ORIGINAL ARTICLE

Selection and identification of lactic acid bacteria that inhibit yeast contaminants isolated from fermented plant beverages

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Abstract

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In order to investigate yeast contamination in finished products of fermented plant beverages (FPBs), 27 FPBs samples were collected from northern Thailand. Nine samples from finished products were contaminated with yeast and 36 yeast isolates were isolated and identified to the genera level by conventional methods. These included 12 isolates of *Rhodotorula* sp., 9 isolates of *Pichia* sp., 9 isolates of *Hansenula* sp., 3 isolates of *Saccharomyces* sp. and 3 isolates of *Candida* sp. *Rhodotorula* sp. was chosen to use as a target organism for the primary screening of lactic acid bacteria (LAB) with antiyeast activity, using a dual culture overlay assay. Fifteen of the 72 LAB cultures isolated from Thai fermented foods and the FPBs produced a strong inhibition against the *Rhodotorula* sp. Ten of these also had a broad antiyeast spectra (at least 5 genera inhibited). Three of the isolates that gave the best inhibition (DW1, 3 and 4) were identified as *Lactobacillus plantarum* strains based on conventional identification methods.

Key words : fermented plant beverages, yeast contaminants, lactic acid bacteria

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การคัดเลือกและเทียบเคียงแบคทีเรียแลกติกที่สามารถยับยั้งยีสต์ซึ่งปนเปื้อนในน้ำหมักชีวภาพ

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เพื่อตรวจสอบการปนเปื้อนของยีสต์ในผลิตภัณฑ์สุดท้าย (finished product) ของน้ำหมักชีวภาพจำนวน 27 ตัวอย่างที่เก็บจากภาคเหนือของประเทศไทย พบว่ามี 9 ตัวอย่างที่พบการปนเปื้อนด้วยยีสต์ ซึ่งสามารถแยกยีสต์ได้ 36 ไอโซเลท ตามความแตกต่างของโคโลนี เมื่อนำมาเทียบเคียงตามวิธีดั้งเดิม พบว่าเป็นยีสต์ในสกุล *Rhodotorula* sp. 12 ไอโซเลท *Pichia* sp. 9 ไอโซเลท *Hansenula* sp. 9 ไอโซเลท *Saccharomyces* sp. 3 ไอโซเลท และ *Candida* sp. 3 ไอโซเลท ดังนั้น *Rhodotorula* sp. ถูกเลือกเป็นยีสต์เป้าหมายในการคัดเลือกเบื้องต้นเพื่อหาแบคทีเรียแลกติก (72 ไอโซเลท ที่แยกได้จากอาหารหมักและน้ำหมักชีวภาพ) ที่มีกิจกรรมการยับยั้งยีสต์ด้วยวิธี dual culture overlay assay พบว่ามี 15 ไอโซเลทให้ผลการยับยั้งดีมาก และในการคัดเลือกขั้นที่สองเพื่อดูความสามารถในการยับยั้งยีสต์หลายชนิดพบว่ามีอยู่ 10 ไอโซเลทที่ให้ผลการยับยั้งยีสต์ได้อย่างน้อยที่สุด 5 สกุล แต่มีเพียง 3 ไอโซเลท (DW1, 3 และ 4) ที่ให้ผลการยับยั้งดีเด่น และผลการเทียบเคียงแบคทีเรียแลกติกทั้ง 3 ไอโซเลทโดยวิธีดั้งเดิม พบว่าต่างก็เป็น *Lactobacillus plantarum*

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Molds and yeasts cause major problems in food and feed as spoilage organisms. Molds are particularly important because they produce mycotoxins (Pitt and Hocking, 1997). Biopreservation, i.e. the use of microorganisms to preserve food and feed, has been considered as an alternative to the use of chemical preservatives in the expectation that they could be safer. (Ström *et al*., 2002). Lactic acid bacteria (LAB) are of particular interest as biopreservation organisms due to their production of lactic acid, acetic acid, hydrogen peroxide and other antimicrobial compounds (Magnusson and Schnürer, 2001). There are many reports on the production of antibacterial compounds by LAB but reports on the inhibition of yeasts and molds are comparatively few (Magnusson and Schnürer, 2001). Magnusson *et al*.(2003) reported that *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produced proteinaceous compounds with a broad spectrum inhibitory action against several molds such as *Aspergillus fumigatus, A. nidulans, Penicillium roqueforti, Mucor hiemalis, Talaromyces flavus, Fusarium poae, F. graminearum, F. culmorum*, and *F. sporotrichoides*. However, a weaker activity was observed against the yeasts *Debaryomyces hansenii, Kluyveromyces marxianus,* and *Saccharomyces cerevisiae*.

