Isolation and Selection of Anti−*Candida albicans* Metabolites Producing Lactic Acid Bacteria from Various Sources

Tanes Sungsri[1], Monthon Lertcanawanichakul[2] and Patcharaporn Siwayaprahm[1]*

[1]   *Department of Microbiology, Faculty of Science, Kasetsart University, 50 Ngamwongwan Road, Chatuchack, Bangkok 10900.*
[2]   *Division of Medical Technology, School of Allied Health Sciences and Public Health Walailak University Nakhon Si Thammarat 80160.*
[*]   *Correspondent author: fscippsp@ku.ac.th*

Abstract

Five hundred and fifty-two isolates of lactic acid bacteria (LAB) have been isolated and screened from fermented foods, natural sources and dairy effluents on De Mann Rogosa Sharpe (MRS) agar. Fifty-one isolates, in the percentile of 9.24, produced the secondary metabolites that could inhibit the growth of *Candida albicans* BCC6120 by using dual culture overlay assay. The culture broth of LAB, moreover, showed anti-*C. albicans* activity in acidic condition at pH range of 3.0-5.0 by using agar well diffusion method. Interestingly, the isolate L47-2 showed much more colonization surrounding the surface of sterile toothpick and test tube when growing in MRS broth. The identification of isolate L47-2 by morphological and biochemical characteristics using API 50 CHL Test Kit and further confirmed by 16S rRNA gene sequence analysis revealed that isolate L47-2 was similar to *Lactobacillus paracasei* with 99% nucleotide identity.

Keywords: anti-*C. albicans* activity, lactic acid bacteria

1. Introduction

Candida albicans is a commensal of the human oral, gastrointestinal, vaginal, cutaneous and mucosal surfaces. In immunocompetent as well as immunocompromised individuals, *C. albicans* causes cutaneous or subcutaneous infections such as vaginitis or oral thrush or infections of the nails and skin (1). It can also cause opportunistic infections, especially in immunocompromised patients. Oropharyngeal candidiasis (OPC), which is frequently observed in patients infected with the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS), in patients receiving broad-spectrum antibiotics or undergoing cancer chemotherapy (2). *C. albicans* can enter the bloodstream to cause serious systemic invasive disease, commonly treated with the antimycotic agent fluconazole (3). Unfortunately, *C. albicans* and other species can develop resistance to fluconazole, especially during long-term treatment of OPC (4, 5). The management of *Candida* infections faces a number of problems including limited number

of effective antifungal drugs, toxicity of the available antifungal drugs, resistance of *Candida* to commonly use antifungal drugs, relapse of *Candida* infections and the high cost of antifungal drugs (6, 7, 8). The alternative management of *Candida* infections was concerned by using phytochemicals from herbs and/or metabolites from antimicrobial producing microorganisms, such as lactic acid bacteria (LAB) (9, 10, 11).

LAB have long been used in fermentations to preserve the nutritive qualities of various foods. The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (12). In addition, they also prolong the microbiological shelf-life of final products by producing several antimicrobial metabolites such as organic acids, fatty acids, cyclic dipeptides, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, bacteriocins and antibiotics (13). Among organic acids, lactic, acetic, phenyllactic (PLA) and p-OH-phenyllactic acids (OH-PLA) produced by LAB play a role in inhibiting fungal and bacterial growth (14, 15). There are many reports on the production of antibacterial compounds by LAB but reports on the inhibition of yeasts and molds are comparatively few (16, 17, 18, 19). The antifungal agents from LAB are mostly low-molecular-mass organic compounds. For example, capric acid and several short-chain fatty acids have been described in an antifungal metabolites producing *Lactobacillus sanfrancisco* CBI strain from sourdough (13). Benzoic acid, methylhydantoin, mevalonolactone and cyclo-(glycyl-L-leucyl), synergists of lactic acid have been isolated from *L. plantarum* and are active against *Fusarium avenacum* (20). Phenyl-lactic acid and 4-hydroxy-phenyl-lactic acid produced by *L. plantarum* 21B were determined to have broad fungicidal activity (21). Early studies discovered antifungal activities produced by a *L. casei* strain which inhibited both growth and aflatoxin production of *Aspergillus parasiticus* (22).

