Diversity and bioprospecting potential of rhizo and endophytic bacteria from two mangrove plants in Saudi Arabia

Fehmida Bibi¹, Ikram Ullah², Sana Akhtar¹, Muhammad Yasir¹, Eman Ahmed Kensarah¹, Ahmed Abdullah Khalaf Al-Ghamdi³, Esam I. Azhar¹,³

¹Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.
²Sulaiman Bin Abdullah Aba Al-Khail-Centre for Interdisciplinary Research in Basic Sciences (SA-CIRBS), International Islamic University, Islamabad, Pakistan.
³Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia.

Received 3 April, 2017; Accepted 26 April, 2017

Mangrove plants are located on coastal area of sea and harbor diverse communities of microorganisms. The aim of our present study was to isolate bacteria from two different mangroves collected from the coastal area of Thuwal, Saudi Arabia and to further screen them for their antimicrobial activities. We have isolated 317 different rhizo and endophytic bacteria from mangroves using soil, roots and leaf tissues. Bacteria were screened for their antifungal activities against oomycetes pathogens, Phytophthora capsici, Pythium ultimum. Only 25 bacterial strains found to be active against oomycetes fungal pathogens. These bacteria were tested further against other fungal pathogens like Magnaporthe grisea, Altenaria malli, and Fusarium oxysporum. Antagonistic bacteria were further screened for antibacterial activities against human pathogenic bacteria. Only few isolates exhibited antagonistic activity against these pathogenic bacteria. Hydrolytic enzymes production (cellulase, protease, lipase, and amylase) was also assessed. Most of the active isolates exhibited amylase and protease activities. Identification based on 16S rRNA gene revealed 92.8 to 99.9% sequence, similar to type strains of related species. Antagonistic bacteria belong to 5 different classes that is Gammaproteobacteria (γ-Proteobacteria), Alphaproteobacteria (α-Proteobacteria), Firmicutes, Bacteroidetes and Actinobacteria.

Our results provide evidence that, mangroves plants harbor potentially useful bacteria producing active metabolites and enzymes.

Key words: Mangrove plants, antagonistic bacteria, enzymatic activities, 16S rRNA gene sequence, phylogenetic analysis.

INTRODUCTION

Mangroves, the coastal ecosystems, are found in transitional zones between rivers, sea and land (Schaeffer-Novelli et al., 2000; Kathiresan and Bingham, 2001; Walters et al., 2008) in tropical and subtropical regions all around the world. Their distribution lies in 123 countries covering about 152,000 km² (Spalding et al., 2010). Mangrove forests also have diverse microbial communities, which play critical role in maintenance and functioning of these complex and sensitive ecosystems (Sahoo and Dhal, 2009).
Mangroves sedimentation is the actual base for mangrove forests and organisms inhibiting them. In turn, these microorganisms release nutrients from the sediments and provides base for an enormous food web (Holguin et al., 2001; Spalding et al., 2010). Mangrove plants can tolerate a wide range of environmental factors. This tolerance is facilitated by rhizospheric and endophytic microorganisms, which play vital role in biogeochemical cycles and nutrient transformations (Kathiresan and Bingham, 2001). Bacteria are primary decomposers of organic matter (Saxena et al., 1988) and key players in nitrogen fixation (Abraham et al., 2004; Miransari, 2011). Mangrove sediments and organisms controlling these ecosystems are good targets to study (Kathiresan and Bingham, 2001). High salinity, organic matter, low aeration in mangroves provides conditions, favorable for the growth of diverse microbes of biotechnological importance (Sivaramakrishnan et al., 2006; Dias et al., 2009). Organisms present in mangrove ecosystems remains largely unexplored therefore, there is an excellent source for finding new and novel bioactive secondary metabolites with distinct functions such as enzymes, antibiotics and antitumor compounds (Das et al., 2014). Both rhizo and endophytic bacteria performing diverse functions have been isolated previously from mangrove ecosystem (Hong et al., 2015).

Endophytes are microorganisms harboring internal tissues of plants and are important source of secondary metabolites for development of novel drugs against different diseases of human (Strobel et al., 2004). From biotechnological perspective, bacteria from mangrove plants are an important source of functional metabolites including enzymes and antibiotics (Dias et al., 2009; Thatoi et al., 2013). Several previous studies highlighted importance of beneficial bacteria isolated from mangrove habitats (Vazquez et al., 2000; Soares Junior et al., 2013; Zainal Abidin et al., 2016).

