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Isolation, diversity, and biotechnological potential of rhizo- and endophytic bacteria associated with mangrove plants from Saudi Arabia

F. Bibi[1], I. Ullah[2], S.A. Alvi[1], S.A. Bakhsh[1], M. Yasir[1], A.A.K. Al-Ghamdi[3] and E.I. Azhar[1,3]

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

Corresponding author: F. Bibi
E-mail: fehmeedaimran@yahoo.com

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ABSTRACT. Marine bacteria have been exceptional sources of halotolerant enzymes since decades. The aim of the present study was to isolate bacteria producing hydrolytic enzymes from seven different mangroves collected from the coastal area of Thuwal, Jeddah, Saudi Arabia, and to further screen them for other enzymatic and antifungal activities. We have isolated 46 different rhizo- and endophytic bacteria from the soil, roots, and leaves of the mangroves using different enzymatic media. These bacterial strains were capable of producing industrially important enzymes (cellulase, protease, lipase, and amylase). The bacteria were screened further for antagonistic activity against fungal pathogens. Finally, these bacterial strains were identified on the basis of the16S rDNA sequence. Taxonomic and phylogenetic analysis

revealed 95.9-100% sequence identity to type strains of related species. The dominant phylum was Gammaproteobacteria (γ-Proteobacteria), which comprised 10 different genera - *Erwinia*, *Vibrio*, *Psychrobacter*, *Aidingimonas*, *Marinobacter*, *Chromohalobacter*, *Halomonas*, *Microbulbifer*, and *Alteromonas.* Firmicutes was the second dominant phylum, which contained only the genus *Bacillus*. Similarly, only *Isoptericola* belonged to Actinobacteria. Further these enzyme-producing bacteria were tested for the production of other enzymes. Most of the active strains showed cellulytic and lipolytic activities. Several were also active against fungal pathogens. Our results demonstrated that the mangroves represent an important source of potentially active bacteria producing enzymes and antifungal metabolites (bioactive products). These bacteria are a source of novel halophilic enzymes and antibiotics that can find industrial and medicinal use.

Key words: Mangroves; Enzyme-producing bacteria; Antagonistic activity; 16S rDNA sequence; Phylogenetic analysis

INTRODUCTION

Isolation of novel bioactive molecules from the diverse marine ecosystem has rendered marine microbiology as one of the most interesting modern fields of research. Although there is extraordinary biodiversity in the terrestrial environment, the greatest biodiversity occurs in the marine ecosystems (Donia and Hamann, 2003). The ocean occupies more than 70% of the total surface of the earth and is the habitat of myriad microorganisms (Wang et al., 2016). Such ecosystems thrive under special conditions, such as low temperature, high salinity, high pressure, and low light, and are an exciting area of research for marine microbiologists. Owing to the high adaptability towards extreme and complex environmental conditions of temperature, pressure, and pH, marine extremophiles are also popular research objects (Zhang and Kim, 2010). Particularly, rhizophytic and endophytic bacteria isolated from these conditions are a major source of novel enzymes and other metabolites, and some of them have already been used as food additives or potential drugs (Rahman et al., 2010; Lee et al., 2011; Martins et al., 2014).

Mangroves are halophytes inhabiting intertidal areas of the sea and can tolerate salinity, anaerobic conditions, tides, and high temperature. Under these stressful environmental conditions, mangroves are able to produce different kinds of active metabolites with diverse biological functions. Until now, more than 200 active metabolites have been isolated from mangroves and their associated organisms (Bandaranayake, 2002). Both rhizo- and endophytic bacteria play important roles in host plant survival, for example, by colonizing internal plant tissues (for the endophytes) and promoting plant growth and productivity (Lodewyckx et al., 2002; Berg et al., 2014). These endophytic bacteria have been isolated from different plants including citrus, maize, strawberries, and others (Araújo et al., 2000, 2001; Dias et al., 2009). However, the marine endophytes offer a new area of research for the identification and production of new compounds and enzymes of commercial value.

Microbial enzymes are routinely used in several industries, especially because they are economical, environment-friendly, pose no ethical concerns, and can be identified easily by screening microorganisms from various environmental conditions (Hoondal et al., 2002; Dalvi

et al., 2007). Endophytic bacteria produce industrially important enzymes such as amylases, lipases, agarases, cellulases, and proteases (Lodewyckx et al., 2002). Further, microorganisms from mangrove ecosystems are a rich source of industrially important enzymes and antibiotics (Thatoi et al., 2013).

