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Isolation and Selection of Anti-*Candida albicans* Producing Lactic Acid Bacteria

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ABSTRACT

The forty isolates of lactic acid bacteria (LAB) were obtained from various fermented foods. The cross streak plate method was used to preliminary screen for antimicrobial activity. LAB were isolated by selective medium, Mann Rogosa Sharpe (MRS). Most of the isolates showed inhibition against *Staphylococcus aureus* TISTR 517, *Bacillus subtilis* TISTR 008, *Micrococcus luteus* TISTR 884, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 781, and *Candida albicans* DMST 5239. Only sterile culture supernatant of isolate No. L14, later identified as *Lactococcus lactis*, showed antifungal activity by means of agar well diffusion assay. The activity was stable during heat treatment and was retained even after autoclaving at 121°C for 15 minutes. Maximum activity was observed at pH values between 2.5-4.0, and was lost at higher pH values. The anti-*C. albicans* activity was fully regained after readjustment of the pH to the initial value (pH 3.5).

Key words: Lactic acid bacteria - Antifungal activity

INTRODUCTION

Fungi are able to use almost any surface for growth. Unfortunately, they also are proficient at colonizing and using plants, humans and animal as substrates, causing diseases (1,2,3), as well as spoilage in food. In addition they cause great economic losses and public health problems (4), especially involving immunocompromised patients (5). This is in part due to the tremendous advances in medicine that permit the saving of patients with immunocompromising diseases who would otherwise not have survived. Fortunately, it is rare that these patients succumb to fungal infections for which there are few or no drugs available for treatment. Encouragingly, naturally occurring antifungal proteins and peptides, as well as synthetic derivatives, have the potential to be very interesting clinical leads. Plants, bacteria, insects, mollusks, fungi and mammals synthesize a number of proteins and peptides that are antifungal. These proteins appear to be involved in either constitutive or induced resistance to fungal attack, defending plants and animals, including humans, against pathogenic fungi and/or mycotoxins. There are hundreds of antifungal peptides and proteins known, with more being discovered almost daily. However, much of the fungistatic effect has been attributed to lactic and, especially, acetic acids produced by lactic acid bacteria

(LAB) (6). Further studies have confirmed that the acetic acid concentration was strictly related to the antifungal activity and that other bacterial metabolites also have inhibitory activity (7,8,9). While many studies have assessed LAB's antibacterial effects (10), there are very few reports on their specific antifungal compounds. The aim of this study was to isolate and identify LAB from fermented foods for the production of antifungal activity against indicator yeast, *Candida albicans*, its pathogenicity to immunocompromised patients (1,2,3).

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria (LAB)

Samples of fermented foods were collected locally. LAB selective MRS (Mann Rogosa Sharpe) medium (Oxoid) was used for the isolation of bacteria. Each of the isolates was streaked on MRS agar plates to obtain pure cultures. From the isolates the one with the anti-*C. albicans* activity phenotype was selected on the basis of its inhibitory spectrum. An MRS broth containing 15% glycerol was used to preserve the cultures at -80°C.

Identification of LAB

The anti-*C. albicans* producing strain was identified by physiological and biochemical tests (11,12,13). The isolate was examined for production of acid from 49 compounds as sole carbon source using an API 50 CH Test Kit (Bio-merieux, France) according to the manufacturer's instructions. Results from the API test were compared with the APII database, and the fermentation pattern was further evaluated according to the method of Kandler and Weiss (14).

Preparation of Concentrated Culture Filtrate

The Anti-*C. albicans* producing isolate was inoculated to a concentration of 10^8 cells/ml of 100 ml MRS broth in a 500-ml Duran bottle, and incubated as a still culture at 30°C for 48 h. The culture was centrifuged (12,000 rpm, 20 min, 4°C; Hettich Zentrifugens), followed by filter sterilization (0.45-µm poresize; Millipore). The sterile cell-free supernatant was concentrated by using a concentrator tube (1-kDa molecular weight cutoff; PALL Life Sciences) and the volume reduced to 1 ml. Culture filtrate from type strain *L. plantarum* TISTR 080 was used as a control.

