounds produced by

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LAB, low molecular peptides are the most important for food preservation. In recent studies, LAB were extensively evaluated for their potential applications as bio-preservation agents to extend the shelf life of bakery and dairy products, fruits, and vegetables (Saladino et al. 2016; Oliveira et al. 2015; Lynch et al. 2014). Nevertheless, few studies to date have examined the antifungal properties of LAB-derived low molecular peptides and their survival throughout food processing steps that include acidic conditions, high temperatures, and the effects of food matrices. The spoilage of bakery products by fungi is associated with considerable economic loss and health risks, given that bakery products such as bread are consumed by the majority of populations globally. Therefore, in this study, we sought to determine the antifungal activity of 870 LAB isolates from Malaysian fermented foods against six common spoilage fungi associated with bread. Isolates demonstrating antifungal activities were identified by chemical assay and 16S rDNA. The stability of antifungal low molecular peptides was evaluated in simulated food-processing conditions including pH and temperature. In addition, the antifungal low molecular peptides were partially purified by enzymatic hydrolysis and ultra-filtration.

Materials and methods

Isolation of lactic acid bacteria

A total of 870 LAB strains were isolated from Malaysian fermented foods. Ten grams of each sample was mixed with 90 mL of sterile peptone water (0.1% *w*/*v*) and homogenized in a stomacher (Stomacher® 400 Circular Seward). Appropriate dilutions were prepared using sterile peptone water (0.1% *w*/*v*). Diluted samples (100 μL) were inoculated on de Man Rogosa and Sharpe MRS agar modified with 0.8% $CaCO_3$ and incubated under anaerobic conditions at 30 °C for 48 h (Wanchai et al. 2007). The isolates were kept at 4 °C for short term storage or at − 80 °C for long term storage using cryobank tubes (MAST CRYOBANK™, Mast Diagnostica GmbH, Germany).

Fungi preparation

Aspergillus niger fungus was obtained from the Microbiology Laboratory, Department of Food Science, Universiti Putra Malaysia while *A. flavus* MD3, *Penicillium roqueforti* MD4, *Eurotium rubrum* MD5, *Monilia sitophila* MD6, and *Rhizopus nigricans* MD8 were obtained from Faculty of Science, Universiti Sains Islam Malaysia. The selected fungi represented common spoilage fungi for bakery products. Fungi were grown on Potato Dextrose Agar (PDA, Oxoid) plates at 25 °C for 5 days and stored at 4 °C. Spore inoculants were prepared as described by Ström et al. (2002).

Screening for antifungal activity against targeted fungi

Antifungal activity of the 870 isolates was determined by the overlay method as described previously (Magnusson and Schnürer 2001). LABs were inoculated in two 2-cm lines on MRS agar plates and incubated at 30 °C for 24 h under anaerobic conditions. Next, the plates were overlaid with 10 mL of malt extract soft agar (0.05% malt extract and 1% agar Oxoid) containing 10^5 conidia/mL of *A. niger*, the indicator fungus. After a further 48 h of aerobic incubation at 30 °C, the diameter around the zone of inhibition was measured. Inhibition tests were performed in duplicate, and inhibition activity was determined as follows: 20 mm, weak activity; 21–40 mm, moderate activity; 41–60 mm, strong activity; and > 61 mm, very strong activity.

Preparation of cell-free supernatant

The isolates were inoculated into MRS broth and incubated for 48 h at 30 °C. Cell-free supernatant was prepared by centrifuging the broth in a micro-centrifuge (11,500×*g* for 10 min) (MiniSpin® Eppendorf). The supernatant of each isolates was filtrated using a sterile filter (0.45 μm-pore-size filter, Millipore).

Measurement of antifungal activity in heat-treated LAB supernatant

Supernatants of 56 isolates with broad spectrum antifungal activity were heat treated at 121 °C in an autoclave for 60 min and immediately cooled in ice-cold water to approximately 45 °C. Next, the antifungal activity of heat treated supernatants was tested against target fungi in a micro-titer plate assay. Aliquots of Malt Extract Broth (MEB) (100 μL) containing 10^5 conidia/mL were placed in the wells of a 96-well plate with 100 μL of supernatant. Plates were incubated in humid champers at 30 °C for 72 h. Fungal growth was measured visually and as optical density (OD) at 560 nm (BioTek EL 800 Universal Microplate Reader, Winooski, US).

Characterization of antifungal compounds

The effect of proteolytic enzymes on antifungal activity was investigated for 12 isolates of LAB with antifungal activity and heat stability to identify the nature of the active compounds and to determine whether the antifungal activity was related to the acid production or antifungal peptides generated by LAB (Magnusson and Schnürer 2001). The enzymes proteinase K (Sigma), trypsin (Sigma), alcalase (Novoenzyme), and pepsin (Sigma) were added to the supernatant of isolates. The pH of samples was adjusted with 1 M HCl and 2 M NaOH to

7.6, 7.6, 8, and 2.0 for proteinase K, trypsin, alcalase, and pepsin, respectively. Next, 1 mL of supernatant was treated with 100 μL of each enzyme and incubated at 37 °C for 1 h. The mixtures were heated at 65 °C to stop the reaction, and treated supernatants (100 μL) were placed in the wells of a 96-well micro-titer plate, inoculated with 100 μL of MEB containing 10^5 conidia/mL of each target fungi, and incubated at 30 °C for 72 h. Growth inhibition was measured by optical density at 560 nm (Bio-Tek EL 800 Universal Microplate Reader for 96 Well Microplates, Winooski, US), and all experiments were performed in triplicate.