Many rhizo and endophytic bacteria from mangrove plants is a potential producers of important enzymes like amylase, esterase, cellulose and proteases (Dias et al., 2009; Castro et al., 2014). By considering mangroves as an important source of bacteria, these bacteria can produce both enzymes and antimicrobial compounds which are of great interest. Therefore, present study was designed to isolate and characterize rhizo and endophytic bacteria from two mangroves, Haplopeplis perfoliata and Cyperus conglomerates. Their antifungal and enzymatic characteristics have been examined and 16 SrRNA analysis placed them in different groups of bacteria.

**Materials and Methods**

**Sample collection and isolation of bacteria**

Plant samples were collected from the carbonated shore of Red sea in Thuwal, (22°15’54” North, 39°6’44” East) located in Jeddah, Saudi Arabia. Both plant samples that are H. perfoliata and C. conglomerates were put in sterile bag after collection and transferred to laboratory for bacterial isolation.

Soil, roots and leaves samples of plants were used for isolation of bacteria. For bacterial isolation from adhering soil, dipped roots in filtered autoclaved sea water (FAS) to remove adhering soil and serial dilutions were made (10⁻³, 10⁻⁴ and 10⁻⁵) in a filtered autoclaved sea water (FAS), to spread on different media used for culturing of bacteria for maximum isolation.

Half strength R2A (½ R2A) [0.25 g yeast extract, 0.25 g proteose peptone No. 3 (Difco), 0.25 g casamino acid, 0.25 g dextrose, 0.25 g soluble starch, 0.15 g sodium pyruvate, 0.15 g K₂HPO₄, 0.03 g MgSO₄], half Tryptic soy agar (½ TSA) [Pancreatic digest of casein, 7.5 g Papac Digest of soybean, 2.5 g sodium chloride, 2.5 g agar, 15.0 g], marine agar (MA) [peptone, 5.0 g yeast extract, 1.0 g ferric citrate, 0.1 g sodium chloride, 19.45 g magnesium chloride, 8.8 g sodium sulfate, 3.24 g calcium chloride, 1.8 g potassium chloride, 0.55 g sodium bicarbonate, 0.16 g potassium bromide, 0.08 g strontium chloride, 34.0 mg boric acid, 22.0 mg sodium silicate, 4.0 mg sodium fluoride, 2.4 mg ammonium nitrate, 1.6 mg disodium phosphate, 8.0 mg agar, 15.0 g] and half nutrient agar (½ NA) [bacto extract, 1.5 g peptone, 2.5 g agar, 15.0 g] (Difco Laboratories, Detroit, MI) for bacterial culturing.

**Surface sterilization and isolation of endophytic bacteria**

Roots and leaves tissues were also used for isolation of bacteria. To isolate endophytic bacteria from plant, roots and leaves samples were washed several times with tap water and was further sterilized by washing with disinfectants as described previously (Bibi et al., 2012).

To check sterilization, washed roots and leaf segments were placed on ½R2A agar to check the growth of bacteria, from these plant parts after incubation at 28°C for 5 days. After confirming sterilization of root and leaf segments, small pieces of sterilized root and leaf segments were ground in FAS using sterile mortar and pestle. Aliquots were further serially diluted (10⁻³, 10⁻⁴ and 10⁻⁵) and plated in duplicate on different media mentioned above.

To inhibit fungal contamination, 50 μg/ml cycloheximide was mixed to the medium before pouring. The plates were incubated at 25°C for 2 weeks for bacterial growth. Individual colonies were streak to check purity of the strains and all bacterial isolates were further subculture and stored in, 15% (v/v) glycerol stock of strains at -70°C.