Microbial Inocula

The microbial inocula strains, ie., *Staphylococcus aureus* TISTR 517, *Bacillus subtilis* TISTR 008, *Micrococcus luteus* TISTR 884, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 781, and fungus *Candida albicans* DMST 5239, were used as indicating strains in this study. The bacterial inocula strains or fungus *C. albicans* were grown on TGE (tryptone-glucose-yeast extract) (15) or SD-agar plate (Sabouraud Dextrose), respectively. The bacterial inocula were grown at 37°C for 1 day, while the *C. albicans* was grown at 30°C for 2 days and then stored at 4°C. Microbial cells inocula were prepared from washed cultures grown in TGE or SD

broth (Oxoid) and still cultured at 30°C for 1 day. Microbial cell concentrations were determined using Mcfarland No.0.5, and adjusted to 10^6 per ml of sterile peptone water (2%).

Antimicrobial Activity Assay

Two different assays, the cross streak method and the agar-well diffusion methods were used to detect antimicrobial activity. All experiments performed in duplicate. The cross streak method was performed using TGE (15) agar plates on which selected isolates were inoculated as 7.5-cm long lines, 0.6-cm in width, and incubated at 37°C for 3 days in 5% CO_2 atmosphere. The plates were then cross streaked with microbial inocula strains, incubated aerobically at 37°C for 24 h. The plates were examined to inhibit growth of microbial inocula strains around the streak line of selected isolates.

For the agar well diffusion assay, MRS agar plates containing 10^6 selected strain per ml agar were prepared. Wells, with a diameter of 7 mm, were then cut in the agar using a sterile pipette tip. 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-incubation period at room temperature, followed by aerobic incubation at 37°C for 24-48 h. The antimicrobial effects recorded were reported as follows: -, no suppression; +, suppression, with detectable clear zones around the wells.

Effects of Temperature and pH on Anti-*C. albicans* Activity

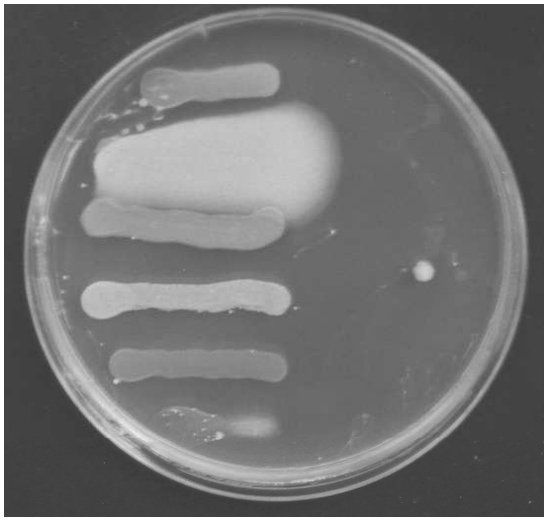
The anti-*C. albicans* activity remaining after exposure to high temperature, different pH values using the agar well diffusion assay. Aliquots (1 ml) of 5-fold concentrated culture filtrate were heated to 121°C for 15 min. The samples were allowed to cool and then tested for anti-*C. albicans* activity. The pH effect was investigated with 5-fold-concentrated culture filtrate, adjusted to pH values of 2.5, 3.0, 4.0, 4.5, 5.0, 6.0, 7.0, and 9.0 with 1 M HCl and 2 M NaOH before evaluating the anti-*C. albicans* activity. MRS broth, concentrated 5-fold and adjusted to the same pH values, served as a control.

RESULTS AND DISCUSSION

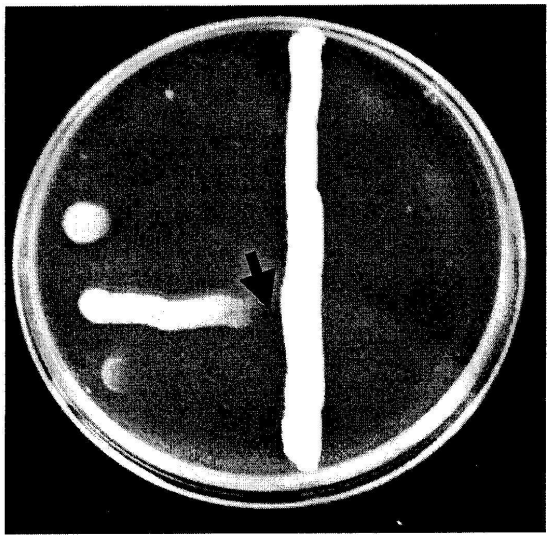
Isolation and Identification of Lactic Acid Bacteria