The production of extracellular enzymes from marine endophytic bacteria is limited and requires investigation (Martinez et al., 1996; Indarmawan et al., 2016). A previous study reported the isolation and identification of important enzymes, such as amylase, esterase, cellulose, and protease from endophytes of mangroves (Castro et al., 2014). Further, production of exo- and endoglucanases have been reported in different groups of bacteria isolated from mangrove sediments (Soares Júnior et al., 2013). In a recent study, cellulase-producing bacterial strains of genus *Bacillus* and *Brucella* were isolated from mangrove soil (Behera et al., 2016). Despite their biotechnological importance, little is known about bacterial communities of mangroves. Therefore, the present study aimed to isolate and screen industrially important bacteria from mangrove plants. We isolated 46 enzyme-producing rhizophytic and endophytic bacteria from seven different mangroves growing in a coastal area of Thuwal, Jeddah, Saudi Arabia. Furthermore, these enzyme-producing bacteria were characterized for additional enzyme production and antifungal activity.

MATERIAL AND METHODS

Sample collection and isolation of bacteria

Plant specimens were collected from the coastal area of Thuwal, Jeddah, Saudi Arabia (22°15'54"N, 39°6'44"E). All the plant specimens were placed in a sterile bag after collection and transferred to the laboratory for bacterial isolation. We used soil, roots, and leaves of the plants for the isolation of enzyme-producing bacteria. For the isolation of bacteria from the adhering soil, we dipped the roots in sterile distilled water and made serial dilutions (10^{-3}, 10^{-4}, and 10^{-5}) in autoclaved filtered sea water (AFS). The dilutions were then spread on four different enzymatic media (mentioned below) for isolating enzyme-producing bacteria. One-tenth strength R2A (1/10 R2A) medium with agar (Difco Laboratories, Detroit, MI, USA) was added separately to each substrate, namely, 1% carboxymethylcellulose (CMC), 1% skim milk, 1% tributyrin, and 1% starch for the isolation of cellulase, protease, lipase, and amylase-producing bacteria, respectively. The roots and leaves were also used for the isolation of bacteria after sterilization following a procedure described previously (Bibi et al., 2012). Cycloheximide (50 µg/mL) was added to the medium to avoid contamination. The plates were incubated at 26°C for almost 1 week and enzyme activities were monitored. To detect cellulase activity, the plates were flooded with a solution of 0.1% Congo red and incubated on an orbital shaker for 15 min and washed with 1 M NaCl (Hendricks et al., 1995). The positive activity was detected as a halozone around bacterial colonies on CMC agar. Skim milk ½ R2A agar plates were used for the isolation of bacteria producing proteases, which formed a clear zone on skim milk agar plates. On tributyrin ½ R2A agar plates, clear zones were detected around bacteria after hydrolysis of tributyrin. Amylase-producing bacteria showed starch hydrolysis as a clear zone on starch ½ R2A agar plates (Kumar et al., 2012). The bacteria positive for the production of any enzyme were further evaluated for other enzymatic activities. All the bacterial strains were further sub-cultured and stored as 15% (v/v) glycerol stock of media at -70°C.

Screening of antifungal activity

The antifungal potential of all hydrolase-producing bacteria isolated from the soil, roots, and leaves of the mangroves were determined. We used four different tests for fungal pathogens. *Phytophthora capsici* and *Pythium ultimum* were present in our laboratory, whereas *Alternaria malli* (KCTC 6972) and *Fusarium moniliforme* (KCTC 6149) were obtained from the Korean Collection for Type Cultures (KCTC). The antagonistic activity against fungal pathogens was conducted using a previously described method (Bibi et al., 2012). All the strains were checked twice for antagonistic activity. The antagonistic activity was then evaluated by measuring the inhibition zone of the fungal mycelia around the bacterial colony.

Extraction of bacterial DNA and 16S rDNA sequencing

The isolated bacteria were further used for genomic DNA extraction using a GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, USA). Briefly, a loop full of bacteria from overnight grown culture on R2A agar was used for the isolation of 5-10 µg DNA. 16S rDNA sequencing was performed to identify the bacterial strains. The 16S rDNA fragment was amplified using bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplifications were performed as described previously (Bibi et al., 2012). The PCR products were purified using a GeneJET PCR Purification Kit (Thermo Scientific, Waltham, USA) and stored at 4°C until they were sequenced commercially by Macrogen (Seoul, Korea). The bacteria were identified by performing a BLAST analysis with the 16S rDNA sequences using the EzTaxon server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). The 16S rDNA sequences of related type strains were obtained from the National Center for Biotechnology Information (NCBI) to determine the phylogenetic placement of the enzymatic bacteria and related type strains. For the phylogenetic analysis, CLUSTALX (Thompson et al., 1997) multiple alignments of the sequences were performed and the BioEdit software (Hall, 1999) was used to edit the gaps. The neighbor-joining method in the MEGA6 program with bootstrap values based on 1,000 replicates was used for construction of the phylogenetic tree (Tamura et al., 2013).

