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Identification and characterization of lactic acid bacteria isolated from fruit tree rhizosphere in MARDI, Malaysia

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ABSTRACT

Aims: Lactic acid bacteria (LAB) have promising applications in the biotechnology industry. As their diversity in soil is largely unexplored, a study was undertaken to collect LAB from the soil, and to characterize the isolates.
Methodology and results: Soil samples from around various fruit trees in MARDI were collected for LAB isolation by accumulation/incubation method. The isolates were examined for their morphological and biochemical characteristics, and their 16S rDNA sequences. Morphological and biochemical analysis showed that all isolates are Gram positive with different characteristic for each isolates. Further identification were performed and soil samples was found to contain diverse genera and species of LAB, including *Lactobacillus* sp. and *Bacillus* sp. Selected isolates were tested for resistance against six antibiotics using agar dilution method. Widespread antibiotic resistance among the strains tested was found towards ampicillin, kanamycin, rifampicin and penicillin.
Conclusion, significance and impact study: LAB have the ability in producing acid and antimicrobial compound which are useful in various industry. Fastidious characteristics of LAB are among the limitations and challenges in industrial applications. Diversity of lactic acid-producing bacteria was encountered in the soil which may be useful and have different characteristics from LAB isolates elsewhere.

Keywords: morphology, soil LAB, antibiotic susceptibility, 16S rRNA, 16S rDNA

INTRODUCTION

Lactic acid bacteria (LAB) have received considerable attention as probiotics over the past few years. LAB are non-sporeforming bacteria that produce lactic acid, and are generally recognized as safe (GRAS) organisms. LAB are “aerotolerant anaerobes" which grow anerobically but, unlike the most anaerobes, they can also thrive in the presence of oxygen. LAB are found in carbohydrate-rich environments such as milk and fermented foods (Fernandez *et al.*, 2003) and they play a role in promoting health as probiotic agents.

Extensive research has been undertaken on potential probiotic LAB strains that are relevant to various aspects of human living, from food processing to health care. In agriculture, LAB is recognized as being among the effective microorganisms beneficial to plant growth and development (Higa *et al.*, 1994). LAB are also well known as biocontrol agents (Lutz *et al.*, 2012). Research by Hamed *et al.* (2012) shows that seed treatment with LAB, or its application as a soil drench could enhance subsequent plant growth. Their results confirm LAB as another group of plant growth-promoting bacteria that provide bioprotection to plants. Kantha *et al.* (2011) find that photosynthetic bacteria and lactic acid bacillus enter into a synergistic relationship that serves as a powerful tool in the production of biofertilizer for use in organic saline paddy fields. Lactic acid bacteria are also known for their ability to preserve vegetation as silage. This group of bacteria promotes fermentation activity that lowers the pH of the soil, thus inhibiting unwanted microorganisms in the crop rhizosphere (Vazquez *et al.*, 2008). Nevertheless, the antibiotic resistance of LAB strains used in food, feed, and probiotic applications are considered dangerous by some agencies as the resistant genes can be transferred to dangerous pathogens (Karapetkov *et al.*, 2011). In order to mitigate this possibility, tests for antibiotic susceptibility should be performed for all novel LAB.

Hitherto, data concerning LAB isolated from soil and their characteristics remain scarce. Wild strains of LAB may be good producers of various growth factors (Fhoula *et al.*, 2013). Thus, this research was undertaken to isolate and characterize new strains of LAB from the soil in Malaysia.

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MATERIALS AND METHODS

Soil samples

Eight soil samples were collected in the rhizospheres of various fruit trees (Table 1) in the vicinity of the Malaysia Agricultural Research and Development Institute (MARDI), Serdang, Selangor. The soil were collected where the ripe fruits fall which provide nutritious value to microorganisms growth. Soil from various fruit trees was collected using sterile spoons, and saved into clean bags.

Table 1: Source of soil samples.