Effect of pH treatment on LAB supernatant activity

The pH of LAB supernatants was adjusted to pH 3, 5, and 7 using 1 N HCl and/or 1 M NaOH. The adjusted supernatant was then evaluated against the target fungi in a micro-titer plate assay. Briefly, 100 μL of MRS broth containing 10^5 conidia/mL was placed into the wells of a 96-well plate, and 100 μL pH-adjusted supernatant was added per well. Plates were incubated at 30 °C for 72 h before fungal growth was measured by OD at 560 nm (Bio-Tek EL 800 Universal Microplate Reader for 96 Well Microplates, Winooski, US). All tests were performed in triplicate.

MIC of lyophilized supernatant

The minimum inhibitory concentration (MIC) of the isolates was evaluated to determine the minimum concentration that can be used to control the growth of target fungi, as described by Coda et al. (2008) with some modifications. Cell-free supernatant from each sample was freeze-dried and diluted in deionized water to obtain concentrations of 10, 20, 30, 40, and 50 mg mL^{-1}, and then sterilized by filtration. Samples were added to wells of a 96-well micro-titer plate, inoculated with 100 μL MEB containing 10^5 conidia/mL, and incubated at 30 °C for 72 h. Fungal growth inhibition was determined by measuring OD at 560 nm using an ELISA plate reader (Bio-Tek EL 800 Universal Microplate, Winooski, US).

Optimization of antifungal compound production

The four selected isolates were inoculated in MRS broth (pH 6.2 ± 0.2), and incubated in a shaker incubator at 100 rpm and 10, 30, 37, and 45 °C to determine the optimum incubation temperature for antifungal compound production at a fixed incubation time of 48 h. Subsequently, the optimum incubation temperature of 37 °C for the four strains and incubation times of 24, 48, and 72 h were evaluated for optimization of incubation time. *Aspergillus niger* was selected the indicator fungus as it showed the highest resistance compared with the other five fungi evaluated.

Identification of LAB isolates

The identification of strains was initially carried out using a chemical assay with API strips to identify strains according to their sugar fermentation profile. The API strips are made of 50 wells that contain different types of sugars for the fermentation of the LAB, and the change of color is recorded for positive and negative results. Molecular characterization of isolates was subsequently performed in bacterial DNA extracted from 1.5 mL of overnight cultures grown in MRS broth at 30 °C using a Presto™ Mini gDNA Bacteria Kit (Taiwan). Purified DNA from each sample was subjected to PCR using a master mix kit (REDiant. 1st Base, Singapore). The PCR settings were as follows; initial denaturation at 95 °C for 2 min, followed by 35 cycles at 92 °C for 45 s, at 54 °C for 1 min, and finally at 72 °C for 1 min. The primer sequences were as follows: 16S forward (5′-AGAG TTTGATCCTGGCTC-3′) and 16R reverse (5′-CGGG AACGTATTCACCG-3′) (Magnusson et al. 2003). Partial 16S rDNA sequencing was performed by NHK Bioscience Solutions (Sdn Bhd Malaysia), and the resulting sequences were used in database searches (NCBI, Gen-Bank).

Purification of antifungal peptides

The method of Gerez et al. (2013) was followed with modifications in which cell-free supernatant was fractionated using 15 mL ultra-filtration tubes of 10 KDa membrane (Vivaspin 15R VS15RH01, Germany). Samples were loaded onto tubes and centrifuged at 6000×*g* for 60 min. Peptides > 10 KDa and peptides < 10 KDa were filtrated at 0.22 μm and evaluated against target fungi in 96-well micro-titer plates. As a control, 10 KDa peptides of MRS broth were used.

Protein profile

Protein profiling was performed using the SDS-PAGE buffer system described by Laemmli (1970) and Schägger (2006) to confirm the presence and molecular weight of proteins in the isolates. Gels were stored in polyethylene bags containing distilled water and analyzed using a GS-800™ Densitometer (Biorad, USA) equipped with PDQuest™ software. The density and molecular weight of the bands in gels were determined by using densitometer (GS-800™ Densitometer, Biorad, USA) equipped with PDQuest™ software.

Data analysis

All the antifungal experiments carried out were performed in triplicate. The symbol ± refers to the data which were presented as standard deviations of the three readings of the replications, and all its analyses carried out were performed in Microsoft Excel.

Table 1 The diameter (mm) of the clear zones around the two strikes of lactic acid bacteria on MRS agar plate tested against the growth of target fungi after incubation for 72 h at 30 °C

Isolates	Target fungi					
	A. niger	*A. flavus* MD3	*P. roqueforti* MD4	*E. rubrum* MD5	*M. sitophila* MD6	*R. nigricans* MD8
C5	36.00 ± 0.00	54.66 ± 2.08	69.66 ± 2.08	–	–	–
IS10	46.33 ± 2.08	58.00 ± 10.14	31.00 ± 2.00	23.66 ± 2.51	–	51.33 ± 1.15
H26	74.66 ± 2.51	44.00 ± 3.00	74.66 ± 3.05	38.66 ± 1.52	43.00 ± 4.00	62.33 ± 1.52
IT8	47.00 ± 2.64	69.66 ± 5.50	63.66 ± 5.68	17.33 ± 3.51	–	64.33 ± 4.04
CF1	78.33 ± 1.52	58.00 ± 4.00	56.66 ± 3.51	–	–	–
DU15	74.00 ± 2.00	45.33 ± 5.50	–	35.33 ± 1.52	35.66 ± 1.52	69.00 ± 2.00
TE10	80.00 ± 0.00	53.00 ± 3.60	72.33 ± 1.52	37.66 ± 1.52	57.33 ± 4.50	50.33 ± 7.02
BB12	72.33 ± 0.57	54.33 ± 2.08	49.33 ± 2.08	–	–	–
IT7	54.66 ± 0.57	37.66 ± 2.51	60.33 ± 1.52	–	20.66 ± 1.52	–
IT5	54.33 ± 0.57	47.00 ± 4.58	71.00 ± 2.64	–	22.00 ± 4.00	36.66 ± 3.51
IT10	28.66 ± 1.52	51.33 ± 8.50	53.00 ± 2.64	46.66 ± 3.21	44.66 ± 4.50	–
E.B	77.00 ± 2.00	–	55.33 ± 4.50	–	43.33 ± 1.52	42.66 ± 6.65
H32	60.33 ± 1.15	–	64.66 ± 4.50	–	23.66 ± 2.51	55.33 ± 4.50
IT11	22.66 ± 5.68	–	–	–	40.66 ± 0.57	52.00 ± 1.00
DU19	38.33 ± 1.15	–	30.00 ± 3.00	–	24.33 ± 1.52	–
IT6	57.66 ± 1.15	–	80.00 ± 0.00	–	23.66 ± 3.51	51.33 ± 1.52