Nucleotide sequence numbers

All the nucleotide sequences of the bacterial strains have been deposited in the GenBank database under accession Nos. KY034369-KY034414.

RESULTS

Isolation of rhizophytic and endophytic enzyme-producing bacteria

Seven different mangroves, *Salsola imbricata*, *Avicennia germinans*, *Avicennia marina*, *Halopeplis perfoliata*, *Halocnemum strobilaceum*, *Zygophyllum qatarense*, and *Cyperus conglomeratus*, were used for the isolation of the rhizophytic and endophytic bacteria. Soil attached with plant roots, and leaf tissue samples were used for bacterial isolation. We used 1/10 R2A media supplemented with a 1% substrate as different enzymatic media for the isolation of enzyme-producing bacteria. Forty-six morphologically distinct bacterial colonies

showing activity on media were isolated from the rhizosphere and endosphere of mangroves. Most bacterial strains (28; 60.8%) were isolated from the endosphere of plants, whereas enzyme-producing rhizobacteria were comparatively scarce (18; 39.2%) on enzymatic test media. We identified 14 (endo, 7; rhizo, 7; 30.4%) cellulolytic bacteria, 4 (endo, 2; rhizo, 2; 8.7%) protease producers, 19 (endo, 6; rhizo, 13; 41.3%) lipolytic bacteria, and 9 (endo, 3; rhizo, 6; 19.6%) amylase-positive bacteria (Table 1). Most endophytes isolated from mangroves showed lipolytic activity (13, 68.4% bacteria were positive). In contrast, most rhizobacteria isolated from mangroves (66.7%) were able to produce amylase, whereas equal numbers of rhizophytic and endophytic bacteria were identified to be protease and cellulase positive. All the enzymatic bacteria were further screened for the production of other enzymes. such as bacteria positive for cellulase production were tested for lipase, protease and amylase activities and vice verse. Twenty-three (50%) bacteria were negative for further enzyme production. In the remaining 50%, only 8 (17.4%) bacteria, namely, EA154, EA156, EA157, EA160, EA161, EA171, EA177, and EA179 were able to produce two more hydrolytic enzymes, whereas the other 15 (32.6%) bacteria were positive for the production of only one enzyme (Table 1). The endophytic strains producing high amounts of protease and amylase were EA157, EA161, and EA171 (+++++, clear zone diameter between 11 to 12 mm). The rhizobacterial strains, EA154, EA156, and EA179, also showed strong enzymatic activities.

Screening of enzymatic bacteria against fungal pathogens

All the bacterial strains were further screened for antifungal activity against pathogenic fungi, *P. capsici*, *Py. ultimum*, *A. malli*, and *F. oxysporum* in an *in vitro* assay. Of the 46 bacteria tested, 26 (56.5%) exhibited inhibitory activity against the oomycetes of *P. capsici* (Table 2), whereas 28 (60.8%) bacterial strains were active against *Py. ultimum*. The antagonistic activity of these bacteria against *Py. ultimum* was significantly higher than that towards *P. capsici*. These bacterial isolates also showed activity against two other tested fungi. Sixteen (34.7%) isolates were active against *A. malli.* Among them, only 9 (19.6%) isolates showed moderate to strong activity, whereas others exhibited weak activity. Strong inhibition was detected by endophytic strain EA160 from the genus *Bacillus* (Table 2). This strain also showed antifungal activity against the other tested fungi. Only few isolates showed weak inhibition against *F. oxysporum* (N = 9; 19.6%); two isolates, EA169 and EA188, showed moderate inhibition against this fungus, whereas others showed weak inhibition (Table 2). Among these antagonistic bacteria, *Bacillus* was the dominant genus followed by species of *Microbulbifer*, *Marinobacter*, and *Halomonas*. Strong antifungal activity was observed for the endophytic strains EA154, EA159, EA160, EA174, and EA195 with a 10-20 mm diameter inhibition zone against fungal pathogens (Table 2), whereas the rest of the isolates showed weak to moderate activity (4-9 mm diameter inhibition zone).

Identification of enzyme-producing bacteria and their phylogenetic analysis

Both rhizophytic and endophytic enzyme-producing bacteria were identified by partially sequencing the 16S rDNA. These 46 enzymatic bacteria belong to ten different genera and were further assigned to three major classes: γ-Proteobacteria (N = 32; 69.6%), Firmicutes (N = 13; 28.3%), and Actinobacteria (N = 1; 2.1%) (Figure 1). A phylogenetic tree was constructed from the data using the neighbor-joining method (Figure 2). The 16S rDNA sequences obtained from this study and related type strain sequences retrieved from NCBI were

Table 1. Taxonomic identification and enzyme production on different enzymatic media.