Production of several fungal inhibitory compounds by *L. casei* subsp. *rhamnosus* with molecular masses below 1 kDa was also evidenced (23). The aim of this study was to isolate and identify anti-*C. albicans* metabolites producing LAB from various sources for production of anti-*C. albicans* metabolites insteady use of antimycotic agent in the nearly future.

2. Materials and methods

2.1 Isolation of LAB

A total of 46 samples were collected from various sources such as fermented pork (Nham), fermented fish, fermented vegetables, fermented fruits, flowers, soils and dairy effluent. Ten grams of each sample was added with 90 ml of sterile 0.85% NaCl to initial dilution (10^{-1}). The suspension was used for serial dilutions up to 10^{-8} and 0.1 ml of each suspension was spread on De Mann Rogosa and Sharpe (MRS) agar, incubated for 48 h at 37°C in 5-10% CO_2 atmosphere. Approximately, 6-9 randomly selected colonies were spotted on MRS agar for replica plates. Isolated isolates were maintained as frozen cultures in MRS broth with 20% glycerol at -80°C.

2.2 Microbial inoculum

Candida albicans BCC6120 from National Center for Genetic Engineering and Biotechnology (BIOTEC) was used as indicating strain in this study. *C. albicans* BCC6120 was grown on TGE (tryptone-glucose-yeast extract) (24) or SD (Sabouraud Dextrose) agar plate. Indicating strain was prepared from inoculums of culture grown to the mid-log phase in SD broth at 30°C with constant agitation (25). LAB isolates were grown on MRS agar plates and incubated at 37°C for 18-24 h in 5-10% CO_2 atmosphere. Prior to use, LAB isolates were activated in MRS broth at 37°C for 18-24 h and subcultured in MRS broth at 37°C for 18-24 h (26).

2.3 Preparation of culture filtrate

The anti-*C. albicans* metabolites producing isolates were cultivated in MRS broth and incubated at 37°C for 48 h in 5-10% CO_2 atmosphere. The cells were removed by centrifugation (10,000 rpm, at 4°C, for 15 min), following by filter sterilization (0.45-µm pore size; Millipore).

2.4 Determination of the anti-*C. albicans* activity

The anti-*C. albicans* activity were determined by three different assays, the dual culture overlay, the agar-well diffusion and the cross streak methods. The dual culture overlay and the agar-well diffusion methods were modified from Magnusson & Schnurer (2001) (16). The cross streak method was described by Monthon (2005) (27). All the experiments were conducted in duplicate. The dual culture overlay method was adapted from Fleming *et al*, (1985) (28). Overnight cultures of the LAB isolates were spotted onto surface of MRS agar plates and incubated for 48 h at 37°C in 5-10% CO_2 atmosphere to allow colonies to develop. A layer of 5 ml of SD broth with 0.75% agar (soft SD agar) containing 1 ml of the sensitive test overnight culture (indicator strain) was poured over the plate on which the growing producer. The plates were aerobically incubated at 30°C for 24 h, and checked for inhibition zones.

The cross streak method was performed using TGE agar plates on which selected isolates were inoculated 7.5-cm in long lines, 0.6-cm in width, and incubated at 37°C for 48 h in 5-10% CO_2 atmosphere. The plates were cross streaked with indicating strain, aerobic incubated at 30°C for 24 h, and inhibit growth of indicating strain were examined around the streak line of selected isolates.

For the agar well diffusion assay, MRS agar plates containing approximately 1.5×10^8 cell selected isolate per milliliter were prepared. Wells, with a diameter of 7 mm, were then cut in the agar using a sterile pasteur pipette. A droplet of agar was added to each well in order to seal it to avoid leakage. Then, 100 µl samples were added to wells and allowed to diffuse into agar during a 5 h pre-incubated period at room temperature, followed by aerobic incubated at 30°C for 24-48 h. Inhibition was detected by a clear zone around the wells.

2.5 Colonization assay

Isolates of LAB showed anti *C. albicans* BCC6120 activity were carried out to assay for the colonization. All selected isolates were inoculated in MRS broth containing sterile toothpick, incubated at 37°C in 5-10% CO_2 atmosphere (do not shake) for 2-5 days. Colonization around the sterile toothpick and surface of test tube were observed.

2.6 Effect of pH on anti-*C. albicans* activity

The culture filtrate was adjusted to pH values of 3, 5, 7, 9 and 11 with 1 N HCl and 1 N NaOH before evaluating the anti-*C. albicans* activity by agar-well diffusion method as previously described above. MRS broth, adjusted to the same pH values, served as a control.