Sampling site (Tree rhizosphere)	Scientific names of tree	Isolate code
Soil from rhizosphere of mangosteen trees	*Garcinia mangostana*	MS
Soil from rhizosphere of rambutan trees	*Nephelium lappaceum*	R1
Soil from rhizosphere of passion fruit trees	*Passiflora edulis*	MA
Soil from rhizosphere of starfruit tree	*Averrhoa carambola*	B
Soil from rhizosphere of limau madu tree	*Citrus suhuiensis hort*	L2
Soil from rhizosphere of pulasan tree	*Nephelium mutabile*	P
Soil from rhizosphere of kaffir lime tree	*Citrus hystrix*	21
Soil from rhizosphere of kaffir lime tree	*Citrus hystrix*	11
Soil from rhizosphere of buluh madu tree	*Gigantochloa albociliata*	31

Isolation of lactic acid bacteria

Microbial enrichment and bacterial isolation were performed on Glucose-Yeast-Peptone (GYP) agar medium and de Man, Rogosa, and Sharpe (MRS) (Oxoid) agar plates. The GYP medium, prepared with some modifications, contained 2% glucose, 1% yeast extract, 1% peptone, 1% sodium acetate, 0.5% (v/v) salt solution, pH 7.0 and with a final agar concentration of 2%. The salt solution contained 4% $MgSO_4 \cdot 7H_2O$, 0.16% $MnSO_4 \cdot 4H_2O$, 0.2% $FeSO_4 \cdot 7H_2O$ and 0.2% NaCl (Chen *et al.*, 2005).

LAB were isolated using accumulation with incubation method. The accumulation method of LAB isolation was carried out by incubating 1 g of soil sample in 5 mL of GYP and MRS broth. The mixtures were incubated under anaerobic conditions at 30 °C for 2 to 3 days (BBL GasPack, $H_2 + CO_2$) (Chen *et al.*, 2005). After incubation, each mixture was diluted and spread onto GYP and MRS agar media with further incubation under the same conditions.

Identification of isolated bacteria

Pure cultures of LAB were characterized using the Gram staining protocol. Further phenotypic identification was performed based on morphology and biochemical tests on the isolated bacteria (Collin and Lyne, 1990). Biochemical testing that were used for bacterial isolates characterization process are oxidase, catalase, citrate utilization, lysine decarboxylase, H_2S production and lactose fermentation.

The polymerase chain reaction (PCR) was then carried out to confirm the identities of the isolates to the level of the genus based on their 16S rRNA. In this procedure, genomic DNA from the isolated LAB was extracted using the Wizard Genomic Purification Kit (Promega). The PCR reactants were as follows: 2.0 µL $MgCl_2$, 0.5 µL dNTP, 5.0 µL GoTaq 5×, 3.0 µL DNA template, 11.37 µL ddH_20, 1.5 µL reverse and forward universal primers, 0.5 µL Taq DNA polymerase (5 unit/µL) and distilled water making up to 50 µL. The polymerase reaction was performed over 30 cycles on the following protocol: initialization step at 95 °C, within 3 min, denaturation step on 94 °C, within 1 min, annealing step on 55 °C, within 1 min, and elongation step on 72 °C, within 10 min. The PCR products were visualized on agarose gels following electrophoresis and the size of the PCR products determined by comparison with a DNA ladder. A commercial kit (Wizard Genomic DNA Purification kit) was used to purify the PCR products. Following sequencing, a BLAST comparison of the 16S rRNAs of the isolates was carried out using sequences from Genebank.

Antibiotic susceptibility test

Testing for susceptibility to antibiotics of the isolated LAB was based on the method described by Huys *et al.* (2002). Five different antibiotics (Oxoid Ltd) were tested: ampicillin (AMP, 10 µg per disc), tetracycline (TE, 30 µg per disc), kanamycin (30 µg per disc), penicillin (2 µg per disc), rifampicin (2 µg per disc). Standardized culture suspensions (0.5 MacFarland) for each isolate were swabbed onto MRS agar plate using a sterile cotton swab. Antibiotic discs were transferred onto the surface of the inoculated plates, which were then incubated for 36 h at 37 °C. Diameters of the inhibition zones that appeared were measured.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

Lactic acid bacteria can commonly found in fermented

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food. However, there is a need to explore wild lactic acid bacteria from different environment. According to Yanagida *et al.* (2007), environmental and wild LAB strains are good competitors for different growth factors and productions of antagonistic compounds. LAB was isolated from the soil in the rhizospheres of various fruit trees (Table 1). It was felt that the necessary nutrients for growth of LAB might be richer in the vicinity of fruit trees because of fruit fall (Chen *et al*., 2005). Twenty strains of bacteria were isolated from eight soil samples under anaerobic conditions using accumulation with incubation method. Twelve bacteria were rod or short-rod bacteria while eight were coccus bacteria. Among those 20 bacterial isolates, only 9 were able to survive under both anaerobic and aerobic conditions on MRS media. The isolates were coded as shown in Table 1.