Strain lab No.	Accession No.	Closely related type strain[a]	% Identity[b]	Enzymatic activity on isolation media[c]	Cellulase	Protease	Lipase	Amylase
Salsola imbricata								
Soil								
EA151	KY034369	*Bacillus licheniformis* ATCC 14580^T	99.2	Cellulase	++	-	-	-
EA152	KY034370	*Isoptericola salitolerans* TRM F109^T	99.9	Amylase	-	++++	-	++
Avicennia germinans								
Soil								
EA153	KY034371	*Bacillus sonorensis* NBRC 101234^T	99	Lipase	-	-	+++	-
EA154	KY034372	*Bacillus subtilis subsp. inaquosorum* KCTC 13429^T	99.0	Lipase	-	+++++	+	++++
Avicennia marina								
Root								
EA155	KY034373	*Erwinia toletana* CECT 5263^T	99.1	Cellulase	++	-	+++++	-
Halopeplis perfoliata								
Soil								
EA156	KY034374	*Vibrio antiquarius* Ex25^T	98.8	Cellulase	++	-	+++++	++++
Root								
EA157	KY034375	*Bacillus licheniformis* ATCC 14580 ^{T}	99.3	Lipase	-	+++++	++	+++++
EA158	KY034376	*Psychrobacter alimentarius* JG-100 ^{T}	99.6	Amylase	-	-	+++++	+
EA159	KY034377	*Bacillus cereus* ATCC 14579^T	98.4	Lipase	-	++	+	-
EA160	KY034378	*Bacillus licheniformis* ATCC 14580^T	99.6	Amylase	-	++++	++++	++
Leaf								
EA161	KY034379	*Bacillus subtilis subsp. inaquosorum* KCTC 13429^T	100	Lipase	-	+++++	+	++++
Halocnemum strobilaceum								
Soil								
EA162	KY034380	*Aidingimonas halophila* YIM 90637^T	98.3	Cellulase	++	-	-	++++
EA163	KY034381	*Marinobacter daqiaonensis* YCSA40^T	98	Lipase	++++	-	++	-
EA164	KY034382	*Chromohalobacter israelensis* ATCC 43985^T	98.3	Lipase	-	-	+	++++
EA165	KY034383	*Bacillus cereus* ATCC 14579^T	99.8	Protease	-	+++	-	-
EA166	KY034384	*Halomonas anticariensis* FP35^T	96.9	Cellulase	++	-	-	-
EA167	KY034385	*Marinobacter daqiaonensis* YCSA40^T	95.9	Lipase	-	-	++	-
EA168	KY034386	*Halomonas lutea* DSM 23508^T	97.8	Amylase	-	-	-	+
EA169	KY034387	*Aidingimonas halophila* YIM 90637^T	95.5	Amylase	-	-	-	+
EA170	KY034388	*Bacillus licheniformis* ATCC 14580^T	98	Protease	-	+++	-	-
Root								
EA171	KY034389	*Bacillus pumilus* ATCC 7061^T	99.8	Cellulase	++	+++++	-	+++++
EA172	KY034390	*Microbulbifer celer* ISL-39^T	99.4	Cellulase	++	-	-	-
EA173	KY034391	*Bacillus licheniformis* ATCC 14580^T	98.7	Lipase	-	-	+	-
EA174	KY034392	*Bacillus safensis* FO-36b^T	100	Amylase	-	+++++	-	+
EA175	KY034393	*Marinobacter zhanjiangensis* JSM 078120^T	98	Lipase	-	-	+	-
EA176	KY034394	*Microbulbifer halophilus* YIM91118^T	98.4	Amylase	-	-	-	+
Zygophyllum qatarense								
Soil								
EA177	KY034395	*Microbulbifer celer* ISL-39^T	99.4	Cellulase	++	+++	w	++++
EA178	KY034396	*Halomonas sinaiensis ALO Sharm*^{T}	96.5	Cellulase	+	-	-	-
EA179	KY034397	*Alteromonas macleodii* ATCC 27126^T	98.9	Cellulase	=	+++++	-	++++
Root								
EA180	KY034398	*Halomonas smyrnensis* AAD6^T	99.7	Lipase	-	-	+	++++
EA181	KY034399	*Microbulbifer elongatus* ATCC 10144^T	99.4	Lipase	-	-	+	-
EA182	KY034400	*Marinobacter xestospongiae* UST090418-161I^T	99.9	Cellulase	++	-	+++	-
EA183	KY034401	*Chromohalobacter israelensis* ATCC 43985^T	96.4	Lipase	-	-	+	-
EA184	KY034402	*Microbulbifer celer* ISL-39^T	99.4	Protease	-	+++	-	-
EA185	KY034403	*Microbulbifer elongatus* DSM 6810^T	99.5	Lipase	+++	-	+	+++
EA186	KY034404	*Marinobacter lacisalsi* FP2.5^T	95.2	Amylase	-	-	-	++
EA187	KY034405	*Chromohalobacter israelensis* ATCC 43985^T	96.6	Cellulase	++	-	-	-
EA188	KY034406	*Marinobacter lacisalsi* FP2.5^T	94.7	Lipase	-	-	++	++++
EA189	KY034407	*Microbulbifer agarilyticus* JAMB A3^T	99.4	Protease	-	+++	-	-
EA190	KY034408	*Halomonas caseinilytica* DSM 23509^T	99.1	Lipase	-	-	++	-
EA191	KY034409	*Chromohalobacter israelensis* ATCC 43985^T	99.9	Cellulase	++	-	-	-
EA192	KY034410	*Microbulbifer celer* ISL-39^T	99.4	Amylase	-	++++	-	++
Cyperus conglomeratus								
Soil								
EA193	KY034411	*Microbulbifer celer* ISL-39^T	98.5	Lipase	-	-	++	-
Root								
EA194		*Microbulbifer halophilus* YIM91118^T	98.6	Cellulase	++	-	-	-
EA195	KY034413	*Bacillus cereus* ATCC 14579^T	100	Lipase	-	-	++	++++
EA196	KY034414	*Microbulbifer halophilus* YIM91118^T	96.8	Lipase	-	-	+	++++