**Screening for antifungal activity**

Bacteria isolated from soil, roots and leaves of the mangroves were used to check their antifungal potential. Five different test fungal pathogens Phytophthora capsici, Pythium ultimum and Magnaporthe grisea were obtained in our laboratory while Altenaria maili (KCTC...
Antagonistic activity against fungal pathogens was determined by using cross streak method. All isolates were streaked on PDA media supplemented with ½ R2A in sea water. Each 6 mm mycelial disc of 4-day-old test fungal pathogens was placed in center of plate perpendicular to streak of isolates at 4 cm distance from edges of plate and incubated at 28°C for 4 to 6 days. All strains were checked twice for antagonistic activity. The antagonistic activity was then evaluated by measuring the inhibition zone of fungal mycelia around bacterial colony.

Screening of bacteria for antibacterial activity

Using overlay assay, all antagonistic bacteria have been checked for their antibacterial activity. Firstly, bacterial isolates were grown on ½ R2A in sea water at 28°C for 48 h. Pathogenic bacteria were grown in culture media for 24 h, mixed with 0.1% soft agar and overlaid on strains to be screened.

All test pathogenic bacterial strains were diluted to final concentration $A_{600}=0.1$. After applying overlay of soft agar, plates were incubated at 28°C for 36 h and the zone of inhibition was determined. The test strains of bacteria (Escherichia coli ATCC 8739, Enterococcus faecalis ATCC 29212, Enterococcus faecium ATCC 27270, Pseudomonas aeruginosa ATCC 27853 and MRSA ATCC 43300) were grown in LB broth at 37°C.

Evaluation of hydrolitic enzyme activity

Amylase production was checked on starch media. Amylase producing bacteria showed starch hydrolysis as clear zone on starch ½ R2A agar plates (Kumar et al., 2012).

To check cellulase activity, CMC agar (carboxy methyl cellulose agar) media was used. Bacteria were streaked on plates and incubated at 28°C for 2 days. After these, the plates were flooded with solution 0.1% Congo red and put on orbital shaker for 15 min and washed with 1 M NaCl (Hendrick et al., 1995). Positive activity was seen as halo zone around bacterial colonies on CMC agar. Protease activity was checked using skim milk ½ R2A agar plates. Bacteria producing protease made clear zone on skim milk agar plates.

For lipolytic activity, tributyrin ½ R2A agar media was used. After 48 h of incubation at 28°C clear zone was detected around bacteria after hydrolysis of tributyrin.

Bacterial DNA extraction and 16S rRNA gene analysis

Bacteria isolated from two mangrove plants were used for genomic DNA extraction using DNA extraction kit (Thermo Scientific, Waltham, USA). To identify bacterial strain 16S rRNA gene, full gene sequencing was performed. Using bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GTTACCTTGTAGACTT-3'), the 16S rRNA gene fragment was amplified. Amplifications were performed under the following conditions: one cycle of 95°C for 5 min followed by 30 cycles of 94°C for 1 min, an annealing of 58°C for 50s and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min.

Using PCR purification kit (Thermo Scientific, Waltham, USA) PCR products were purified and were sequenced commercially by Macrogen (Seoul, Korea). Sequences obtained after 16S rRNA gene similarity were blast using EzTaxon server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012), for identification of bacteria. To determine the phylogenetic placement of antagonistic bacteria and related type strains, the 16S rRNA gene sequences of related type strains sequences were obtained from National Centre for Biotechnology Information (NCBI).

Phylogenetic analysis

For phylogenetic analysis using CLUSTALX (Thompson et al., 1997), multiple alignments of the sequences were performed and BioEdit software (Hall, 1999) was used for editing of gaps.

The neighbour-joining method in a MEGA6 Program with bootstrap values based on 1000 repetitions was used for construction of phylogenetic tree based on 16S rRNA gene sequences (Tamura et al., 2013).

Nucleotide sequence numbers

All nucleotide sequences of antagonistic strains have been deposited in the GenBank database under accession numbers KY234238- KY234262.

RESULTS

Isolation of rhizo and endophytic enzyme producing bacteria

A total of 317 rhizo and endophytic antagonistic bacteria were isolated from two mangrove plants C. conglomerates and H. perfoliata. All bacterial isolates were isolated from soil, roots and leaves tissues of plants. All bacterial strains isolated from different parts of the plants mentioned above were cultured on four different media ½ TSA and ½ R2A, MA and NA.

Bacterial number was high on ½ TSA and MA as compared to ½ R2A and ½ NA. But more antagonistic bacteria were recovered from ½ R2A culture media. As different parts of plants were used, more number of antagonistic rhizobacteria was isolated from C. conglomerates while endophytes were more in number from H. perfoliata (Table 1).

