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IDENTIFICATION OF LACTIC ACID BACTERIA AND YEASTS FROM FERMENTED RICE PRODUCT (*KHAO-KHAB*)

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INTRODUCTION

Khao-khab, a product in Utaradit province, the northern part of Thailand, is made from rice (*Oryza sativa*) or glutinous rice (*Oryza glutinosa*). *Khao-khab* is comsumed as it is or the vegetables are put on it over a steaming water pot and then wrapped its cooked and comsumed with spicy sauce. The fried noodle was also wrapped with the sheet of *khao-khab* for comsumption. Acetic acid bacteria in *khao-khab*, *Acetobacter indonesiensis, A. lovaniensis, A. pasteurianus, A. syzygii* and *A. farinalis* strains have been isolated and described [1]. This study, lactic acid bacteria and yeasts are isolated and identified based on the phenotypic and genotypic characteristics.

MATERIALS AND METHODS

Isolation of LAB and yeasts Twelve fermented rice products collected in Laplae district, Utaradit province were used for the isolation. The pH of the samples was measured by a pH meter (Mettler Toledo, USA). Ten grams of sample were homogenized with 90 ml of sterile 0.85 % NaCl solution to a uniform suspension and then tenfold serial dilutions in 0.85 % NaCl solution were carried out. For evaluation of LAB, for each serial dilution, 1 ml aliquots were each pipetted into a Petri dish with 20 ml of melted isolation medium, de Man, Rogosa and Sharpe agar (MRS agar, Difco) containing 0.3 g/100 ml $CaCO_3$ and incubated at 30°C for 3 days. The colonies with a surrounding clear zone were counted as lactic acid bacteria and they were picked up for purification. For evaluation of the yeasts, the aliquots of 0.1 ml of each dilution were spread onto the surface of Yeast and Malt Extract agar plates (YM agar) and incubated at 30°C for 3 days. The colonies were counted and their cell morphology were observed. Yeasts were purified by the streaking technique on a YM agar plate.

Identification of LAB

Phenotypic characterization Cells and colony appearance were examined on the cells grown on MRS agar plates after 3 days incubation. The production of gas from D-glucose, arginine hydrolysis, nitrate reduction, slime formation and acid production from carbohydrates were determined [2,3]. All the above tests were carried out by incubating the cultures at 30 °C for 3 days. Growth at 45 °C, at different starting pH values (4.0, 4.5, 8.0 and 8.5) and different NaCl concentrations (4, 6, 8 and 10 g/100 ml) was tested by using half strength of MRS (MRSH) broth. *Meso*-diaminopimelic acid (DAP) in the cell wall peptidoglycan was determined as described previously [4]. The isomer of lactic acid produced by each strain was analyzed enzymatically according to the method of Okada et al.[5].

16S rRNA gene sequencing analysis DNA from LAB isolates was isolated from cells grown in an MRSH broth andwas purified as reported previously [6]. The 16S rRNA gene was sequenced as previously described [7]. The sequences determined (1307–1352 bases) were aligned with the selected putative homolog sequences obtained from BLASTn searches of the Gen-homolog sequences obtained from BLASTn searches of the Gen-Bank/EMBL/DDBJ database by employing CLUSTAL_X [8].

Identification of yeast isolates

Phenotypic characterization Cells and colony appearance of yeasts were determined from cells and colonies grown in YM broth and on YM agar, respectively, after incubation at 30 C for 72 h. For the spore morphology, cells were grown on YM agar, 5 g/100 ml malt agar, and acetate agar were used to induce the sporulation of yeasts whilst the hyphae growth was also determined by using YM agar, according to the methods of Yarrow [9]. Physiological and biochemical characterization of the isolated yeasts were determined by investigating the assimilation reactions of sugars using the ID 32C test

(bioMérieux, France). The isolates were grouped based on their relationships among the phenotypic characteristics by cluster analysis. Hierarchical cluster analysis was conducted by using SPSS for Windows version 13.0.