[a]Identification of strain based on 16S rDNA sequence analyses; [b]Percentage similarity of strain with closely related type strain. [c]Enzymatic activity of bacteria on main isolation media used for isolation. [d]Production of cellulase, protease, lipase, and amylase by enzyme-producing bacteria was determined by plate assay. The activity was measured after 2-4 days incubation at 28°C by measuring the clear zone: -, negative; +, 3 mm; ++, between 5 to 7 mm; +++, between 8 to 9 mm, ++++, between 10 to 11 mm, +++++, between 12 to 16 mm.

used to construct the phylogenetic tree. Most of the strains showed high bootstrap values with significant branching patterns (Figure 2). The bacterial strains exhibited a sequence similarity of 95.9-100%. γ-Proteobacteria was the dominant (N = 32; 69.6%) phylum among all enzyme-producing strains and included 10 different genera, namely, *Microbulbifer* (N = 11; 34.4%), *Marinobacter* (N = 6; 18.7%), *Halomonas* (N = 5; 14.7%), *Chromohalobacter* (N = 4; 12.5%), *Aidingimonas* (N = 2; 6.2%), *Erwinia* (N = 1; 3.1%), *Vibrio* (N = 1; 3.1%), *Psychrobacter* (N = 1; 3.1%), and *Alteromonas* (N = 1; 3.1%). Among these genera, *Microbulbifer* was

the dominant (N = 11; 34.3%) genus. The second dominant phylum was Firmicutes, where *Bacillus* (N = 13; 28.2%) was the only genus found among all mangroves. Actinobacteria consisted of only one genus, *Isoptericola* (Table 1).

Table 2. Antifungal activity of rhizo- and endophytic bacteria isolated from mangroves against different pathogenic fungi.