Forty isolates of LAB obtained from various fermented food samples were isolated and tested to produce antimicrobial activity by means of the cross streak and agar well diffusion methods. The cross streak method was performed on TGE agar plates, while the agar well diffusion method was performed on SD agar plates after selected isolates were cultured in an MRS liquid medium for 2 days and cell-free culture supernatants were aseptically separated. The test showed that four of the 40 isolates were effective in terms of antagonism towards *C. albicans*. LAB strain L14 was the most active against *C. albicans* by using both methods as shown in **Figure 1**, and was selected for further study of its identity. The other 36 isolates of LAB did not show antifungal activity against *C. albicans* but some inhibited bacterial indicator

strains (*Bacillus subtilis* TISTR 008, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 781, *Staphylococcus aureus* TISTR 517, and *Micrococcus luteus* TISTR 884), as tabulated in **Table 1**.



(a)



(b)

Figure 1. Antimicrobial activity of lactic acid bacteria strain L14 using the cross streak method. The *C. albicans* inhibited growth as indicated by the arrow. Indication, bacterial strains could not grow in the plate (b). Bacterial strains are aligned from top (a), ie., *Escherichia coli* TISTR 887, *Bacillus subtilis* TISTR 008, *Pseudomonas aeruginosa* TISTR 781, *Candida albicans* DMST 5239, *Staphylococcus aureus* TISTR 517, and *Micrococcus luteus* TISTR 884.

Table 1. Inhibition of bacteria and yeast by selected lactic acid bacteria using cross streak method, and agar well diffusion methods (20).

Isolate	Name	Antimicrobial activity[1]			
		Indicator bacterial strains		*Candida albicans*	
		1.1	1.2	1.1	1.2
1	L1	+	+	-	-
2	L2	+	+	-	+
3	L3	+	+	-	-
4	L4	+	+	-	+
5	L5	+	+	-	-
6	L7	+	+	-	-
7	L8	+	+	-	-
8	L9	+	+	-	-
9	L10	+	+	-	-
10	L11	+	+	-	-
11	L12	+	+	-	+
12	L13	+	+	-	+
13	L14	+	+	+	+
14	L15	+	+	-	-
15	L16	+	+	-	-
16	L17	+	+	-	-
17	L18	+	+	-	-
18	L19	+	+	-	-
19	L20	+	+	-	-
20	L21	+	+	-	-
21	L22	+	+	-	-
22	L23	+	+	-	-
23	L24	+	+	-	-
24	L25	+	+	-	+
25	L26	+	+	-	+
26	L27	+	+	-	-
27	L28	+	+	-	-
28	L28	+	+	-	-
29	L29	+	+	-	-
30	L30	+	+	-	-
31	L31	+	+	-	+
32	L32	+	+	-	-
33	L33	+	+	-	-
34	L34	+	+	-	-
35	L35	+	+	-	-
36	L36	+	+	-	-
37	L37	+	+	-	-
38	L38	+	+	-	-
39	C3	+	+	-	-
40	C7	+	+	-	+

[1]Antimicrobial activity: +, supression; -, no suppression

1.1 or 1.2 is the method for evaluate antimicrobial activity by cross streak method, and agar well diffusion method, respectively.

Indicator bacterial strains also see Materials and Methods (Microbial inocula) and Figure 1.

LAB strain L14 is a gram positive coccoid, catalase negative, VP positive. The isolate did not produce gas from glucose. The data indicate that the strain belongs to the genus *Lactococcus* (18,19). The isolate fermented glucose, fructose, maltose, lactose, sucrose, trehalose, xylose, ribose, mannitol, amygdalin, but not raffinose. However, the isolate also utilized esculin, in contrast to the strain ATCC 19435, the type strain of *L. lactis* subsp. *Lactis* 11. On the basis of acid production of carbohydrates, the isolate was tentatively identified as *L. lactis* according to APII database identification (Bio-merieux), having close similarity with *L. lactis* subsp. *lactis*.