Further characterization and identification of the isolated bacterial were carried out using the accumulation method only for these 9 strains that were able to survive under both aerobic and anaerobic conditions. Incubation under anaerobic conditions helped in eliminating irrelevant aerobic bacteria from soil samples. LAB are fastidious microorganisms that are unable to grow on minimal media containing a carbon source only (Hebert *et al.*, 2000); supplementation with various amino acids, vitamins, minerals, and fatty acids is required for normal growth and development (Hayek and Ibrahim, 2013). Observations on the isolates in this study suggested that LAB from soil were relatively scarce and that they required appropriate a nutritionally adequate environment to grow well.

Table 2: Characteristic of 9 LAB isolated from soil, based on morphology.

Sample Code	Colony Morphology	Cell Shape	Gram stain
MS	White, convex, smooth	Coccus	Gram positive
R1	White, convex, smooth	Rod	Gram positive
MA	Cream, irregular, flat	Rod	Gram negative
B	Cream, irregular, flat	Rod	Gram positive
L2	White, convex, smooth	Rod	Gram positive
P	White, convex, smooth	Rod	Gram positive
11	White, convex, smooth	Rod	Gram positive
21	White, convex, smooth	Rod	Gram positive
31	White, convex, smooth	Rod	Gram positive

Morphological and biochemical examination of LAB species

The phenotypic characteristics of isolated LAB are shown in Table 2. The morphology of isolated bacteria colonies was observed on MRS media. The bacterial colony pigmentation was white or cream, and the colonies were convex, with smooth elevation and entire margins. The different isolates were of varying cell sizes and morphologies when viewed under the phase contrast microscope. This showed that different soil rhizospheres supported dissimilar LAB species.

Results of the biochemical tests on the isolates are shown in Tables 2 and 3. Gram staining showed that 8 out of 9 isolated bacteria were gram positive. An isolate from soil rhizosphere of the passion fruit tree coded MA tested as Gram negative. All of these isolated bacteria were able to ferment lactose, but they tested negative for H_2S production, lysine decarboxylase and oxidase characterization (Table 3). The test for the enzyme catalase gave positive results in 4 out of 9 isolated bacteria, namely R1 MA, L2 and P.

Identification of lactic acid bacterial by 16SrDNA profiling

The PCR-based method of 16S rDNA profiling allows rapid and precise identification of the isolated bacteria. From a DNA extract of fresh LAB colonies, a PCR product of about 1,500 bp was generated for each isolate. Further PCR reactions were performed on the purified genomic DNA using universal primers. A nucleotide BLAST search of the partial 16S rDNA sequences obtained for the various isolates showed that most of them had varying levels of identity with known bacteria in the database; *Bacillus* species were especially common. Isolates R1, L2, P and 31 were identified as *B. coagulans* (98-99% similarity), whereas isolates MS and MA both showed 99% homology with *Enterobacter* and *Enterococcus* sp. each. The isolates B, 21 and 11 were identified as *L. brevis* (99% similarity), *B. amyloliquefaciens* (98%) and *B. subtilis* (98%) respectively (Table 4).

These results showed that the soil contained a diverse spectrum of species and genera of LAB. The identified species of LAB from soil are differ from species isolated from lakes (Yanagida *et al.*, 2007). Yanagida *et al.* (2007) found the abundance of *L. lactis* isolated from lakes as the main source which are identify from MARDI soil. This shows that different environment contain different strains of LAB presents which need to be explore for their different characterization. Gram positive bacteria dominate soil microbiota (Aislabie and Deslippe, 2013) and most of these bacteria play major roles in mineralizing plant-derived materials such as humus, pesticides, and hydrocarbon in soils (Prescott *et al.*, 2002). Table 4 shows that similar LAB strains could be present in different soil samples. Bayane *et al.* (2010) note an important diversity of spore-forming LAB in soil such as *B. coagulans* that was also encountered in the present study have potential applications in the formulation of probiotics. Another LAB isolated in this study, *Enterobacter,* is among several species of the family *Enterobacteriaceae* that are plant-associated

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Table 3: Biochemical characteristics of LAB isolates.