Antifungal activity[a]						
Strain lab No.	Accession No.	Closely related type strain	*P. capsici*	*Py. ultimum*	*A. mali*	*F. moniliforme*
Salsola imbricata						
Soil						
EA151	KY034369	*Bacillus licheniformis* ATCC 14580^T	++	++	++++	W
EA152	KY034370	*Isoptericola salitolerans* TRM F109^T	++++	++	W	-
Avicennia germinans						
Soil						
EA153	KY034371	*Bacillus sonorensis* NBRC 101234^T	++	++	++++	W
EA154	KY034372	*Bacillus subtilis subsp. inaquosorum* KCTC 13429^T	++++	++++	+++	-
Avicennia marina						
Root						
EA155	KY034373	*Erwinia toletana CECT 5263*^{T}	-	-	-	-
Halopeplis perfoliata						
soil						
EA156	KY034374	*Vibrio antiquarius* Ex25^T	-	-	-	-
		Root				
EA157	KY034375	*Bacillus licheniformis* ATCC 14580^T	++	+++++	++++	-
EA158	KY034376	*Psychrobacter alimentarius* JG-100^T	-	-	++++	-
EA159	KY034377	*Bacillus cereus* ATCC 14579^T	++++	+++++	-	-
EA160	KY034378	*Bacillus licheniformis* ATCC 14580^T	++++	++	+++++	+
Leaf						
EA161	KY034379	*Bacillus subtilis subsp. inaquosorum* KCTC 13429^T	++	++	++	-
Halocnemum strobilaceum						
Soil						
EA162	KY034380	*Aidingimonas halophila* YIM 90637^T	++	+++	-	-
EA163	KY034381	*Marinobacter daqiaonensis* YCSA40^T	+++	++	-	W
EA164	KY034382	*Chromohalobacter israelensis* ATCC 43985^T	-	-	-	W
EA165	KY034383	*Bacillus cereus* ATCC 14579^T	+++	++	-	-
EA166	KY034384	*Halomonas anticariensis* FP35^T	++	+	W	-
EA167	KY034385	*Marinobacter daqiaonensis* YCSA40^T	++	++	-	-
EA168	KY034386	*Halomonas lutea* DSM 23508^T	+	+	W	-
EA169	KY034387	*Aidingimonas halophila* YIM 90637^T	+	+	W	++++
EA170	KY034388	*Bacillus licheniformis* ATCC 14580^T	-	-	W	-
Root						
EA171	KY034389	*Bacillus pumilus* ATCC 7061^T	++	++	-	-
EA172	KY034390	*Microbulbifer celer* ISL-39^T	++	++	-	-
EA173	KY034391	*Bacillus licheniformis* ATCC 14580^T	++	+	-	-
EA174	KY034392	*Bacillus safensis* FO-36b^T	++++++	+++++	+++	W
EA175	KY034393	*Marinobacter zhanjiangensis* JSM 078120^T	-	-	-	-
EA176	KY034394	*Microbulbifer halophilus* YIM91118^T	-	-	-	-
Zygophyllum qatarense						
Soil						
EA177	KY034395	*Microbulbifer celer* ISL-39^T	-	-	-	-
EA178	KY034396	*Halomonas sinaiensis ALO Sharm*^{T}	+	-	-	-
EA179	KY034397	*Alteromonas macleodii* ATCC 27126^T	++	+	-	-
Root						
EA180	KY034398	*Halomonas smyrnensis* AAD6^T	++	+++	-	-
EA181	KY034399	*Microbulbifer elongatus* ATCC 10144^T	-	-	-	-
EA182	KY034400	*Marinobacter xestospongiae* UST090418-1611^T	-	-	++	-
EA183	KY034401	*Chromohalobacter israelensis* ATCC 43985^T	-	+		W
EA184	KY034402	*Microbulbifer celer* ISL-39^T	-	-	-	-
EA185	KY034403	*Microbulbifer elongatus* DSM 6810^T	+	+++	-	-
EA186	KY034404	*Marinobacter lacisalsi* FP2.5^T	+	+	-	-
EA187	KY034405	*Chromohalobacter israelensis* ATCC 43985^T	++	+	-	-
EA188	KY034406	*Marinobacter lacisalsi* FP2.5^T	-	-	W	+++
EA189	KY034407	*Microbulbifer agarilyticus* JAMB A3^T	-	+	-	-
EA190	KY034408	*Halomonas caseinilytica* DSM 23509^T	-	+	-	-
EA191	KY034409	*Chromohalobacter israelensis* ATCC 43985^T	-	-	W	-
EA192	KY034410	*Microbulbifer celer* ISL-39^T	-	-	-	-
Cyperus conglomeratus						
Soil						
EA193	KY034411	*Microbulbifer celer* ISL-39^T	-	-	-	-
Root						
EA194	KY034412	*Microbulbifer halophilus* YIM91118^T	-	-	-	-
EA195	KY034413	*Bacillus cereus* ATCC 14579^T	+++++	++++	-	-
EA196	KY034414	*Microbulbifer halophilus* YIM91118^T	-	-	-	-

[a]Antagonistic activity of all enzyme-producing bacteria isolated in this study. The activity was measured 4-5 days after incubating at 28°C by measuring the clear zone of mycelial growth inhibition: -, negative; W, weak activity; +, 3 mm; ++, between 4 to 6mm; +++, between 7 to 9 mm, ++++, between 10 to 11 mm, +++++, between 12 to 16 mm, ++++++, between 17 to 20 mm.

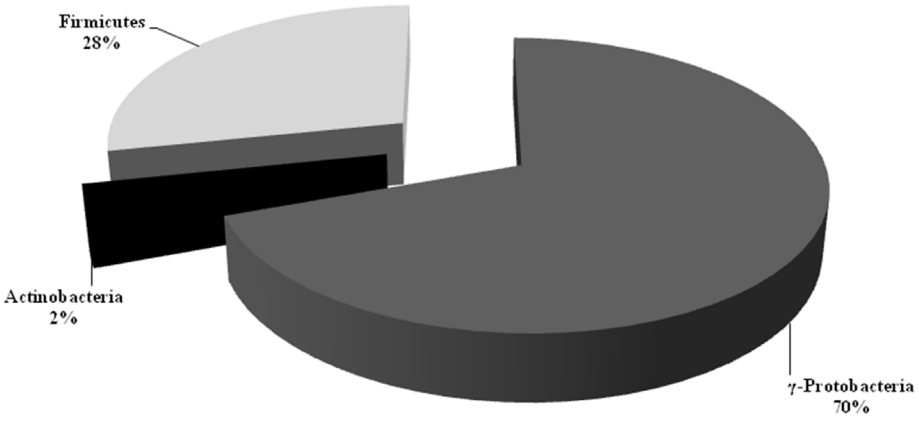

Figure 1. Percentage composition of different phyla of enzyme-producing rhizo- and endophytic bacteria isolated from mangroves on the basis of 16S rDNA sequence similarity.