26S rRNA gene sequence analysis The nucleotide sequences of the D1/D2 variable domains of the 26S rRNA encoding gene were determined by the modified methods of Kurtzman and Robnett [10]. The obtained sequences, were aligned and compared with homolog 26S rRNA gene partial sequences obtained from the DNA databank by BLASTn homology searches.

Analytical procedures Ethanol was analysed by gas chromatography (Hewlett-Packard, HP 5890 Series; USA) using Parapak QS (Carbowax 20 M) column and flame ionizationdetector (FID, 150 °C).

RESULTS AND DISCUSSION

The total count of LAB in the *khao-khab* samples ranged from 3.2 x10^8 to 2.2 x10^{11} colonies/g sample, whilst yeasts were from 5.9 x10^5 to 1.2 x10^8 colonies/g sample. The pH values of the samples ranged from 3.4 to 4.0. In the identification of LAB isolates, all rod-shaped LAB isolates produced DL-lactic acid, were identified as *Lactobacillus* based on morphological, physiological, and biochemical characteristics and they were grouped based on the phenotypic characteristics by cluster analysis. Twenty-three isolates (Group A) were heterofermentative and 7 isolates (Group B) were homofermentative . On the basis of 16S rRNA gene sequence similarity (Table 1), the representative isolates in Group A (FL11-1, FL18-1, FL19-1S and FL21-1) were closely related to *L. fermentum* (99.9-100%) [11]. Group B isolates which contained *meso*-diaminopimelic acid in their cell wall, including isolates FL11-3 and FL22-2 (Group B1), isolate FL17B (Group B2), and isolate FL22-1 (Group B3), were closely related to *L. plantarum* (99.9-100%), *L. pentosus* (100%) and *L. nantensis* (99.1%), respectively [2,12]. Their differential characteristics are shown in Table 2.

In Thailand, *L. plantarum* strains were a common species isolated from sauerkraut, pickled bean sprouts, pickled spring onion, sweetened rice, fermented sausages [2] whilst *L. pentosus* was found in pickled mustard, pickled spring onions, fermented durian, fermented tea leaves, fermented fish and fermented Thai sausages[2]. In addition, *L. plantarum* and *L. casei* strains were isolated from the starter dough of Chinese steamed buns [13].

Table 1 Group, isolate no., similarity (%) and identification of isolates.

Group	Isolate no.	% Similarity	Identification
A	FL18-1, FL11-1, FL19-1S, FL21-1	99.9-100	*L. fermentum*
B1	FL11-3, FL22-2	99.9-100	*L. plantarum*
B2	FL17B	100	*L. pentosus*
B3	FL22-1	99.1	*L. nantensis*

For the identification of 12 yeast isolates, they were identified as *Pichia kudriavzevii* (3 isolates), *P. occidentalis* (3 isolates), *P. manshurica* (2 isolates), *Yarrowia lipolytica* (2 isolates), and each isolate as *Yarrowia* sp. and *Geotrichum* sp., based on the D1/D2 domain sequences of 26S rRNA gene similarity (99-100%) (Table 3). They were grouped based on the phenotypic characteristics by cluster analysis. Six selected yeast isolates could produce ethanol from glucose ranged from 0.132 -7.872 g/l (Table 3). Their differential characteristics are shown in Table 4. The yeasts isolates in this study were different from *Candida tropicalis, Pichia stipitis, C. parapsilosis, Issatchenkia orientalis* and *Saccharomyces cerevisiae* strains that isolated from the starter dough of Chinese steamed buns [13].

CONCLUSION

In this study, 30 lactic acid bacterial isolates included *Lactobacillus fermentum* (23 isolates), *L. plantarum* (3 isolates), *L. pentosus* (2 isolates) and *L. nantensis* (2 isolates) and 12 yeast isolates identified as *Pichia kudriavzevii* (3 isolates), *P. occidentalis* (3 isolates), *P. manshurica* (2 isolates), *Yarrowia lipolytica* (2 isolates), and each isolate as *Yarrowia* sp. and *Geotrichum* sp. are distributed in *khao-khab* collected in Utaradit province. The LAB isolates and yeasts may play the important roles in the fermentation of this product.

