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Isolation and Identification of Lactic Acid Bacteria with Antifungal Activity Against Anthracnose Disease

[1]Asma Saleh W. El-Mabrok, [1]Zaiton Hassan, [2]Ahmed Mahir Mokhtar,
[3]Khaled M.A. Hussain and [1]Mohamed Mustafa Aween
[1]Faculty of Science and Technology, Univerisiti Sains Islam Malaysia (USIM),
Bandar Baru, 71800 Nilai, Negeri Sembilan, Malaysia
[2]Serotech Sdn Bhd. Bagunan Wawasan, Universiti Kebangsaan Malaysia,
43650 UKM, Bangi, Selangor, Malaysia
[3]Faculty of Veterinary of Medicine , University Putra Malaysia (UPM),
43400 UPM, Serdang, Selangor, Malaysia

Abstract: The objective of this study was isolation, identification of lactic acid bacteria from different source and testing their antifungal activity against phytopathogens fungi *C. capsici* and *C. gloeosporioides*. The isolates were identified by their phenotypic and genotypic characteristics and their antifungal activity was performed by the overlay method. Seven LAB isolates were showed good antifungal activity against phytopathogens fungi and were grew at different temperatures, pH and NaCl. However, C5 and G7 have the ability to inhibit the growth of both target phytopathogens fungi compared to other isolates. C5 and G7 identified by API 50CHL kit as *Lactobacillus plantarum* and the identified by used 16S rDNA was *Lactobacillus plantarum* C5 and *L. pentosus* G7. This is the first study that observed LAB isolates *L. plantarum* C5 and *L. pentosus* G7 inhibited both of *C. capsici* and *C. gloesporeioides in vitro*. This study demonstrates that the LAB *L. plantarum* C5 and *L. pentosus* G7 have potential to be used as biological control of this phytopathogen by inhibiting the mycelia growth.

Key words: *C. capsici*, *C. gloesporeioides*, API 50CHL kit, 16S rDNA, lactic acid bacteria, Malaysia

INTRODUCTION

Lactic Acid Bacteria (LAB) have a long history of safe use in fermented foods. Today, several members of the lactic acid bacteria are known to play a role in inhibiting fungal and bacterial growth (Fayol-Messaoudi *et al*., 2005). Biological control by antagonistic microorganisms is widely recognized as a promising method for control of post-harvest plant diseases (Janisiewicz and Koresten, 2002; Andrews and Harris, 2000). Different species of Colletotrichum, namely; *C. capsici* (Sydow) Butler and Bisby and *C. gloeosporioides* (Penz.). Penz., and Sacc., are known to cause anthracnose in chilli. Economic losses caused by the disease are mainly attributed to lower fruit quality and marketability. Although, infected fruits are not toxic to humans or animals, severely affected fruits showing blemishes are generally considered unfit for human consumption. This is because the anthracnose causes an unpleasant colour and taste in chilli products (Nayaka *et al*., 2009).

Selected strains of LAB isolated from fresh fruits and vegetables showed inhibitory activities against phytopathogenic and spoilage bacteria and fungi and can be used as biological control agents to protect seeds and seedlings from various pathogens (Trias *et al*., 2008).

The objective of this study was to evaluate the capabilities of LAB isolated from different sources for their potential as biocontrol agent against fungi causing anthracnose, namely; *C. capsici* and *C. gloeosporioides* and identified using API 50CHL and 16S rDNA.

MATERIALS AND METHODS

Isolation and characterization of LAB isolates: The vegetables (1 g) were cut into small pieces and suspended into 9 mL peptone water (0.1% w/v, Oxoid) in stomacher bags and the bags were agitated in stomacher (400 Circulator, seward). Then 1 mL was added to 10 mL of MRS broth (Oxoid) and incubated at 30°C for 24-48 h. Appropriate serial dilution with peptone water (0.1% w/v) and 0.1 mL was spread plated on modified MRS agar (De Man *et al*., 1960). The pure colonies were again tested

Corresponding Author: Asthma Saleh W. El-Mabrok, Faculty of Science and Technology,
Univerisiti Sains Islam Malaysia (USIM), Bandar Baru, 71800 Nilai, Negeri Sembilan, Malaysia

for catalase activity and gram stained (Mallesha *et al*., 2010). All plates were incubated under anaerobic condition in anaerobic jar at 37°C for 48 h and the growth as determined by turbidity of LAB under different temperatures (10 and 45°C), pH (4.4 and 9.6) and different NaCl concentrations (6.5 and 18%) at 30°C for 48 h were carried out (Sathe *et al*., 2007).