Screening for antimicrobial potential

Further these bacteria were used to check their antifungal potential against two different pathogenic fungi, Pythium ultimum and Phytophthora capsici. From these total 317 bacteria, when screened against these two fungi, 25 (7.8%) isolates were active against Py. ultimum and 21 (6.6%) were active against P. capsici.

These rhizo and endophytic antagonistic bacteria were checked for their antifungal potential against three different fungi that is Magnaporthe grisea, A. malli, and Fusarium oxysporum. From these 317 bacteria, when screened against these two fungi, 25 (7.8%) isolates were active against Py. ultimum and 21 (6.6%) were active against P. capsici. These rhizo and endophytic antagonistic bacteria were checked for their antifungal potential against three different fungi that is Magnaporthe grisea, A. malli, and Fusarium oxysporum. From these 317 bacteria, when screened against these two fungi, 25 (7.8%) isolates were active against Py. ultimum and 21 (6.6%) were active against P. capsici.
Table 1. Antimicrobial activity of bacteria isolated from two mangroves plants against different pathogenic fungi and bacteria.

<table>
<thead>
<tr>
<th>Lab no.</th>
<th>Accession no.</th>
<th>Type strain</th>
<th>% identity</th>
<th>Antifungal activity against</th>
<th>Antibacterial activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P. ultimum</td>
<td>P. capsici</td>
</tr>
<tr>
<td>EA216</td>
<td>KY234238</td>
<td>Bacillus sonorensis NBRC 101234 T</td>
<td>99.4</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>EA217</td>
<td>KY234239</td>
<td>Halomonas smyrniensis AAD(T)</td>
<td>97.5</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>EA218</td>
<td>KY234240</td>
<td>Bacillus amyloliquefaciens subsp. plantarum FZB42 T</td>
<td>99.7</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>EA219</td>
<td>KY234241</td>
<td>Labrenzia aggregata IAM 12614 T</td>
<td>99</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>EA220</td>
<td>KY234242</td>
<td>Labrenzia alexandri DFL-11 T</td>
<td>98.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EA221</td>
<td>KY234243</td>
<td>Mycobacterium bacteremicum ATCC 25791 T</td>
<td>99.7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EA222</td>
<td>KY234244</td>
<td>Celeribacter halophilus ZXM137 T</td>
<td>98</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EA223</td>
<td>KY234245</td>
<td>Chromohalobacter israelensis ATCC 43985 T</td>
<td>98</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>EA225</td>
<td>KY234246</td>
<td>Bacillus subterraneus DSM 13966 T</td>
<td>99.4</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>EA226</td>
<td>KY234247</td>
<td>Roseovarius indicus B108 T</td>
<td>98</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>EA200</td>
<td>KY234248</td>
<td>Halomonas anticariensis FP35(T)</td>
<td>97.8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EA201</td>
<td>KY234249</td>
<td>Pseudalteromonas flavipulchra NCIMB 2033(T)</td>
<td>99.9</td>
<td>W</td>
<td>+</td>
</tr>
<tr>
<td>EA202</td>
<td>KY234250</td>
<td>Microbacter halophilus YIM91118(T)</td>
<td>97.2</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>EA203</td>
<td>KY234251</td>
<td>Salinicola halophilus CG 4.1(T)</td>
<td>97.8</td>
<td>+</td>
<td>W</td>
</tr>
<tr>
<td>EA204</td>
<td>KY234252</td>
<td>Pseudalteromonas rutherina KMM 300(T)</td>
<td>99.1</td>
<td>+</td>
<td>W</td>
</tr>
<tr>
<td>EA205</td>
<td>KY234253</td>
<td>Alteromonas australica H 17(T)</td>
<td>99.5</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>EA206</td>
<td>KY234254</td>
<td>Halomonas stenophila N12(T)</td>
<td>98.7</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>EA207</td>
<td>KY234255</td>
<td>Marinobacter mobilis CN46(T)</td>
<td>97.9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EA208</td>
<td>KY234256</td>
<td>Bacillus licheniformis ATCC 14580(T)</td>
<td>99.1</td>
<td>+ +</td>
<td>+++</td>
</tr>
<tr>
<td>EA209</td>
<td>KY234257</td>
<td>Pseudomonas pachastrellae KMM 330(T)</td>
<td>99.7</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>EA210</td>
<td>KY234258</td>
<td>Sinomicrobium pectinilyticum 5DNS001(T)</td>
<td>94.8</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>EA211</td>
<td>KY234259</td>
<td>Marinobacter mobilis CN46(T)</td>
<td>97.1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
inhibition to *F. oxysporum* (2.5%).