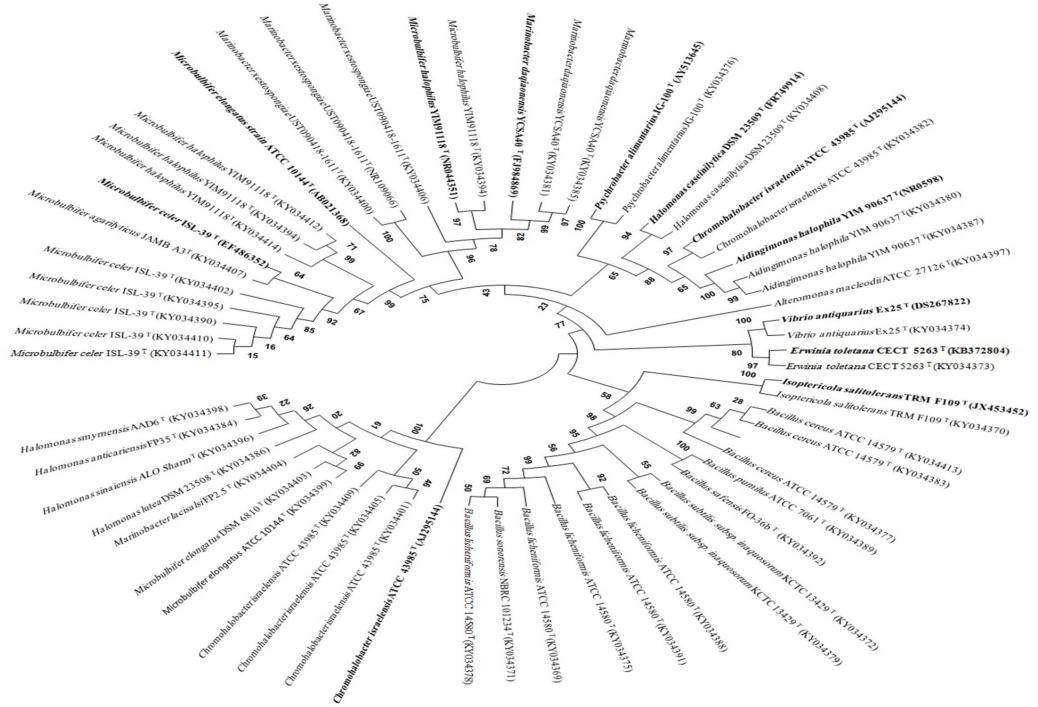

Figure 2. Phylogenetic placement of enzyme-producing bacteria isolated from mangroves on the basis of 16S rDNA sequence similarity with closely related type strains of other species. The phylogenetic relationships were inferred from the 16S rDNA sequence using the neighbor-joining method from distances computed with the Jukes-Cantor algorithm. Bootstrap values (1000 replicates) are shown next to the branches. GenBank accession Nos. for each sequence are shown in parentheses. Bar, 0.01; accumulated changes per nucleotide.

DISCUSSION

Marine bacteria are excellent sources of industrially useful enzymes. We conducted the present study to isolate hydrolytic enzyme-producing bacteria from mangroves using different enzymatic culture media. Both rhizo- and endophytic bacteria were isolated from the soil, roots, and leaves of the plants. Forty-six rhizophytic and endophytic bacteria exhibiting various enzymatic activities were isolated on 1/10 R2A media containing appropriate substrates (CMC, skim milk, tributyrin, and starch). The numbers of hydrolytic enzyme-producing bacteria is summarized in Table 1.

Mangroves are an excellent source of potentially important bacteria. Marine bacteria produce commercially important bio-active metabolites such as antibiotics against various pathogenic microbes and enzymes of industrial importance (Chatellier et al., 2000). The enzymes from marine bacteria are halotolerant and stable under extreme conditions and possess unique features and catalytic activities (Sellek and Chaudhuri, 1999; Gomes and Steiner, 2004). Several previous studies have reported isolation of enzyme-producing halophilic bacteria from different marine sediments, water, crystallizer ponds, and salt lakes (Sánchez-Porro et al., 2003; de Lourdes Moreno et al., 2009; Rohban et al., 2009). Among the 46 active bacteria identified in this study, 14 bacterial strains showed cellulytic activity, 2 were positive for protease, 13 for lipase activity, and six for amylase activity. All the bacteria were further screened for the production of other enzymes, and 2 cellulase positive, 6 lipase positive, 10 protease positive, and 14 amylase positive isolates showed further enzymatic activities (Table 1).