Fungal preparation: *C. capcisi* and *C. gloeosporioides* were obtained from Faculty of Agriculture, University Putra Malaysia. Fungi were grown on Potato Dextrose Agar (PDA, Oxoid) incubated at room temperature for 7 days and the spores concentration was determined using a haemocytometer and adjusted to 10^4 spores/cells per mL (Strom *et al*., 2002).

Screening of LAB isolates for antifungal activity by overlay method: The isolates of LAB were screen for antifungal activity by overlay technique as described by Strom *et al*. (2002) on MRS agar plates using *C. capcisi* and *C. gloeosporioides* as indicator with slight modification. Briefly, the LAB was grown in two 2 cm streaks on MRS agar plates and incubated under microaerobic conditions at 30°C for 48 h. These plates were overlaid with semisolid malt extract agar (0.7%) seeded with 10^4 spores mL^{-1} of *C. capcisi* and *C. gloeosporioides* and incubated aerobically at 30°C for 24-72 h.

Identification of LAB by API 50CHL kit assay: LAB isolates that have been antifungal activity in well method were subjected to API 50CHL kit (API system, Bio-Merieux, l'Etoile, France) assay. Purified LAB were cultivated in 5 mL MRS broth incubated at 30°C over night, after which the culture was washed and resuspended into API®50CHL medium (Bio-Merieux®SA 69280, France). The turbidity of the suspension was determined by the McFarland Method according to the instructions provided by the manufacturer. The results were read after 24 h and verified after 48 h. Colour reactions were score against a chart provided by the manufacture (Tamminen *et al*., 2004). The results were analyzed with API WEB (Bio-Merieux).

Genotypic identification of lactic acid bacteria by 16S rDNA: Total genomic DNA was extracted from an overnight culture in 20 mL MRS broth incubated at 30°C using Master Pure™ gram positive DNA purification kit (USA). About 1 mL of overnight culture was centrifuged 11500 rpm for 10 min at 25°C (Eppendorf centrifuge 5804 R) and the pallet was collected and TE buffer was added and incubated at 37°C overnight. The sample was incubated at 65-70°C for 15 min and vortexed every 5 min followed by placing in ice for 5 min. The pellets were rinsed with 200 µL ethanol 70% and centrifuged at 5000 rpm for 2 min at room temperature and the DNA was resuspended with 35 µL of dionised water and kept at -20°C for further study. The PCR settings were set at 95°C for 2 min, 92°C for 45 sec, 54°C for 1 min and 72°C for 1 min, 35 cycles using two primers 16S.S forward (5′-AGAGTTTGATCCTGGCTC-3′) and 16S. Rreverse (5′-CGGGAACGTATTCACCG-3′). The partial 16S rDNA sequences, the sequences were used for searching in public databases (GenBank) (Jarvis and Hoffman, 2004).

RESULTS

Isolation and characterization of LAB isolates: LAB isolated from different sources of fruits and vegetables, milk, beef, fermented fish and soil, only 7 isolates showed good and antifungal activity against both *C. capsici* and *C. gloeosporioides*. All isolates were gram-positive, catalase negative and were grown at different temperatures, pH and NaCl, except G1 could not grow at 6.5 and 18% NaCl (Table 1).

Screening of antifungal activity of LAB by overlay method: Diameter of growth inhibition zones of the target fungi *C. gloeosporioides* and *C. capsici* by the 7 lactic acid bacteria isolates is shown in Table 2. Good inhibitory activity (>20 mm) was shown by C5 and G7 while other 3 isolates (D10, D11, D1) showed good inhibitory activity (10-18 mm), 2 isolates (G1 and B3) showed lowest activity (<6 mm) against both fungi *C. gloeosporioides* and *C. capsici*. However, C5 and G7 have the ability to inhibit the growth of both target phytopathogens fungi compared to other isolates.