Table 2 Differential characteristics of *Lactobacillus* isolates.

Characteristics	Group A (23)	Group B1 (3)	Group B2 (2)	Group B3 (2)
Growth at pH4.0	+	+	+	+
pH 4.5	+ (-2)	+	+	+
pH 8.0	- (+11)	+	+	+
pH 8.5	- (+11)	+	+ (-1)	+
Growth in 4%NaCl	+ (-1)	+	+	+
6% NaCl	+ (-10)	+	+	+
8% NaCl	-	+ (-1)	+	-
Growth at 45 C	+	+	+	+
Arginine hydrolysis	- (+4)	-	w	-
Gas from glucose	+	-	-	-
Acid from:				
D-Amygdalin	-	+	+	+
L-Arabinose	+ (-11)	+	+	-
D-Cellobiose	-	+	+	+
D-Galactose	+ (-4)	+	+	+
Gluconate	- (4w)	+	+	+ (-1)
Glycerol	-	w	-	-
Lactose	+ (-7)	+	+	+
D-Maltose	+ (-1)	+	+	+
D-Mannitol	-	+	+	-
D-Mannose	+ (-11)	+	+	+
D-Melibiose	+	+	+	+
Methyl-glucoside	- (+2)	-	-	-
Raffinose	+ (-1)	+	+	-
D-Ribose	+ (-2)	+	+	+
Salicin	-	+	+	+
D-Sorbitol	-	+	+	-
Sucrose	+ (-1)	+	+	+
D-Xylose	- (+3)	- (+1)	-	-
D-Trehalose	-(+10)	+	+	+

+, positive; w, weak positive; -, negativreaction
Numbers in parentheses indicate the number of isolates showing the reaction.

Table 3 Isolate no., ethanol (g/l), similarity (%) and identification of isolates.

Group	Isolate no.	Ethanol (g/l)	% Similarity	Identification
A1	FY19	ND	100	*Pichia kudriavzevii*
	FY20	7.872	100	*Pichia kudriavzevii*
A2	FY18	3.374	99	*Pichia occidentalis*
	FY21	ND	99	*Pichia occidentalis*
A3	FY11	ND	100	*Pichia manshurica*
	FY15	0.132	98	*Pichia manshurica*
A4	FY14	ND	100	*Pichia kudriavzevii*
B	FY12	0.075	100	*Yarrowia lipolytica*
	FY13	0.055	100	*Yarrowia lipolytica*
	FY16	ND	99	*Yarrowia* sp.
C	FY17	0.275	100	*Geotrichum* sp.
D	FY22	ND	100	*Pichia occidentalis*

ND, not determined.

Table 4 Differential characteristics of yeasts isolates.

Assimilation of	A1 (2)	A2 (2)	A3 (2)	A4 (1)	B (3)	C (1)	D (1)
Cycloheximide	-	-	-	-	+	+	-
Erythritol	-	-	-	-	+	-	-
Gluconate (K)	-	-	-	-	-(+1)	-	-
N-Acetyl-Glucosamine	+	+	-	-	+	-	-
Glucosamine	+	+(-1)	+	+	+	+	-
Glycerol	-(+1)	-	+(-1)	+	+	+	-
Lactic acid	+	-	+(-1)	+	-(+1)	-	+
D-Mannitol	-	-	-	-	+	+	-
D-Ribose	-	-	-	-	+(-1)	-	-
D-Sorbitol	-	-	-	-	-(+1)	+	-
L-Sorbose	-	+	-	-	-(+1)	+	+
D-Xylose	-	-	-	-	-	+	-

+, positive; -, negative reaction. Numbers in parentheses indicate the number of isolates showing the reaction.

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