2.7 Identification of LAB

The selected isolates were subjected into MRS broth for activation before use. The anti-*C. albicans* metabolites producing isolate was identified by morpholological and biochemical tests (gram's stain and catalase activity) (29). The selected isolate was identified by API 50 CHL system (BioMerieux, France) according to the manufacturer's instructions. The results from the API 50 CHL test were compared with the API database. Finally, the identification of the selected isolate was confirmed by sequencing of 16S rDNA (30).

3. Results and Discussion

Five hundred and fifty-two isolates of LAB were isolated from various sources samples and tested anti-*C. albicans* activity by means of the dual culture overlay, the cross streak and agar well diffusion methods. The overlay method was performed on MRS agar plates, the cross streak method was performed on TGE agar plates and the agar well diffusion method was performed on SD agar plates. The results showed that 51 isolates in the percentile of 9.24 were effective in terms of anti-*C. albicans* BCC6120. Some characteristic results are shown in Figure 1.
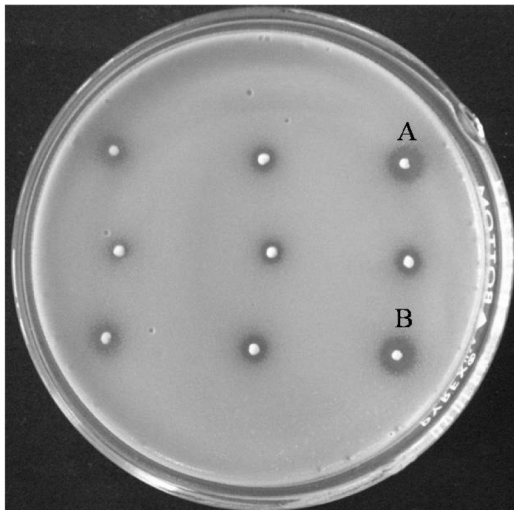

Figure 1. Anti- *C. albicans* activity of LAB isolates L46-3 (A) and L47-2 (B) by means of dual culture overlay method. The clear zone indicated *C. albicans* BCC6120 activity.

In colonization assay, 2 of 51 isolates showed adhesion surrounding the surface of sterile toothpick and test tube. The LAB isolates, L46-3 and L47-2, were isolated from fermented bamboo shoots and dairy effluent respectively. While the latter isolate exhibit the highest adhesion as shown in Figure 2.

Figure 2. Colonization of anti-*C. albicans* LAB isolates in the MRS broth containing sterile toothpick for 5 days, 1: medium no culture; 2: medium culturing L47-2; 3: medium culturing L46-3; and 4: negative control. The colonization showed adhered surrounding surface of sterile toothpick and test tube.

All isolates did not showed to inhibit the growth of *C. albicans* BCC6120 on TGE agar, due to the TGE medium contained only one percent of glucose while MRS medium has two percent in the experiments. LAB isolates, L46-3 and L47-2, were the most active against *C. albicans* BCC6120 by using overlay and agar well diffusion methods as shown in Table 1.

Table 1. Inhibition of *C. albicans* BCC6120 by selected LAB using dual culture overlay, cross streak and agar well diffusion methods.

LAB isolates	Anti-*C. albicans*			LAB isolates	Anti-*C. albicans*		
	Dual culture Overlay	Cross streak	Agar well diffusion		Dual culture Overlay	Cross streak	Agar well diffusion
L8-7	++	-	++	L41-7	+	-	+
L8-8	+	-	+	L42-7	+	-	+
L8-9	+	-	+	L42-9	+	-	+
L8-10	+	-	+	L42-12	+	-	+
L8-11	+	-	+	L43-1	+	-	+
L8-12	+	-	+	L43-2	+	-	+
L24-1	+	-	+	L43-3	+	-	+
L24-2	+	-	+	L43-4	+	-	+
L24-3	+	-	+	L43-5	+	-	+
L24-4	+	-	+	L43-6	+	-	+
L24-5	+	-	+	L43-9	+	-	+
L24-6	+	-	+	L43-11	+	-	+
L24-7	+	-	+	L43-12	+	-	+
L24-8	+	-	+	L45-1	+	-	+
L24-9	+	-	+	L45-4	+	-	+
L24-10	+	-	+	L45-8	+	-	+
L24-11	+	-	+	L45-10	+	-	+
L24-12	+	-	+	L45-11	+	-	+
L40-1	+	-	+	L46-1	+	-	+
L40-3	+	-	+	L46-3	+++	-	+++
L40-5	+	-	+	L46-8	+	-	+
L40-6	++	-	++	L46-9	+	-	+
L40-7	+	-	+	L46-10	+	-	+
L40-8	+	-	+	L46-12	+	-	+
L40-10	++	-	++	L47-2	+++	-	+++
L41-4	+	-	+				