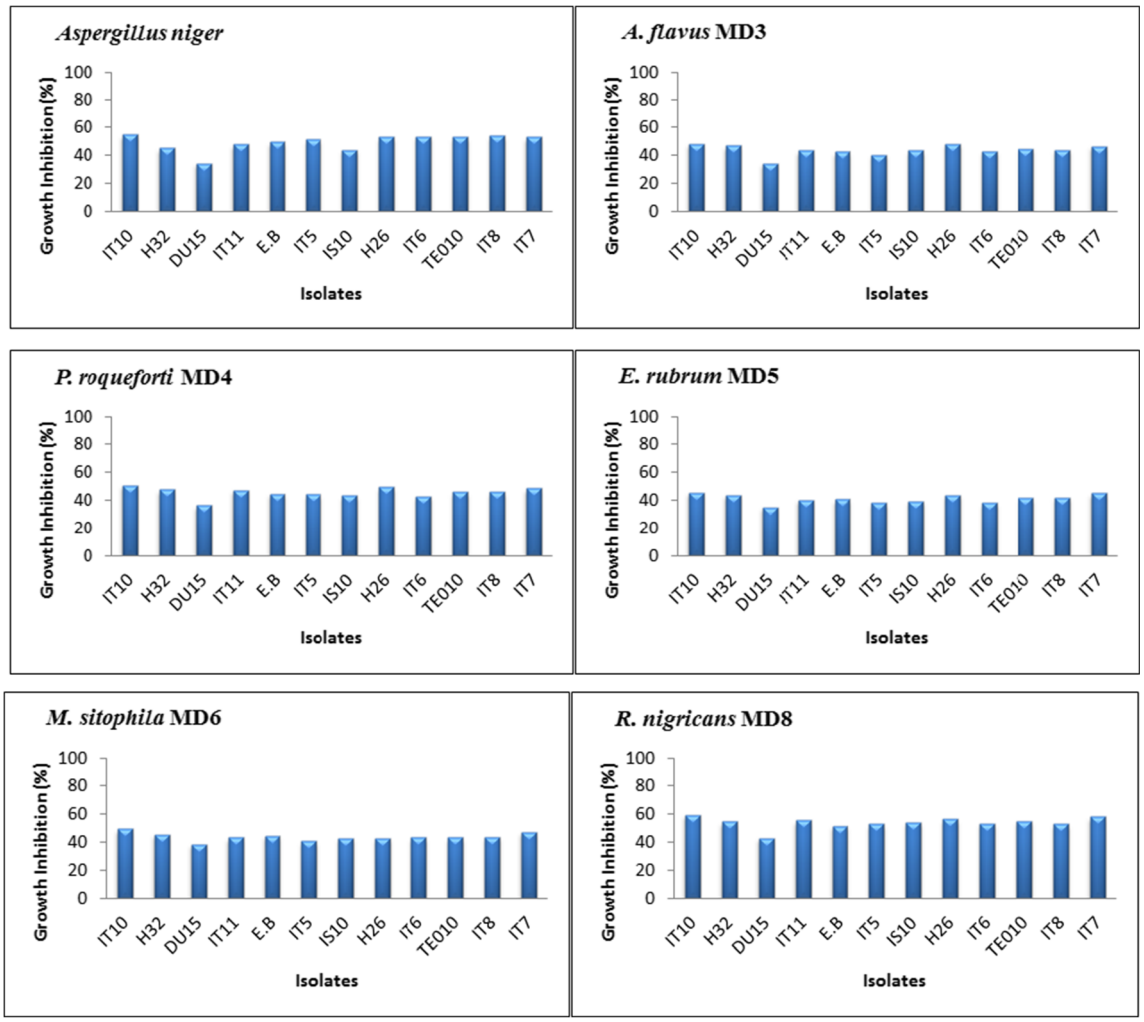

Fig. 1 Growth reduction percentages of target fungi after incubation with heat-treated free-cell supernatant of LAB isolates

Results

This study was performed to evaluate the antifungal activity of LAB isolated from Malaysian fermented foods against bakery spoilage fungi, particularly *Aspergillus niger*, the most common fungi found in bakery products. Initial screening for the antifungal activity of LAB isolates against the spoilage fungi demonstrated that out of 870 isolates, only 56 (6.4%) inhibited the growth of the *Aspergillus niger* indicator strain. Further screening with five spoilage fungi selected for this study revealed that only 16 (28%) out of the 56 LAB isolates exhibited broad-spectrum antifungal activity (Table 1). The antifungal activity of the isolates ranged from weak to strong. The diameter of the clear zone around the two LAB lines varied from as low as 15.66 mm up to 80 mm. The MRS control plate containing fungal spores without LAB showed increased growth after 48 h at 30 °C, and the plates were fully covered by the fungi. Plates were maintained for 2 weeks to observe fungal growth, and seven isolates, IS10, IT8, H26, IT10, DU15, IT5, and TE10, showed delayed fungal growth for 14 days.