Fermented plant beverages (FPB or FPBs) are non alcoholic beverages produced from a variety of plants such as cereals, fruits and vegetables. The FPB production is catalyzed by lactic acid bacteria and the lactic fermentation is normally contaminated with yeast (Kantachote *et al*., 2005a). Our previous study (Kantachote and Charernjiratrakul, 2004) demonstrated that a non-contaminated starter culture of LAB is necessary to reduce the amount of yeast in the finished FPB product. Therefore, the aims of the present study were to partially identify yeasts that contaminated FPBs and to isolate antiyeast lactic acid bacteria from Thai fermented food and FPBs.

Materials and methods

1. Isolation and identification of yeast

A total of 27 samples of FPBs were collected from various sources in northern Thai-

land. Each sample of FPBs was fermented from different raw materials such as noni (*Morinda coreias* Ham.), *Phyllanthus emblica* Linn., *Aegel marmelos* Corr., *Kaempferia parviflora* Wall., *Cyperous rolundus* Linn., *Musa sapientum* Linn., *Tinospora crispa* Miers ex Hook., *Allium sativum* Linn., etc. To isolate yeast, 0.1 ml of each sample was spread on Potato Dextrose Agar (PDA) and then incubated at 30°C for 48-72 h. Single colonies were further purified and checked by using a microscope. Pure cultures were maintained on a PDA slant at 4°C and subcultured at intervals. Identification was conducted to genus level following the methods described in a Standard Taxonomic Manual (Deak and Beuchat, 1996) using cell shape, colony morphology, productions of pigment and spores, and biochemical tests. Sugar assimilation was examined in yeast nitrogen base medium containing 5% of the following single sugars: maltose, galactose, sucrose, lactose, raffinose and inulin, whereas sugar ferment-ation was also investigated using 6% of the following single sugars: maltose, trehalose, xylose, cellobiose, starch, raffinose, lactose, sucrose, galactose and glucose in a basal medium. Ability to grow in YM (yeast extract malt extract) medium with the addition of a compound such as: 0.01 or 0.10% cycloheximide, 10 or 16% NaCl was tested. Utilizations of ethanol methanol, urea and citrate were also conducted.

2. Isolation and identification of lactic acid bacteria

Seventy-two cultures of lactic acid bacteria were isolated from fifty varieties of Thai fermented food samples such as Nham, fermented vegetables, fermented fish, fermented milks, FPBs, and 22 isolates obtained from a previous study (Kantachote *et al*., 2005b). In the case of solid samples, roughly 2.5 g of each was soaked in 25 ml sterile 0.85% NaCl and then treated for 2 min in a stomacher. Each suspension was spread on de Man Rogosa and Sharpe (MRS) agar. After incubation at 30°C for 24 h, single colonies were transferred to a new MRS plate and further purified. Working cultures were kept on MRS agar slants at 4°C. The LAB were identified following the methods in Bergey's Manual of Systematic Bacteriology, vol. 2 (Kandler and Weiss, 1986) and The lactic Acid Bacteria vol. 2 (Hammes and Vogel, 1995). Carbohydrate fermentation profiles were conducted in MRS fermentation broth with 0.004% bromocresol purple but without glucose and containing 2% of each of the investigated sugars.

3. Yeast cell inocula

Each isolate of yeast was grown in malt extract broth (2%, Difco Laboratories) and incubated at 30°C for 24 h. Yeast cell counts were determined using a haemacytometer, and adjusted to 10^5 cells/ml with sterile peptone water (0.2% w/v) to use as an inoculum for testing.

4. Determination of the antiyeast activity of LAB against *Rhodotorula* sp.

Isolated LAB strains were primarily screened for antiyeast activity using a dual culture overlay assay (adapted from Magnusson *et al*., 2003). LAB were inoculated in two 2-cm lines on MRS agar plates and allowed to grow at 30°C for 48 h. The plates were then overlaid with 10 ml of malt extract soft agar (0.05% malt extract) containing 10^5 cells per ml of *Rhodotorula* sp. After 48 h of aerobic incubation at 30°C, the radius of inhibition zone was measured. The inhibition activity was graded by following scales: no visible inhibition (-), no yeast colony growth of 1-3 mm, weak (+); no yeast growth of 3-10 mm, moderate (++); no yeast growth of > 10 mm, strong (+++); next to the LAB inoculation. Inhibition tests were done in duplicate.