The 16S rDNA sequence was used for the identification of the enzyme-producing rhizophytic and endophytic bacteria. A phylogenetic analysis of these bacterial isolates using the 16S rDNA sequences grouped them into three phyla, namely, γ-Proteobacteria (*Microbulbifer*, *Marinobacter*, *Halomonas*, *Chromohalobacter*, *Aidingimonas*, *Erwinia*, *Vibrio*, *Psychrobacter*, and *Alteromonas*), Firmicutes (*Bacillus*), and Actinobacteria (*Isoptericola*) (Figure 1). More enzyme-producing endophytic bacteria were isolated from mangroves compared to rhizobacteria.

γ-Proteobacteria was the most dominant group identified in our study and included ten different genera. Marine bacteria from this group are already known for the production of different antibiotics (Radjasa et al., 2007). Previous studies have also reported production of different hydrolases from marine bacteria belonging to genera *Erwinia*, *Vibrio*, *Psychrobacter marinobacter*, *Chromohalobacter*, *Halomonas*, *Microbulbifer*, and *Alteromonas* (Dalmaso et al., 2015), whereas the production of enzymes by marine *Aidingimonas* have not been previously reported. The second dominant phylum Firmicutes comprised of *Bacillus*, which has been reported as a dominant genus among all the marine enzyme-producing bacteria (Divya et al., 2010). This is because *Bacillus* is easy to culture and can endure harsh environmental conditions. In a previous study on mangroves (Tabao and Moasalud, 2010), four different species of *Bacillus* were reported to produce cellulase, which is similar to the results of our study where several *Bacillus* spp exhibited strong enzymatic activities. Most of the *Bacillus* spp in our study was rhizobacteria.

The Actinobacteria identified in this study contained only one species, *Isoptericola.* This species was isolated from the soil surrounding the roots of the marine plant *S. imbricata*. Actinobacteria from the marine environment play an important role in bioremediation and production of antibiotics and enzymes. A multitude of antibiotics have been previously

isolated from marine sources, especially from Actinobacteria (Manivasagan et al., 2013). In this study, only one strain of Actinobacteria exhibited amylase activity, which was also positive for protease production when further assessed for other enzyme production. Furthermore, these bacteria were positive for other enzyme activities, especially lipase and cellulase.

Finally, we assayed the antagonistic potential of these bacteria against four different fungal pathogens. Most of the isolates were active against *Py. ultimum* and *P. capsici*, whereas few inhibited the growth of *A. mali* and *F. moniliforme*. Most of the isolates that inhibited the growth of fungal pathogens in our study were related to *Bacillus*. Marine bacteria from this genus are already known for their antimicrobial activity against different pathogens and synthesize different classes of antibiotics (Fan et al., 2011). In addition, marine *Bacillus* produces different bioactive metabolites with novel structures and modes of action, which are pivotal for treatment of various human infections (Mondol et al., 2013). Certain bacterial strains identified in this study exhibited strong enzymatic and antifungal activities. Strains EA154, EA160, and EA 161 belonging to *Bacillus* spp. produced hydrolytic enzymes and were antagonistic to fungal pathogens (Tables 1 and 2). The dominance of these bacteria from the genus *Bacillus* in mangrove plants indicates a role in plant defense against pathogens. *Bacillus* spp. from mangrove plants is already known for producing diverse extracellular enzymes (Dias et al., 2009; Khianngam et al., 2013). Recently, a metagenomic study from Saudi Arabia reported the identification of microbial communities in Red Sea mangrove (*Avicennia marina*) (Alzubaidy et al., 2016). However, it was not a functional study, unlike the present study. Another study from the same region reported the presence and antimicrobial properties of bacterial communities in Red Sea sediments (Al-Amoudi et al., 2016). In the present study, isolates of γ-Proteobacteria were prevalent and demonstrated immense biotechnological potential. Our data corroborate previous results regarding the potential of mangrove bacterial communities, especially those of genus *Bacillus*, which was predominant among other isolates, produced hydrolytic enzymes, and exhibited antimicrobial activity (Ando et al., 2001; Tabao and Moasalud, 2010). This is the first study in Saudi Arabia that isolated hydrolytic enzyme-producing bacteria from mangroves and screened them for antifungal activity.

In conclusion, isolation of bacteria from mangroves on enzymatic media resulted in the identification of a large number of enzyme-producing isolates with antifungal activity. These observations suggest a potential role of these bacteria in host plant defense against different pathogens. Finally, mangrove plants could be important sources of industrially and pharmaceutically useful bacteria that can be used for enzyme and antibiotic production.

Conflicts of interest

The authors declare no conflict of interest.

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