Inhibition pattern against different fungi was different where strongest inhibition was seen against oomycetes (Figure 1). Rhizobacteria EA208 from *C. conglomerates* showed strong activity with 7 to 9 mm mycelial inhibition against both pathogenic fungi tested. This strain has similarity to *Bacillus licheniformis* ATCC 14580(T). Another rhizobacteria EA216 from *Halopeplis perfoliata* showed strong inhibition against all fungi tested.

An endophytic isolate EA218 from same host plant has similar strong inhibition against pathogenic fungi. This strain belongs to class Firmicutes and has closest 16S rRNA similarity to *Bacillus licheniformis* ATCC 14580(T). Seventeen different genera were encountered which in turn belong to four major classes: *γ*-Proteobacteria (n=15; 60%), *α*-Proteobacteria (n=1; 4%), *Firmicutes* (n=4; 16%), *Bacteroidetes* (n=1; 4%) and *Actinobacteria* (n=1; 4%) (Figure 3).

**Phylogenetic analysis of endophytic bacteria on the basis of 16S rRNA gene sequence**

All antagonistic bacteria were then identified by partial 16S rRNA gene sequence analysis. Seventeen different genera were encountered which in turn belong to four major classes: *γ*-Proteobacteria (n=15; 60%), *α*-Proteobacteria (n=1; 4%), *Firmicutes* (n=4; 16%), *Bacteroidetes* (n=1; 4%) and *Actinobacteria* (n=1; 4%) (Figure 3).

Phylogenetic analysis was performed and tree was generated from the distance data using the neighbor-joining method with the Jukes and Cantor model in a MEGA6 Program (Figure 4).
Figure 1. Antifungal activity of bacteria isolated from mangroves against pathogenic fungi. (a) *Fusarium oxysporum* (F.O), (b) *Magnaporthe grisea* (M.G), (c) *Phytophthora capsici* (P.C), and (d) *Alternaria mali* (A.M).

Table 2. Enzyme production of different bacteria isolated from mangrove plants.

<table>
<thead>
<tr>
<th>Lab No</th>
<th>Accession No</th>
<th>Type strain</th>
<th>Amylase</th>
<th>Cellulase</th>
<th>Lipase</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA216</td>
<td>KY234238</td>
<td><em>Bacillus sonorense</em> NBRC 101234&lt;sup&gt;T&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>EA217</td>
<td>KY234239</td>
<td><em>Halomonas smyrnensis</em> AAD6(T)</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>EA218</td>
<td>KY234240</td>
<td><em>Bacillus amyloliquefaciens</em> subsp. <em>plantarum</em> FZB42&lt;sup&gt;T&lt;/sup&gt;</td>
<td>++++</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>EA219</td>
<td>KY234241</td>
<td><em>Labrenzia aggregata</em> IAM 12614&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA220</td>
<td>KY234242</td>
<td><em>Labrenzia alexandrii</em> DFL-11&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA221</td>
<td>KY234243</td>
<td><em>Mycobacterium bacteremicum</em> ATCC 25791&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>EA222</td>
<td>KY234244</td>
<td><em>Celeribacter halophilus</em> ZXM137&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA223</td>
<td>KY234245</td>
<td><em>Chromohalobacter israeliensis</em> ATCC 43985&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA225</td>
<td>KY234246</td>
<td><em>Bacillus subterraneus</em> DSM 13966&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>EA226</td>
<td>KY234247</td>
<td><em>Roseovarius indicus</em> B108&lt;sup&gt;T&lt;/sup&gt;</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Haploplepis perfoliata

Soil

Leaves

Cyperus conglomeratus

Soil
Table 2. Contd.