5. Determination of antiyeast spectra

The following 8 yeasts *Pichia* sp., *Rhodotorula* sp., *Candida* sp., *Saccharomyces* sp., *Hansenula* sp., *Endomycopsis* sp., *Schizosaccharomyces* sp. and *C. neoformans* were used to investigate the spectrum of each LAB isolate. The last 3 strains were obtained from the Department of Microbiology; Faculty of Science, Prince of Songkla University and the remaining yeasts were isolated from the FPBs in this study. The spectrum

of LAB isolates able to inhibit growth of 8 yeast test strains was determined by the overlay method as described above.

Result

1. Isolation and identification of yeast

From 27 samples of FPBs, only 9 samples were seen to be contaminated by yeasts. Thirty six colonies with different morphology were isolated and were identified to a genus level. The results based on their properties of morphology, physiology and biochemical tests (Table 1), according to the identification key, were *Rhodotorula* sp. 12 isolates (33.3%), *Pichia* sp. 9 isolates (25%), *Hansenula* sp. 9 isolates (25%), *Saccharomyces* sp. 3 isolates (8.3%) and *Candida* sp. 3 isolates (8.3%). As *Rhodotoula* sp. were the most commonly detected yeast species in the FPB samples, it was chosen to use as an indicator for primary screening of

Table 1. Identification of yeast isolates found as contaminants in fermented plant beverages.

Test	Group A	Group B	Group C	Group D	Group E
Number of isolate	12	9	9	3	3
Pink colony	+	-	-	-	-
Spore	Chlamy.	Asco.	Asco.	Chlamy	Asco.
Sugar assimilation					
Maltose	+	+	-	+	+
Galactose	+	+	-	+	+
Sucrose	+	+	-	+	+
Lactose	-	-	-	-	-
Raffinose	+	+	-	-	+
Growth in					
Ethanol	+	+	+	+	+
Methanol	-	-	-	-	-
10%NaCl	+	+	-	+	+
16%NaCl	+	+	-	-	-
0.01%Cyclohexamide	+	d/-	+	+	-
0.10%Cyclohexamide	+	-	+	+	-
Citrate	+	+	+	+	-
Urea	+	-	-	-	-
Sugar fermentation					
Xylose	-	-	-	-	-
Raffinose	-	+	-	-	+
Maltose	-	+	-	+	+
Inulin	-	-	-	-	d
Glucose	-	+	+	+	+
Galactose	-	+	-	+	+
Sucrose	-	+	-	+	+
Lactose	-	-	-	-	-
Cellobiose	-	+	-	-	-
Starch	-	d	-	d	+
Trehalose	-	d	-	-	d
Unknown result	*Rhodotorula*	*Pichia*	*Hansenula*	*Candida*	*Saccharomyces*

Chlamy. = chlamydospore, Asco. = Ascospore
d/- (some isolates gave a delayed positive result and some gave a negative result)

antiyeast LAB isolates.

2. Isolation of antiyeast LAB

31 LAB isolates (43%) inhibited *Rhodotorula* sp. (Some characteristic results are shown in Figure 1). However, only 15 LAB isolates were able to inhibit *Rhodotorula* sp. with moderate or strong inhibition (data not shown). Hence, these 15 isolates were selected for secondary screening. The selected LAB isolates were coded as follows: DW1, 3, 4, 6, 7, 18, 25, 27, 31, 37, 38, 42, 47, 54, and 55 (DW refers to Duangporn and Wilawan).

3. Determination of antiyeast spectra

The ability to inhibit 8 different yeast species was then tested. Only 12 LAB isolates (DW1, 3, 4, 6, 7, 18, 25, 38, 42 and 54) had a broad spectrum of inhibition by inhibiting at least 5 genera of the target organisms (Table 2). It was found that *Schizosaccharomyces* sp. and *Candida neoformans* were resistant to all selected LAB isolates. After consideration of the antiyeast spectra

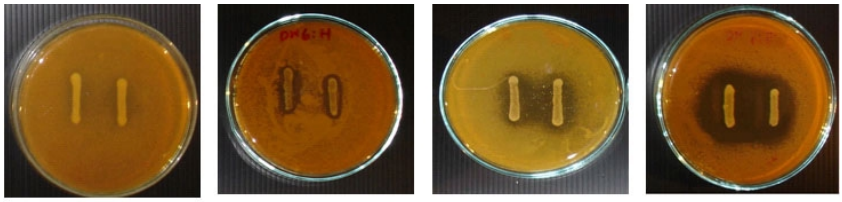

Figure 1. Degree of inhibition of *Rhodotorula* sp. by LAB isolates: DW8, no inhibition (-); DW2, weak inhibition (+); DW6, moderate inhibition (++); and DW4, strong inhibition (+++).

[Color figure can be viewed in the electronic version]

Table 2. Antiyeast spectra of Lactic acid bacteria isolated from fermented Thai foods and fermented plant beverages.