The inhibition was graded by the radius of inhibition zone using the following scales: - = no inhibition, + = no *C. albians* BCC6120 growth for 1-3 mm, ++ = no *C. albicans* BCC6120 growth for 3-10 mm, +++ = no *C. albians* BCC6120 growth of more than 10 mm from the LAB inoculum.

3.1 Effect of pH on anti-*C. albicans* activity

The anti-*C. albicans* activity was stable at pH range of 3.0-5.0. The activity was loss when the pH of the antifungal substance was adjusted to above 5.0, which according to the result of previous report (16). LAB have long been known to produce organic acids into their production medium (31). The antifungal metabolites could be acids like lactic and acetic acids, as

well as hydrogen peroxide, formic acid, propionic acid, and diacetyl (32). Nevertheless lactic acid is the major LAB metabolite, causing pH reductions that inhibit many microorganisms (33). Similar to lactic acid, acetic acid and propionic acid interact with cell membranes to neutralize the electrochemical proton gradient, but the effect of acetic and propionic acid is often dependent on the decrease in pH caused by lactic acid (33, 34). Propionic acid reduces fungal growth, especially at lower pH and affect fungal membranes at pH values below 4.5 (35, 36). Ten randomly isolates, L8-7, L8-10, L24-2, L24-10, L40-6, L40-10, L41-7, L45-11, L46-3 and L47-2 were selected to determine the pH value by the inhibition zone. The pH value in the inhibition zone was 3.0 to 5.0 as shown in figure 3, suggesting a limited contribution of undissociated lactic acid to the inhibitory effect. However, the observed reduction in anti-*C. albicans* activity of the culture filtrates at pH value exceeding 5.0 indicates synergistic effects between lactic acid and other antifungal compounds (16).
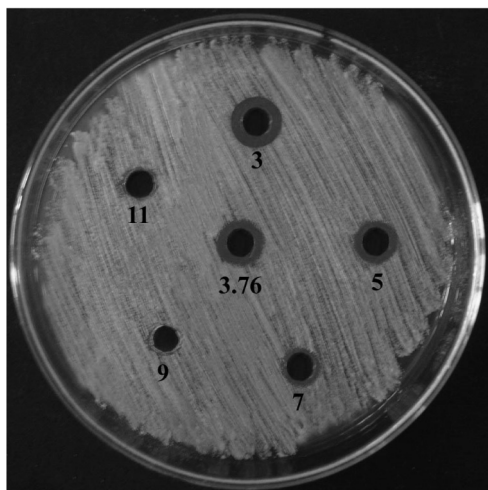

Figure 3. Effect of pH on anti-*C. albicans* activity by agar well diffusion method. The culture filtrates were adjusted to pH 3, 5, 7, 9 and 11, the pH value 3.76 was the starting value.

3.2 Identification of LAB

The isolate L47-2 showed the highest activity against the growth of *C. albicans* BCC6120 and colonization, therefore this isolate was selected to identification. The isolate L47-2 is a gram positive rod and catalase negative. The morpholological and biochemical data indicated that the isolate belongs to the genus *Lactobacillus* (26). The carbohydrate fermentation patterns identified as *L. paracasei* (results not shown) according to API database (Bio-merieux). This identification was confirmed by the 16S rRNA gene sequence data corresponding of the *L. paracasei* showed 99% identity (GenBank accession no. AB362764).

4. Conclusions

The aim of this study was to evaluate the anticandidal metabolites of lactic acid bacteria. Only isolate L47-2 showed the most effective against *C. albicans* BCC6120 and colonization. Furthermore, their activity is also shown to be pH-dependency and later identified as *L. paracasei*. This isolate is expected to the new biopreservative compounds. Further studies, this promising isolate L47-2 can be used as a bioactive compound to cure the *C. albicans* infection in immunocompromised patients and is an option for both of prophylaxis and human health promotion.

5. Acknowledgments

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