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

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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. gloeosporioides 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. gloeosporioides, 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 (Pers.) 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
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 (Satho et al., 2007).

**Fungal preparation:** C. capisi 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 10^6 spores/cells per mL (Strom et al., 2002).

**Screening of LAB isolates for antifungal activity by overlay method:** The isolates of LAB were screened for antifungal activity by overlay technique as described by Strom et al. (2002) on MRS agar plates using C. capisi 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 10^6 spores mL^-1 of C. capisi 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 overnight, after which the culture was washed and resuspended into API50CHL 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 scored against a chart provided by the manufacturer (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 MasterPure™ 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 pellet 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'-AGGATTGGATCGTTGGCTC-3') and 16S. Reverse (5'-CGGAACGTATTCACCGG-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, bean, fermented fish and soil, only 7 isolates showed good and antifungal activity against both C. capisi 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**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Source</th>
<th>Shape</th>
<th>Gram</th>
<th>Catalase</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>NaCl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>45</td>
<td>4.4</td>
</tr>
<tr>
<td>D1</td>
<td>Peach</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C5</td>
<td>Durian</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G1</td>
<td>Guava</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B3</td>
<td>Dragon fruit</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D10</td>
<td>Star fruit</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D11</td>
<td>Melon</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G7</td>
<td>Ginger</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 2: Antifungal activity of LAB isolates against C. gloeosporioides and C. capsici after 48 h incubation at 30°C determined by dual agar overlay method

<table>
<thead>
<tr>
<th>LAB isolate</th>
<th>Inhibitory activity against C. gloeosporioides</th>
<th>Inhibitory activity against C. capsici</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5</td>
<td>27.00±1.00*</td>
<td>27.00±1.00*</td>
</tr>
<tr>
<td>G7</td>
<td>26.00±1.00*</td>
<td>25.66±0.57*</td>
</tr>
<tr>
<td>D10</td>
<td>15.66±0.57*</td>
<td>16.33±0.57*</td>
</tr>
<tr>
<td>D11</td>
<td>16.66±0.57*</td>
<td>15.66±0.57*</td>
</tr>
<tr>
<td>D1</td>
<td>15.66±0.57*</td>
<td>13.33±1.52*</td>
</tr>
<tr>
<td>G1</td>
<td>5.66±0.57*</td>
<td>3.33±0.57*</td>
</tr>
<tr>
<td>B3</td>
<td>6.66±0.57*</td>
<td>4.33±0.57*</td>
</tr>
</tbody>
</table>

*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 mm

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

<table>
<thead>
<tr>
<th>LAB isolate</th>
<th>API similarity index</th>
<th>Identification</th>
<th>16S rDNA similarity (%)</th>
<th>Identification</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5</td>
<td>95.9</td>
<td>L. plantarum</td>
<td>97</td>
<td>L. plantarum</td>
<td>NR042394.1</td>
</tr>
<tr>
<td>G7</td>
<td>95.9</td>
<td>L. plantarum</td>
<td>95</td>
<td>L. plantarum</td>
<td>HQ884301.1</td>
</tr>
<tr>
<td>D10</td>
<td>99.9</td>
<td>L. plantarum</td>
<td>96</td>
<td>L. plantarum</td>
<td>AB603684.1</td>
</tr>
<tr>
<td>D11</td>
<td>99.9</td>
<td>L. plantarum</td>
<td>97</td>
<td>L. plantarum</td>
<td>AB603680.1</td>
</tr>
<tr>
<td>G1</td>
<td>99.9</td>
<td>L. plantarum</td>
<td>95</td>
<td>L. plantarum</td>
<td>AB603684.1</td>
</tr>
<tr>
<td>B3</td>
<td>99.2</td>
<td>L. parasaisi</td>
<td>97</td>
<td>L. casei</td>
<td>HQ344100.1</td>
</tr>
<tr>
<td>B3</td>
<td>95.9</td>
<td>L. plantarum</td>
<td>92</td>
<td>L. plantarum</td>
<td>HQ884301.1</td>
</tr>
</tbody>
</table>

1400 bp 1 2 3 4 5 6 7
250 bp

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, 1 kb DNA Ladder (ng/0.5ug): 25, 25, 25, 60, 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 D1 was as Lactobacillus parasaisi 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. plantosus 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. gloeosporioides, an important phytopathogen that normally infect chili, apple, avocado, guava, papaya, mango and passion fruit, sour cherry, carrot and yam hosts among others (Wattad et al., 1994; Abung 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. plantosus 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; Lavenirnoca et al., 2000; Karunanithi et al., 1990). L. plantarum isolated strains E76 and E95 isolated from beer and pickled cabbage, respectively showed antifungal activity on F. slopeaceum, F. culmorum, F. graminearum and F. oxysporum that infect malted 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. gloeosporioides 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 Dieguez, 2004). Fungi Trichoderma longibrachiatum was reported to significantly inhibit 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. gloeosporioides.

**CONCLUSION**

LAB isolated from fruits inhibited fungi C. capsici and C. gloeosporioides 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. gloeosporioides 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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