The cell-free supernatants from 16 isolates containing antifungal compounds with broad-spectrum inhibition were evaluated for heat stability. Twelve (75%) out of the 16 isolates were heat-stable and maintained inhibition activity after heat treatment at 121 °C for 60 min (Fig. 1). Supernatants from the isolates exhibited different responses to the heat treatment. For example, the DU15 strain showed a partial loss of antifungal activity while the TE10 and IT8 strains were highly stable following heat treatment and maintained their antifungal activity. The inhibition of fungal growth was further confirmed by inoculating 50 μL from each well of the test plates onto PDA agar. No growth or very low growth was observed for wells that had been treated with heated supernatant. However, the growth of the control was very high on the PDA agar plates (data not shown). In addition, the treatment of supernatant with proteolytic enzymes showed that the antifungal activity of four isolates (DU15, TE10, IS10, and IT10) was partially decreased due to the hydrolysis of antifungal proteins present in the supernatants (Fig. 2). The antifungal activity of the four isolates was decreased following enzymatic treatment, while the other LAB isolates were not affected by the enzymes' activity and maintained their antifungal properties.

The antifungal activity of LAB supernatants was evaluated at pH 3, 5, and 7 to determine their suitability for use in food systems. Supernatants from the four selected isolates showed high activity at pH 3, and the activity slowly decreased at pH 5 and was further decreased at pH 7 (Fig. 3). *A. niger* and *A. flavus* MD3 were highly sensitive to the supernatant of LAB isolates at pH 3 and 5. In comparison, the growth of *M. sitophila* MD6 and *R. nigricans* MD8 was inhibited at pH 3, but high growth of both fungi was observed at pH 5 and 7. The TE10 strain exhibited strong antifungal activity against *A. niger*, *A. flavus* MD3, *P. roqueforti* MD4, and *E. rubrum* MD5 at pH 3 and 5. In addition, the strain IS10 showed best antifungal activity at pH 3 and 5, but the activity was reduced at pH 7. However, the DU15 strain showed 65–75% growth inhibition against all target fungi at pH 3 and 5, and good activity (above 50%) at pH 7 towards *A. niger* and *A. flavus* MD3.

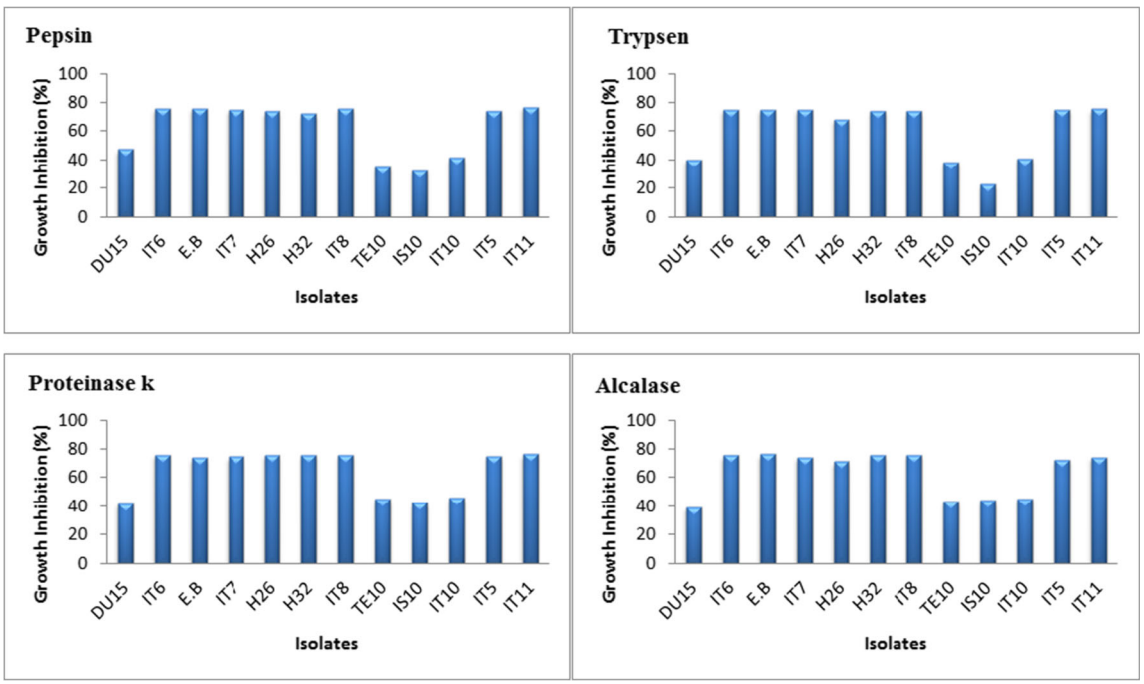

Fig. 2 Sensitivity of antifungal compounds from lactic acid bacteria towards different proteolytic enzymes