Table 1: Characteristics of LAB with antifungal activity isolated from different sources

					Temperature (°C)		pH		NaCl (%)	
Strains	Source	Shape	Gram	Catalase	10	45	4.4	9.6	6.5	18
D1	Peach	Rod	+	-	+	+	+	+	+	+
C5	Durian	Rod	+	-	+	+	+	+	+	+
G1	Guava	Rod	+	-	+	+	+	+	-	-
B3	Dragon fruit	Rod	+	-	+	+	+	+	+	+
D10	Star fruit	Rod	+	-	+	+	+	+	+	+
D11	Melon	Rod	+	-	+	+	+	+	+	+
G7	Ginger	Rod	+	-	+	+	+	+	+	+

Table 2: Antifungal activity of LAB isolates against *C. gloeosporioides* and *C. capsici* after 48h incubation at 30°C determined by dual agar overlay method

LAB isolates	Inhibitory activity against *C. gloeosporioides*	Inhibitory activity against *C. capsic*
C5	27.00±1.00^{a}	27.00±1.00^{a}
G7	26.00±1.00^{a}	25.66±0.57^{a}
D10	15.66±0.57^{bc}	16.33±0.57^{b}
D11	16.66±0.57^{b}	15.66±0.57^{bc}
D1	15.66±0.57^{bc}	13.33±1.52^{cde}
G1	5.66±0.57^{efg}	3.33±0.57^{h}
B3	6.66±0.57^{ef}	4.33±0.57^{gh}

$^{a-h}$Inhibitory activity of selected lactic acid bacteria isolates against *C. gloeosporioides* and *C. capsici* after 48 h incubation at 30°C by dual agar overlay method; the results are mean values of triplicate determinations ±SD antifungal activity: (-) = No growth; (+) = Inhibition zone of <6 mm; (++) = Inhibition zone of 6 -10 mm; (+++) = Inhibition zone of 10-18 mm; (++++) = Inhibition zone of >20 mm

Table 3: Similarity index of LAB isolated from Malaysian fermented vegetables and fruits as determined by API 50CHL and 16S rDNA

LAB	Source	API similarity index	Identification	16S rDNA similarity (%)	Identification	Characteristics
C5	Durian	95.9	*L. plantarum*	97	*L. plantarum*	NR042394.1
G7	Ginger	95.9	*L. plantarum*	95	*L. pentosus*	HQ384301.1
D10	Star fruit	99.9	*L. plantarum*	96	*L. plantarum*	AB603684.1
D11	Melon	99.9	*L. plantarum*	97	*L. plantarum*	AB603680.1
G1	Guava	99.9	*L. plantarum*	95	*L. plantarum*	AB603684.1
D1	Peach	99.2	*L. parasasei*	97	*L. casei*	HQ534100.1
B3	Dragon fruit	95.9	*L. plantarum*	92	*L. pentosus*	HQ384301.1

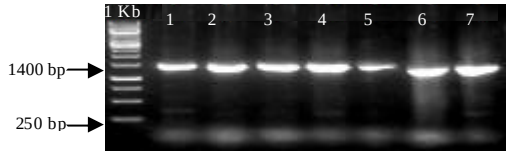

Fig. 1: Identification of LAB by API 50CHL assay and 16S rDNA: 1 kb = DNA ladder; Lane 1 = D1; Lane 2 = C5; Lane 3 = G7; Lane 4 = G1; Lane 5 = B3; Lane 6 = D10; Lane 7 = D11; Condition: 1% agarose gel; volume of DNA ladder/sample loaded per lane: 1 uL each, 1 kb DNA Ladder (bp): 250, 500, 750, 1000, 1400, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000, 1kb DNA Ladder (ng/0.5ug): 25, 25, 25, 60, 25, 25, 25, 70, 30, 30, 30, 70, 30, 30

Identification of LAB by API 50CHL assay and 16S rDNA: Identification of the seven LAB isolates that showed antifungal activity against the target phytopathogens fungi is presented in Table 3. The API 50CHL kit identified D1was as *Lactobacillus parasasei* and the other six as *Lactobacillus plantarum.* However, 16S rDNA sequence identified four isolates (C5, D10, D11 and G1) as *L. plantarum* while G7 and B3 as *L. pentosus* and D1 as *L. casei* (Fig. 1).