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EA205</td>
<td>KY234253</td>
<td><em>Alteromonas australica</em> H 17(T)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>EA206</td>
<td>KY234254</td>
<td><em>Halomonas stenophila</em> N12(T)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>EA207</td>
<td>KY234255</td>
<td><em>Marinobacter mobilis</em> CN46(T)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA208</td>
<td>KY234256</td>
<td><em>Bacillus licheniformis</em> ATCC 14580(T)</td>
<td>-</td>
<td>++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>EA209</td>
<td>KY234257</td>
<td><em>Pseudomonas pachastrellae</em> KMM 330(T)</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>EA210</td>
<td>KY234258</td>
<td><em>Sinomicrobi um pectinilyticum</em> 5DNS001(T)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA211</td>
<td>KY234259</td>
<td><em>Marinobacter mobilis</em> CN46(T)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA213</td>
<td>KY234260</td>
<td><em>Microbulbifer halophilus</em> YIM91118(T)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>EA214</td>
<td>KY234261</td>
<td><em>Microbulbifer variabilis</em> Ni-2088(T)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EA215</td>
<td>KY234262</td>
<td><em>Salinimonas lutimaris</em> DPSR-4(T)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Enzymatic activity of bacteria isolated in this study on different enzymatic media. Production of amylase, cellulase, lipase and protease was determined by plate assay. The activity was measured after 2-4 days incubation at 28°C by measuring the clear zone: +, 3 mm; ++, between 5 to 7 mm; +++, between 8 to 9 mm; ++++, between 10 to 11 mm.

Figure 2. Screening of antagonistic bacteria for enzymatic activities. (a) Amylase activity (b) Cellulase activity (c) Protease activity and (d) Lipase activity.

High bootstrap values have been seen which resulted in significant branching points of phylogenetic tree. Phylogenetic analysis on the basis of 16S rRNA gene sequences has revealed five main clusters. First include species of class γ-Proteobacteria, second include species related to class α-Proteobacteria, third include species from class Firmicutes and fourth and fifth cluster comprises of only one spp. which belongs to both class Bacteroidetes and Actinobacteria, respectively.

Bacterial isolates showed 16S rRNA gene sequence
Figure 3. Percentage composition of different phyla of antagonistic rhizobacteria and endophytic bacteria isolated from mangroves on the basis of 16S rRNA gene sequence similarity.

Figure 4. Phylogenetic placement of antagonistic bacteria isolated from mangroves on the basis of 16S rRNA gene sequences and closely related sequences of the type strains of other species. The phylogenetic relationships were inferred from the 16S rRNA gene by using the neighbor-joining method from distances computed with the Jukes-Cantor algorithm. Bootstrap values (1,000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Bar, 0.01 accumulated changes per nucleotide.
similarity of 92.8 to 99.9% with their closest type strain. High bootstrap values have been observed for all species making four different clusters. \textit{γ-Proteobacteria} comprised of 10 different genera, clearly identified as separate cluster with bootstrap values of 89 to 100\% (Figure 4). Representative of class \textit{α-Proteobacteria} comprised of four different genera with high bootstrap values (98 to 100\%). \textit{Bacteroidetes} comprise only one strain EA210 \textit{Sinomoniscum pectinilyticum} SDNS001 (T) with low 16S rRNA gene sequence similarity (94\%), which were also recovered. \textit{Actinobacteria} consist of one strain (EA221) which is an endophyte, \textit{Celenibacter halophilus} ZXM137 (T).

**DISCUSSION**

Mangrove plants are an excellent source of chemical compounds of medicinal and agricultural use (Miles et al., 1998). However, studies related to isolation and screening of antagonistic rhizo and endophytic bacteria from mangrove plants, from Saudi Arabia is not done before. To our knowledge, our study is the first report regarding isolation, screening and identification of rhizo and endophytic bacteria from \textit{H. perforata} and \textit{C. conglomeratus} in Saudi Arabia.

In microbial antagonism, different types of metabolites are secreted by microbes especially growth inhibitor due to competition for nutrients and living space (Whipp et al., 2001; Riley and Wertz, 2002). Endophytic bacteria produce such metabolites in defence of host plants against different plant pathogens (Strobel and Daisy, 2003). We have used different combinations of culturing media for isolation of bacteria. Great diversity have been seen in bacteria as well as their biological activities isolated on different culturing media. This variation in growth of bacteria on different media is also seen in previous studies, where specific group of bacteria recover on specific media and conditions (Vieira and Nahas, 2005; Chang et al., 2015).