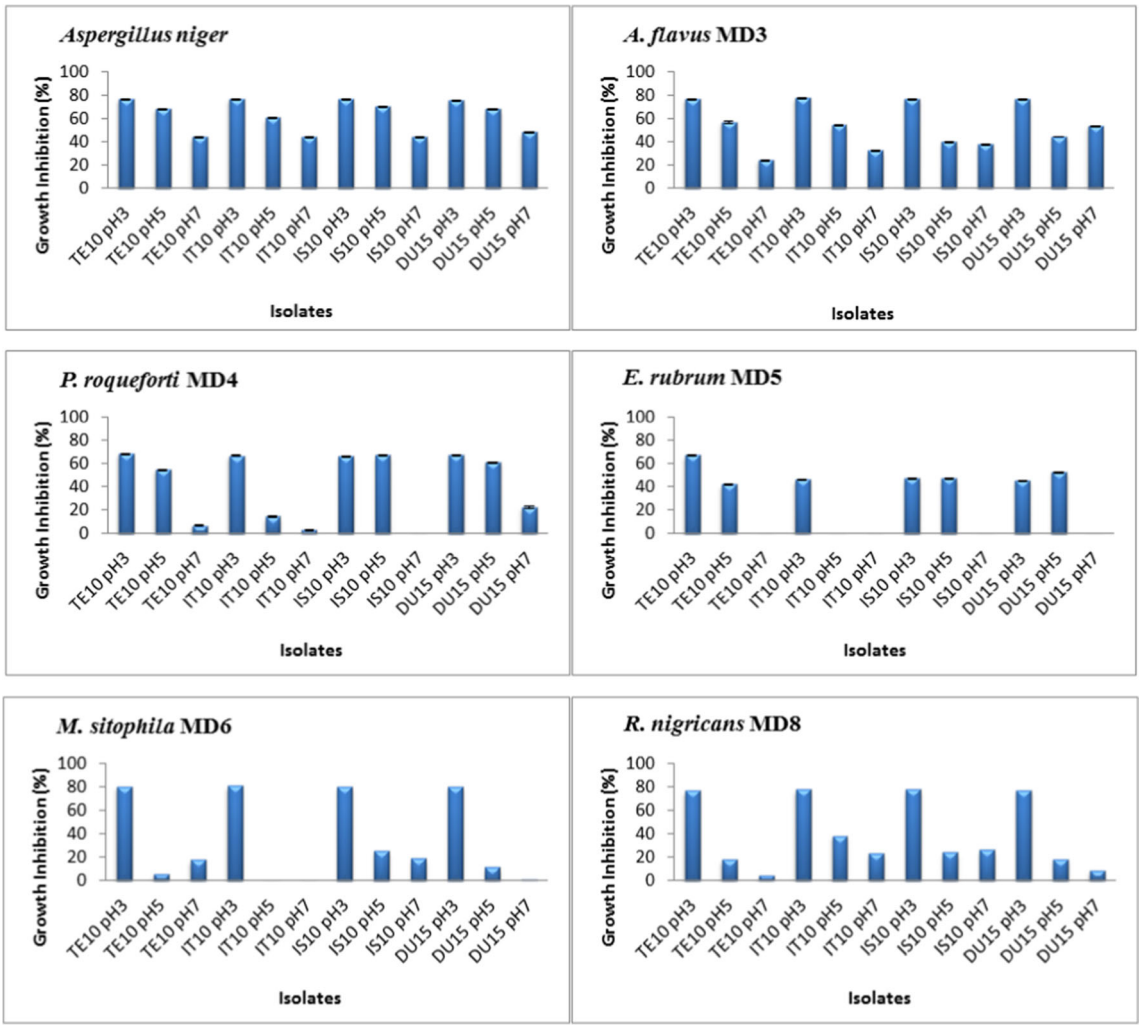

Fig. 3 Effects of pH on the antifungal activity of LAB supernatants against *A. niger*, *A. flavus* MD3, *P. roqueforti* MD4, *E. rubrum* MD5, *M. sitophila* MD6, and *R. nigricans* MD8 after incubation for 72 h at 30 °C

The minimum inhibition concentration (MIC) of the lyophilized supernatant of the four LAB isolates was evaluated to optimize the dose of concentrated supernatant that could fully inhibit the growth of the tested spoilage fungi. The four isolates were selected for their high potential to produce antifungal compounds that are protein-like in nature. The TE10 strain inhibited all target fungi at a concentration of 30 mg/mL, and this was the lowest MIC observed in this study. However, the MIC for the IT10 and IS10 strains was 50 mg/mL, while the DU15 MIC was 40 mg/mL (Fig. 4). The production of antifungal compounds was evaluated at different temperatures and incubation times to determine the optimum conditions (Table 2). The production of antifungal substances was low for the DU15 strain at 10 °C and 37 °C; however, this strain showed the highest antifungal activity at 30 °C for 24 h. The optimum incubation time for the production of antifungal compounds by IT10, IS10, and TE10 at 37 °C was 48 h.

API sugar profile analysis determined the species of the four selected LAB strains. Furthermore, the sugar profile of the four strains demonstrated differences among the strains via their fermentation patterns in the API strips, which contained 50 wells with different types of sugar. The identification of the four strains was further confirmed by sequencing and searching for matching strains on NCBI database by highest similarity ranking to determine the species identity of the isolates. The results from 16S rDNA sequencing identified DU15 as *Leuconostoc mesenteroides*, TE10 as *Lactobacillus plantarum*, IT10 as *Lactobacillus plantarum*, and IS10 as *Lactobacillus plantarum* (Table 3). However, the NCBI database-matching results showed differences in accession number among the three *Lactobacillus plantarum* identified in this study, with slight differences in genomic profiles. The supernatants of the four strains were subjected to ultra-filtration to purify the low molecular peptides < 10 KDa, and these low molecular peptides