DISCUSSION

Lactic acid bacteria can be isolated from different food sources and reported to have antimicrobial activity. While, most report was on antibacterial activity of LAB, reports on the antifungal activity of LAB are few. This study observed that 9.25% of LAB isolated from fruits showed antifungal activity against *C. capsici* and *C. glosporeoides,* an important phytopathogen that normally infect chilli, apple, avocado, guava, papaya, mango and passion fruit, sour cherry, carrot and yam hosts among others (Wattad *et al*., 1994; Abang *et al*., 2002; Svetlana *et al*., 2010). Sathe *et al.* (2007) observed 10% of the LAB isolated from vegetables possesses antifungal property against *Aspergillus flavus*, *Fusarium graminearum*, *Rhizopus stolonifer* and *Botrytis cinera*. Recently, Hamed *et al.* (2011) reported that LAB isolated from yogurt and milk and *L. plantarum* NRRL B-4524 showed inhibitory activity against *Fusarium oxysporum* and provide protective effect to tomato plants.

The LAB with fungal activity could grow between 10 and 45°C, pH 4.4 and 9.6 and NaCl 6.5 and 18%. Except D3 could not grow at 6.5 and 18% NaCl.

16S rDNA identified the isolates C5, D10, D11 and G1 as *L. plantarum* while G7 and B3 as *L. pentosus* and D1 as *L. casei.* The results agreed with other studies that the kit is not accurate enough to identify to the species level in some cases (Yin and Zheng, 2005). The antifungal activity of *L. plantarum* strains has also been reported by other investigators (Gourama and Bullerman 1995; Lavermicocca *et al*., 2000; Karunaratne *et al*., 1990). *L. plantarum* isolated strains E76 and E98 isolated from beer and pickled cabbage, respectively showed antifungal activity on *F. avenaceum, F. culmorum, F. graminearum* and *F. oxysporum* that infect malting barley (Laitila *et al*., 2002).

Gerez *et al.* (2009) observed that the used of LAB isolates were able to inhibit the conidial germination and mycelia growth. The conidia germination is the growth stage that is most sensitive to inhibition. In fact, the precise mechanism of antimicrobials can often not be defined because of a complex interaction between the

different compounds produced during cell growth and the frequently synergistic effects among them (Legan, 1993). In this study both the isolates *L. plantarum* C5 and *L. pentosus* G7 successfully inhibited the mycelia growth and conidia germination of *C. capsici* and *C. glosporeoides* as demonstrated by agar overlay method that were used to assess the inhibitory of selected LAB. Reports indicated that *Bacillus subtilis* and *Candida oleophila* inhibited Colletotrichum ssp. (Wharton and Diegue, 2004). Fungi *Trichoderma longibrachiatum* was reported to significantly inhibite the growth of *C. gloeosporioides* and the suggested to be used as biological control (Sobowale *et al*., 2010). *Trichoderma* species are reported able to effectively control *C. capsici* infection in chilli (Sharma *et al*., 2005; Maymon *et al*., 2004). To the best of knowledge, this is the first study that *L. pentosus* G7 isolated from ginger inhibited both *C. capsici* and *C. glosporeoides*.

CONCLUSION

LAB isolated from fruits inhibited fungi *C. capsici* and *C. gloesporeioides* that often caused anthracnose disease in chilli. This is the first report that observed LAB isolates *L. plantarum* C5 and *L. pentosus* G7 inhibited both of *C. capsici* and *C. gloesporeioides in vitro*. This study demonstrates that the LAB *L. plantarum* C5 and *L. pentosus* G7 have potential to be used as biological control of this phytopathogen by inhibiting the mycelia growth and conidia germination.

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