Effects of pH and Temperature on Anti-*C. albicans* Activity

The anti-*C. albicans* activity was found to be heat stable. Concentrated supernatant autoclaved for 15 min at 121°C retained full inhibitory activity against *C. albicans* growth. The activity was stable at pH values that were between 2.5-4.0 but rapidly decreased between pH 4.0-4.5. No inhibitory activity was detected at a pH above 4.5. The activity was regained after readjustment of the pH to the starting value (pH 3.5). Initially, a cross streak method was used to evaluate the anti-*C. albicans* activities. The pH value in the zone was approximately 3.5 to 3.8, suggesting a limited contribution of undissociated lactic acid to the inhibitory effect. However, the reduction in anti-*C. albicans* activity of the culture filtrates at pH values exceeding 4.0 indicates synergistic effects between lactic acid and other antifungal substances. The possibility of increased desorption of antifungal substances from the bacterial cells at very low pH values suggests further very complex interactions between the antifungal effects of LAB and the pH (20).

Stability

The anti-*C. albicans* activity of cell-free culture supernatant was lost during prolonged storage. No activity could be recovered after storage for 1 day at -30°C. The poor stability of anti-*C. albicans* activity might be not only due to irreversible precipitation-denaturation processes, but also during unintentional thawing of culture supernatant during the freeze-drying procedure (20).

CONCLUSIONS

The new strain appears to have anti-*C. albicans* activity at acidic pH values. Stability of the anti-*C. albicans* substances to heat is considered to be very important. *L. lactis* is a food-grade microorganism, widely used in food industries and has GRAS statis (generally regarded as safe) (21). Such, the anti-*C. albicans* substances produced by the strain L14, identified as *L. lactis*, may be used as a bioactive compound to cure the *C. albicans* infection in immunocompromised patients and is an option for both the prophylaxis and human health promotion.

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บทคัดย่อ

มณฑล เลิศคณาวนิชกุล

การแยกและคัดเลือกแลคติคแอซิดแบคทีเรียที่ผลิตสารต้าน *Candida albicans*

สามารถคัดเลือกแลคติคแอซิดแบคทีเรียจำนวน 40 ไอโซเลตจากอาหารหมักชนิดต่างๆ โดยการใช้วิธีขีดไขว้เชื้อเป็นวิธีทดสอบเบื้องต้นในการคัดเลือกคุณสมบัติในการยับยั้งจุลินทรีย์ด้วยอาหารเลี้ยงเชื้อ Mann Rogosa Sharpe (MRS) ปรากฏว่าเชื้อทุกไอโซเลตสามารถยับยั้งการเจริญของ *Staphylococcus aureus* TISTR 517, *Bacillus subtilis* TISTR 008, *Micrococcus luteus* TISTR 884, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 781 และ *Candida albicans* DMST 5239 แต่พบว่ามีเฉพาะส่วนของอาหารเลี้ยงเชื้อที่ใช้เลี้ยงแลคติคแอซิดแบคทีเรียหมายเลขแอล 14 เท่านั้นที่มีคุณสมบัติในการต้าน *Candida albicans* ด้วยวิธี agar well diffusion ซึ่งสารที่มีคุณสมบัติดังกล่าวมีความทนทานต่ออุณหภูมิสูงถึง 121 องศาเซลเซียส เป็นเวลา 15 นาที และยังคงมีฤทธิ์อยู่ในช่วง pH 2.5-4.0 แต่จะสูญเสียฤทธิ์ไปเมื่อปรับ pH ให้สูงขึ้น และจะมีฤทธิ์กลับมาอีกครั้งเมื่อปรับ pH ให้เท่ากับค่าตั้งต้น (pH 3.5)

สำนักวิชาสหเวชศาสตร์และสาธารณสุขศาสตร์ มหาวิทยาลัยวลัยลักษณ์ อำเภอท่าศาลา จังหวัดนครศรีธรรมราช 80160