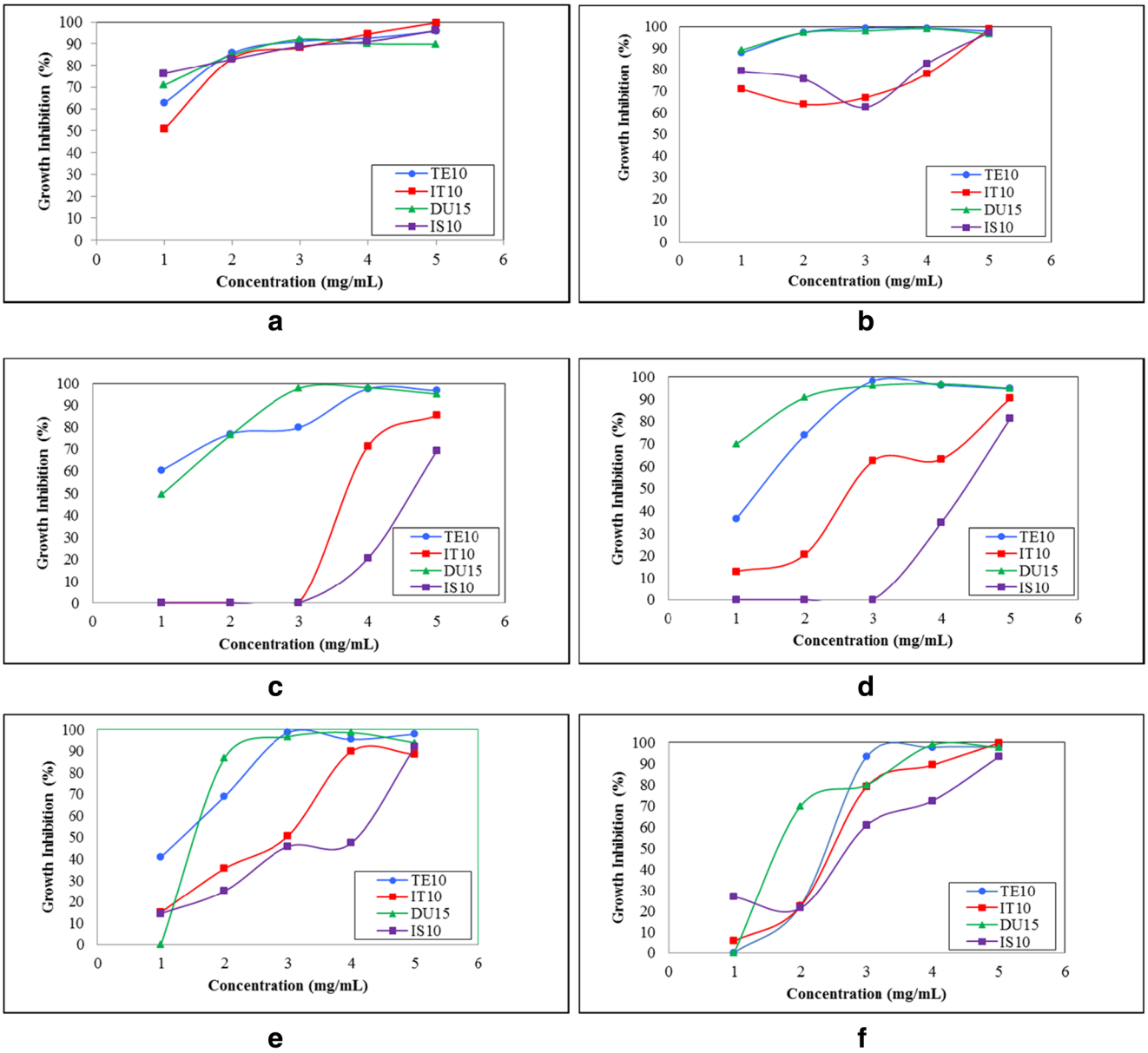

Fig. 4 Minimum inhibition concentration of the supernatant of the LAB isolates against **a**. *A. niger*, **b**. *A. flavus* MD3, **c**. *P. roqueforti* MD4, **d**. *E. rubrum* MD5, **e**. *M. sitophila* MD6, and **f**. *R. nigricans* MD8 after a 72-h incubation at 30 °C

demonstrated strong antifungal activity against all target fungi evaluated in this study (Table 4). Antifungal activity was significantly increased by ultra-filtration, and the growth inhibition of fungi was as high as 94%. The TE10 strain inhibited all fungi and showed high activity between 73 and 94%, while the antifungal activity of IS10, DU15, and IT120 was 72–95%, 69–93%, and 69–93%, respectively. In addition, SDS-PAGE was performed to confirm the presence of low molecular peptides in in the 10 KDa fractions of ultra-filtrated supernatant, with molecular weights in the range of 2.7–5.2 KDa identified by densitometer (Fig. 5).

Discussion

In this study, the antifungal activity of 870 LAB isolates was screened and the associated antifungal compounds partially characterized. Antifungal activity screening demonstrated that certain LAB isolated from Malaysian fermented foods have broad spectrum activity against bakery spoilage fungi, namely *Aspergillus niger*, *A. flavus* MD3, *P. roqueforti* MD4, *E. rubrum* MD5, *M. sitophila* MD6, and *R. nigricans* MD8. In a previous study, Lan et al. (2012) evaluated the antifungal activity of 85 LAB isolates from fermented wax gourd against

Table 2 Effects of temperature and time on the production of antifungal peptides by IT10, IS10, DU15, and TE10 against *A. niger*

Parameters	Relative percentage of antifungal activity of LAB isolates			
	IT10	IS10	DU15	TE10
Incubation temperature				
10	0%	0%	33%	0%
30	46%	42%	75%	60%
37	82%	68%	53%	87%
45	0%	45%	0%	54%
Incubation time				
24 h	31%	65%	72%	67%
48 h	84%	58%	56%	88%
72 h	67%	42%	53%	72%

the *P. oxalicum*, *A. flavus*, *A. sydowii*, *and Mucor racemosus* fungi; and two LAB isolates *Weissella cibaria* 861006 and *W. paramesenteroides* 860509 showed broad spectrum activity against selected fungi. Several studies have recently reported the isolation and identification of LAB strains with antifungal activity, and these finding are of interest due to the important role of LAB in the bio-preservation of processed foods.