317 rhizo and endophytic bacteria have been isolated and screen for their antifungal potential against five different pathogenic fungi mentioned above. Several previous studies also reported isolation of antagonistic bacteria from mangroves. Mainly endophytic bacteria, which produce secondary metabolites were isolated (Hu and Wu, 2010; Hu et al., 2010; Ding et al., 2012; Eldeen et al., 2015). These active bacterial isolates were further checked for their antibacterial activity where only few bacteria were active against human pathogenic bacteria. Mostly, strains of \textit{Bacillus} in our study showed antibacterial activity which is similar to finding reported previously (Hu et al., 2010; Eldeen et al., 2015), where strain of \textit{Bacillus} isolated from mangrove plant exhibit antibacterial activity against different pathogenic bacteria. Microorganisms from mangrove plants are capable of producing various groups of enzymes of industrial and biotechnological significance (Thatoi et al., 2013; Saravanakumar et al., 2016). Antagonistic bacteria from two mangroves were checked for ability to produce hydrolytic enzymes.

In a recent study from Thailand, endophytic bacteria isolated from mangrove plants were screened for hydrolytic enzymes production. Many bacteria were positive for production of proteases, lipases, amylases or cellulases where one strain of \textit{Bacillus safensis} was able to produce all enzymes (Khianggam et al., 2013). In another study, enzymatic potential of the bacteria from mangroves have been evaluated, where four strains of \textit{Bacillus} sp. exhibited strong enzyme production (Tabao and Moasalud, 2010).

In this study, \textit{Bacillus} sp. showed strong enzymatic activities. From Saudi Arabia, mangroves were used for isolation of bacteria producing a polymer Polyhydroxybutyrate (PHB) (Alarfaj et al., 2015). A strain of \textit{B. thuringiensis} from this study showed maximum production of PHB. In another study, 12 different fungi have been isolated from mangroves growing on Red Sea Coast of Saudi Arabia. These fungal isolates were able to produce different enzymes and help in biodegradation of diesel fuel (Ameen et al., 2016). This study is the first report on isolation, screening on the basis of antimicrobial activity and enzyme characterization of bacteria from \textit{C. conglomerates} and \textit{H. perforata} in Saudi Arabia.

In this study, \textit{Bacillus} was the dominant genus that is similar to many previous studies where different strains of \textit{Bacillus} were isolated from mangrove plants during screening for their antifungal potential (Liu et al., 2010; Ando et al., 2001). Two endophytic strains of \textit{Bacillus}, \textit{B. thuringiensis} and \textit{Bacillus pumilus} were isolated from mangrove plants from India (Ravikumar et al., 2010). These two endophytic strains of \textit{Bacillus} were able to inhibit many bacterial and fungal pathogens. Feng et al. (2009) have evaluated bacterial communities from mangroves for their antagonism. One endophytic strain \textit{Bacillus amyloliqufaciens} was able to control Phytophthora blight caused by \textit{Phytophthora capsici} in \textit{capsicum} in an \textit{in vitro} assay. In our work, we have many strains from \textit{α-Proteobacteria} and \textit{Firmicutes}, showing strong antagonistic activity against \textit{Py. ultimum} and \textit{P. capsici} suggest their application as biocontrol agent in future.

Twenty-five antagonistic rhizo and endophytic bacteria from mangroves exhibit both antifungal and antibacterial activities. Furthermore, bacteria exhibited different types of enzymatic activities of industrial importance. It is concluded from the study that rhizo and endophytic bacteria isolated from soil, roots and leaves of mangrove plants produce enzymes, antibacterial and antifungal metabolites, pointing their significant role in host plant.
Further study is in progress to identify these active metabolites responsible for antimicrobial activity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH)—King Abdulaziz City for Science and Technology-the Kingdom of Saudi Arabia-award number (12BIO-2724-03). The authors also, acknowledge with thanks Science and Technology Unit, King Abdulaziz University for technical support.

REFERENCES


Strobel G, Daisy B (2003). Bioprospecting for microbial endophytes and...