Sample Code	MS	RI	MA	B	L2	P	21	11	31
Oxidase	-	-	-	-	-	-	+	+	-
catalase	+	+	-	-	+	+	+	+	+
Citrate	+	-	-	-	-	-	-	+	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	-
H2S production	-	-	-	-	-	-	-	-	-
Lactose fermentation	+	+	+	+	+	+	-	-	+

+, Positive results; -, Negative results

Table 4: Identification of soil LAB isolates based on 16SrDNA.

Code	Genus	Identity score (% of similarity)
11	*B. subtilis subsp. Subtilis*	98%
21	*B. amyloliquefaciens subsp. Plantarum*	98%
31	*B. coagulans*	99%
MS	*E. faecium*	99%
RI	*B. coagulans*	99%
MA	*Enterobacter cloacae*	99%
B	*L. brevis*	99%
L2	*B. coagulans*	98%
P	*B. coagulans*	99%

Table 5: Susceptibility of soil LAB to various antibiotics.

Isolates	Inhibition zone (mm)					
	AMP	KM	TC	VA	RIF	PCN
B. coagulans	0	20 ± 0.27	25± 0.5	0	0	0
E. faecium	28± 0.20	19.8± 0.71	13.7±0.35	11 ± 0.29	8.6± 0.58	12± 0.90
B. subtilis	0	17 ± 0.12	19 ± 0.12	0	0	0
E. cloacae	0	15± 0.40	17± 0.17	0	0	0
L. brevis	0	0	0	0	0	0

All the isolates were tested against ampicillin (AMP), kanamycin (KM), tetracycline(TC), vancomycin (VA), rifampicin (RIF) and penicillin (PCN).

bacteria found in the soil, such as in the rhizosphere of rice plants (Hayat *et al*., 2010).

Antibiotic susceptibility test

The disc diffusion method was used to assess the susceptibility of isolated soil LAB to six antibiotics, *viz.* ampicillin, kanamycin, tetracycline, vancomycin, rifampicin, and penicillin. The bacterial strains were characterized as either resistant or sensitive to a specific antibiotic according to the specifications of the Clinical and Standard Laboratory Institute, (2009). Table 5 shows the inhibition zone of LAB isolates with respect to the antibiotic tested. *Bacillus coagulans*, *B. subtilis* and *E. cloacae* were susceptible to kanamycin and tetracycline. However, a number of LAB showed resistance toward the antibiotics used. *Lactobacillus brevis* was resistant to all the antibiotics tested. This may reflect the presence of resistance genes carried by *L. brevis* isolated. These results were in agreement with the finding of Korhonen *et al*. (2008) who observed resistance to tetracycline being more common among the *Lactobacillus* species. The resistance of *Lactobacillus* species against tetracycline is due to the extensive variability of tetracycline mechanisms that give diverse levels of susceptibility to the antibiotic (Roberts, 2005). *Enterococcus faecium* was the isolate most susceptible to antibiotics, being sensitive to all six antibiotics tested (Table 5). The increasing

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presence of antibiotics in the natural ecosystem due to their over-use in human activities such as farming is one of the causes of the emerging resistance of microorganisms to antibiotics (Martinez, 2008). According to Martinez (2009), such antibiotic resistance among microorganisms in the environment could influence the natural microbial population structure and their physiological characteristics.

CONCLUSION

A variety of LAB strains was discovered from the soil in the vicinity of MARDI, reflecting their diversity. The accumulation method with incubation aided in the culture of LAB, making it possible for further identification of the isolates. The soil LAB isolated required enrichment of the medium beyond a carbon source for their growth. Antibiotic resistance in the isolates was observed, and these resistant LAB might differ in characteristics from resistant strains isolated elsewhere.

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