The stability of antifungal activity at high temperatures was determined to simulate actual food processing conditions. A total of 12 isolate supernatants were found to be heat-stable and to therefore have high potential as food bio-preservatives. However, few studies to date have evaluated the heat stability of antifungal compounds for food preservation (Ogunbanwo et al. 2014; Coda et al. 2013; Muhialdin et al. 2011; Rouse et al. 2007; Magnusson and Schnürer 2001). In this study, supernatant was exposed to 121 °C for 60 min to simulate the conditions that preservatives undergo during the food manufacturing processes. Albano et al. (2007) found that the antimicrobial activity of *P. acidilactici* was decreased after heating the supernatant for 60 min at 100 °C, and the supernatant of that strain was therefore not deemed suitable for food-processing applications requiring extreme heat conditions. In another study, antifungal compounds produced by *P. pentosaceous* 54 were found to be heat-stable at 100 °C for 15 min, and no reduction of the inhibition zone was observed (Crowley et al. 2013). This finding indicated that the heat sensitivity of antifungal compounds produced by LAB vary according to the strain and the nature of these compounds, whether organic acids, volatile compounds or proteins, and that this can affect the heat stability of the supernatant.

Table 3 Similarity index of LAB isolated from Malaysian fermented food and fruits as determined by 16S rDNA

Code	Identification	Similarity	Accession	Source
DU15	*Leuconostoc mesenteroides*	99%	JF756172.1	*Tempoyak*
TE10	*Lactobacillus plantarum*	99%	GU644444.1	*Tempeh*
IT10	*Lactobacillus plantarum*	99%	HM058789.1	Buffalo milk
IS10	*Lactobacillus plantarum*	99%	HE646362.1	*Rebung*

The antifungal compounds were characterized by enzymatic treatment studies. The antifungal activity of *L. plantarum* IT10, *L. plantarum* IS10, *L. plantarum* TE10, and *Leuconostoc mesenteroides* DU15 was found to be partially diminished after the treatment of supernatant with selected enzymes. The partial loss of antifungal activity is attributable to the hydrolysis of low molecular peptides present in the supernatant. Roy et al. (1996) reported the loss of antifungal activity of *Lactococcus lactis* subsp. *lactis* CHD-28.3 supernatant following treatment with several enzymes and suggested that the loss of inhibition is an indicator for the presence of low molecular proteins. The treatment of LAB free-cell supernatant by proteolytic enzymes to determine the presence of low molecular peptides in the supernatant was recommended by several previous studies (Cizeikiene et al. 2013; Crowley et al. 2013).

A broad pH range is an essential characteristic for antifungal compounds suitable for use in food applications. The four selected LAB strains demonstrated good antifungal activity at pH 3, moderate activity at pH 5, and low activity at pH 7. The majority of chemical preservatives are biologically active at pH < 4.5 and have been shown to be inactivate at high pH as they partially dissociate in water (Schnürer and Magnusson 2005; Barbosa-Canovas et al. 2003; Theron and Lues 2007). In a previous study, Magnusson and Schnürer (2001) observed the stability of the antifungal activity of *L. coryniformis* subsp. *coryniformis* strain Si3 supernatant at pH values ranging from 3.0 to 4.5, but this activity was highly reduced at pH 4.5 and 6.0 and totally absent at pH values above 6; the active compounds in this instance were low molecular peptides with a molecular weight of 3 KDa. However, antifungal activity was fully restored after the adjustment of pH to 3. The authors suggested that observation was attributable to the isoelectric point (pI) of the antifungal activity of the low molecular peptides, as when the pH reached the pI, these peptides will have a neutral charge (zero net charge) and would eventually lose activity.

LAB supernatants were concentrated by freeze-drying, and the initial volume of 125 mL of supernatant resulted in an average of 3.72 g of compound, which was concentrated approximately 34-fold. However, the freeze-dried LAB supernatant powder inhibited the growth of selected fungi at average concentration of 40 mg/mL. In a previous study, the water-soluble extract of sourdough fermented using *L. brevis* AM7 strain had an MIC of 40 mg peptide/mL and 30.9 mg

Table 4 Antifungal activity of LAB supernatants obtained by ultrafiltration with 10 KDa cut-off against spoilage fungi

Isolate	Inhibition against target fungi (%)						Range (%)
	A. niger	*A. flavus* MD3	*P. roqueforti* MD4	*E. rubrum* MD5	*M. sitophila* MD6	*R. nigricans* MD8	
IT10	93	91	69	74	81	92	69–93
IS10	94	92	73	72	85	95	72–95
DU15	74	93	92	75	84	94	69–93
TE010	94	92	73	78	84	95	73–95

protein/mL (Coda et al. 2008). In earlier study, Yang and Clausen (2005) determined the MIC for freeze-dried supernatant of *L. casei* by diluting it with MRS broth and testing it against targeted fungi, and the optimum ratio was found to be 2:1 supernatant to MRS broth. Ström et al. (2002) studied the MIC for the antifungal cyclic peptide cyclo (L-Phe-L-Pro) produced by *L. plantarum* MiLAB 393 and the MIC for the cyclic peptide cyclo (L-Phe-L-Pro) was 20 mg/mL. The MIC of the lyophilized cell-free supernatant of the isolates identified in this study demonstrated good activity and high potential to inhibit the growth of spoilage fungi in bakery products.