Yeasts \ LAB	DW 1	DW 3	DW 4	DW 6	DW 7	DW 18	DW 25	DW 27	DW 31	DW 37	DW 38	DW 42	DW 47	DW 54	DW 55
Saccharomyces sp.	+++	++	++	-	-	-	++	++	-	-	++	-	-	+++	-
Candida sp.	-	+	++	+	++	+++	++	++	-	-	++	+++	-	++	++
Pichia sp.	+++	+++	++	++	+++	+++	++	-	-	-	++	++	-	++	+++
Hansenula sp.	+++	+++	+++	+	+++	+++	++	-	-	-	++	++	-	+++	-
Rhodotorula sp.	++	++	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	++	+++
Endomycopsis sp.	+++	+++	+++	++	+	++	++	-	--	++	-	++	-	+	+
Schizosaccharomyces sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Candida neoformans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total inhibition score	14+	14+	15+	8+	11+	13+	12+	7+	3	5	11+	12+	3	13+	9+

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

Table 3. Identification of lactic acid bacteria isolated from fermented beverages produced from phomnang seaweed and wild forest noni that showed promise as biopreservatives.

Characteristic	*Lactobacillus plantarum*	DW1	DW3	DW4
Shape	Rod	Rod	Rod	Rod
Gram Stain	+	+	+	+
Catalase test	-	-	-	-
Gas from glucose	-	-	-	-
Growth at 15/45°C	+/-	+/-	+/-	+/-
Carbohydrate fermentation				
Amygdalin	+	+	+	+
Arabinose	d	+	+	+
Cellobiose	+	+	+	+
Esculin	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Glucose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
Raffinose	+	+	+	+
Rhamnose	-	-	+	-
Ribose	+	+	+	+
Sorbitol	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+

Symbols: + = 90% or more strains positive, - = 90% or more strains negative, d = 11-89% strains positive

and the total extent of inhibitory activity it seemed that the 3 LAB isolates with scores of 14+ and 15+, 3 LAB (DW1, 3 and 4) were likely to be the most promising strains for further study (Table 2). Isolate DW1 was obtained from phomnang seaweed (*Gracilaria fisheri*), whereas the isolates DW3 and DW4 were obtained from wild forest noni (*Morinda coreia* Ham). The results of the identification tests indicated that isolate DW1 and DW4 were closely related to *Lactobacillus plantarum*. Isolate DW3 was similar to *L. plantarum* but it was able to utilize rhamnose (Table 3). As more than 90% of the tests identified the 3 strains as *Lactobacillus plantarum* we therefore believe that all 3 were *Lactobacillus plantarum* isolates.

Discussion

In this study, we isolated and identified yeast contaminants in FPBs and they were characterized as *Rhodotorula* sp., *Pichia* sp, *Hansenula* sp., *Saccharomyces* sp. and *Candida* sp. These yeasts are commonly found in fruit and vegetables as spoilage organisms (Pitt and Hocking, 1997; Jay, 2000). Fortunately, most of the isolated yeast contaminants of the FPBs were strongly inhibited by some LAB isolates, particularly isolates (DW1, 3 and 4). In contrast, none of the LAB isolates could inhibit *Schizosaccharomyces* sp. and *Candida neoformans*. The original habitat of these 2 species is not fruit or vegetables. Our work gave similar results to that of Savard *et al*. (2002) who isolated

and characterized yeast from fermented vegetable products. In their work *Saccharomyces* sp. were the most frequently detected followed by *Hansenula* sp. and *Debaryomyces* sp. Adams and Moss. (2002) reported the isolation of *Pichia guilliermondii* and *Saccharomyces fibuligera* from a number of tropical fermented products, whereas *Saccharomyces cerevisiae* was the most frequently encountered yeast in fermented beverages and foods based on fruit and vegetables.

Several researchers have reported that *Lactobacillus plantarum* has an ability to control fungi (Ström *et al*., 2002; Sjögren *et al*., 2003). *L. plantarum* strain MiLAB 393 from grass silage could produce compounds with a broad spectrum of antifungal activity against the food- and feed-borne filamentous fungi and yeasts; *Pichia anomala, Kluyveromyces marxianus, Rhodotorula mucilaginosa, Debaromyces hansenii, Candida albicans* and *Saccharomyces cerevisiae*.

In conclusion, yeast contamination in the FPBs was investigated. Most of the yeast isolates were inhibited by *L. plantarum* strains DW1, DW3 and DW4. Work is now in progress to determine the nature of the antiyeast compounds produced by the 3 *L. plantarum* isolates in the hope of identifying biopreservative compounds. Further work will also test if these promising LAB strains could be used as starter cultures for improving the quality of the FPB products.

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