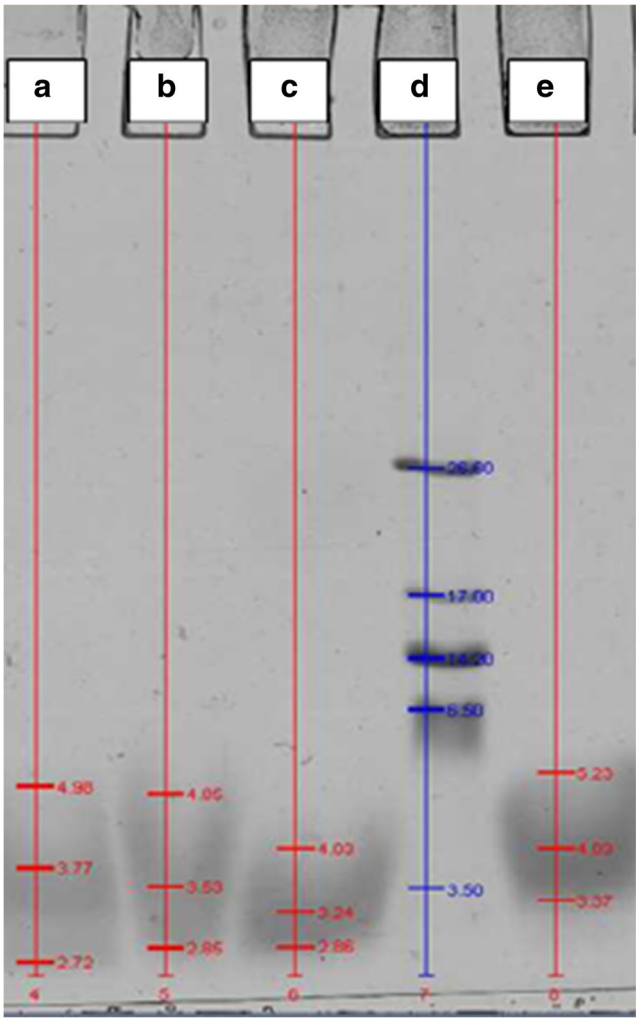

Fig. 5 Protein profiles of LAB supernatant ultra-filtrated with 10 KDa molecular weight cut-off as determined by SDS-PAGE **a**. *L. plantarum* IT10, **b**. *L. plantarum* IS10, **c**. *Leuconostoc mesenteroides* DU15, **d**. Protein ladder, and **e**. *L. plantarum* TE10

The conditions for production of the antifungal compounds were optimized for incubation temperature and time. Isolates IT10, IS10, and TE10 exhibited the highest activity when incubated at 37 °C for 48 h, while the highest antifungal activity of DU15 was observed when incubated at 30 °C for 24 h. In the same way, Rouse et al. (2007) studied the effects of incubation temperature on the growth and production of antifungal substances by four LAB isolates and observed that the isolates did not grow at 10 or 42 °C. The optimum incubation temperatures were 25–30 °C for the production of antifungal compounds, while *L. coryniformis* subsp. *coryniformis* strain Si3 exhibited maximum antifungal activity at 30 °C for 40 h. The authors suggested that the production of antifungal compounds begins in the exponential growth phase and reaches a maximum level early in the stationary phase (Magnusson and Schnürer 2001). The previous studies indicated the significance of incubation time and temperature for the production of antifungal compounds and the relationship with growth stages of bacteria. It is imperative to determine the optimum growth conditions to maximize the production of inhibitory compounds.

Ultra-filtration of the cell-free supernatant increased antifungal activity towards the fungi selected in this study. The antifungal activity of low molecular peptides (< 10 KDa) was markedly higher in comparison with high molecular proteins (> 10 KDa). The strong antifungal activity of low molecular peptides is attributable to the ability of peptides to diffuse through the cell wall of spoilage microorganisms and cause growth inhibition (Li et al. 2017). The results of this study were in agreement with previous reports showing that smaller peptides are more likely to exhibit strong antifungal activity. Antifungal activity increased up to 95%, and this finding supports the advantages of concentrating the low molecular peptides by ultra-filtration. The molecular weights of active peptides were below 10 KDa, representing a mixture of low molecular peptides. The presence of low molecular peptides and the range of their molecular weights were confirmed by SDS-PAGE. The molecular weight of the

peptides of the four LAB isolates showed significant differences by LAB strain. The differences in molecular weights could be due to differences in the proteolytic enzyme activities governed by the genomic profiles of each LAB isolate as shown in Table 3. The LAB strain *L. fermentum* CRL 251 has recently been reported to produce low molecular peptides with strong antifungal activity, and this activity was significantly improved after ultra-filtration of the supernatant using 10 KDa membranes (Gerez et al. 2013). In a previous study, Coda et al. (2008) reported similar results for LAB supernatant found to have strong antifungal activity towards *P. roqueforti* DPPMAF1 after ultra-filtration with three membranes (50, 30 and 10 KDa) in comparison with non-filtrated supernatant.

In conclusion, low molecular peptides from LAB have high potential as natural alternatives to the chemical preservatives currently used in bakery products. In this study, four LAB isolates showed broad-spectrum antifungal activity towards spoilage fungi commonly associated with bakery products. The four isolates were identified as *Leuconostoc mesenteroides* DU15, *Lactobacillus plantarum* TE10, *Lactobacillus plantarum* IT10, and *Lactobacillus plantarum* IS10. The supernatants of selected LAB strains were heat-stable and active in acidic pH. Low molecular peptides of the supernants obtained by ultra-filtration demonstrated high antifungal activity in comparison with crude supernatant. The peptides with high heat stability are recommended to be used for extending the shelf life of bakery products. Further studies are required, however, to further characterize the low molecular peptides and determine the mechanism of their antifungal activity using biochemical and molecular approaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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