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Diversity and antagonistic potential of bacteria isolated from marine grass *Halodule uninervis*

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Abstract
The aim of this study was to isolate bacteria from sea grass, *Halodule uninervis* collected from the coastal area of Jeddah, Saudi Arabia and to screen them for antifungal and enzymatic activities. We have isolated 162 rhizo and endophytic bacteria from soil, roots, and leaves of the sea grass. Antifungal screening of isolated bacteria revealed 19 strains (11.7%) capable to inhibit growth of four pathogenic fungi, *Pythium ultimum*, *Phytophthora capsici*, *pyricularia oryzae,* and *Rhizoctonia solani* in an in vitro assay. Taxonomic and phylogenetic analyses on the basis of 16S rRNA gene sequence revealed 97–99.9% sequence identity to recognized species. *Bacillus*, *Staphylococcus*, *Jeotgalicoccus,* and *Planococcus*, within the Phylum Firmicutes, *Kocuria*, *Arthrobacter*, *Ornithinimicrobium* and *Corynebacterium* (Actinobacteria), *Sulfitobacter*, *Roseivivax*, *Ruegeria* (α-Proteobacteria), *Moraxella,* and *Vibrio* (γ-Proteobacteria), were isolated. Strains belong to Phylum Firmicutes remain dominant antagonistic bacteria in this study. Further hydrolytic enzyme production was determined for these antagonistic bacteria. Our results demonstrated that the sea grass represents an important source of diverse antagonistic bacteria capable of producing antifungal metabolite.

Keywords Sea grass · Antagonistic bacteria · Pathogenic fungi · 16S rRNA gene sequence · Phylogenetic analysis

Introduction

Marine environment is a reservoir of many novel bioactive compounds. Until now, thousand of unique compounds have been isolated from marine environment (Ireland et al. 1993; Newman and Cragg 2004; Hu et al. 2015). In past, for the discovery of a new compound, different marine samples from marine environment have been searched. Many studies have reported the antagonistic bacterial communities associated with marine plants suggesting their beneficial role in plants (Shin et al. 2007; Bibi et al. 2012). In this mutualistic relationship, microbial communities in marine environment help plant in different processes such as growth promotion, nitrogen fixation, remediation of different contaminants, and defense against different pathogenic microbes and in turn plant provide shelter to microbes (Singh and Reddy 2014). Antagonistic microbes associated with marine plants are believed to produce some bioactive compounds to protect host plant from diseases and predators (Bhatnagar and Kim 2010). Therefore, marine environment is widely considered as promising source of identifying new bacteria and their novel metabolites.

Marine bacteria are distributed widely in marine areas from intertidal to deep sea and also in extreme places such as hydrothermal and polar sea. This describes their implausible tendency to adapt to different environment by developing different approaches to survive. These approaches include their ability to produce different metabolic pathways in competitive environment of sea (Harvey 2008). This is the reason that most of the marine antimicrobial compounds previously reported are originated from marine host surface-attached bacteria (Shin et al. 2007; Chen et al. 2012).

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Sea grasses provide habitat to various coastal marine organisms. Microbial communities of sea grasses play an important ecological role in marine environment (Pereg et al. 1994; Marhaeni et al. 2011). Many previous studies have reported rhizo and endophytic population of bacteria from sea grasses (García-Martínez et al. 2005; Jensen et al. 2007; Bagwell et al. 2002; Garcias-Bonet et al. 2016). There are several studies related to secondary metabolites production by sea grasses to protect themselves from marine pathogens (Zidorn 2016). Whereas, studies related to isolation of bacteria producing antagonistic metabolites from sea grasses are very few.

The Red Sea is characterized by a unique epipelagic zone with high temperature and salinity, unique coral reef systems, and marine biota. Hence, these components help in the production of unique microbial communities and secondary metabolites. In the Kingdom of Saudi Arabia, the Red sea has not been explored enough for biotechnological and biopharmaceuticals purposes. Focusing on this, and considering the importance of sea grasses and their antagonistic bacterial communities, we have selected sea grass *Halodule uninervis* for isolation of bacteria from the Red sea coast in Jeddah. Therefore, this study was undertaken to isolate and screen bacteria for their antifungal activity from sea grass *H. uninervis,* and furthermore, these bacteria were assayed for their potential to produce hydrolytic enzymes.

Materials and methods

Sample collection and isolation of bacteria

Plant specimens were collected from the coastal area of Obhur, Jeddah, Saudi Arabia (N21°42′57″; E39°4′48″). Plant specimens were placed in sterile bag and transferred to laboratory for bacterial isolation. For the isolation of bacteria from adhering soil, root was dipped in sterile distilled water to remove adhering soil and serially dilutions were made (10^{-3}, 10^{-4}, and 10^{-5}) in autoclaved filtered sea water (AFS) and spread on one-tenth strength R2A (1/10 R2A), nutrient agar in 50% FS, and Zoebell marine agar. Roots and leaves were also used for isolation of bacteria by washing several times with sterile distilled water to remove any contamination with soil. For isolation of endophytic bacteria from roots and leaves, sterilization procedure was carried out as described before (Bibi et al. 2012). After the sterilization of roots and leaves, about 1.0 g of each segments of roots and leaves were crushed using sterile mortar and pestle. Aliquots (0.1 ml) were serially diluted (10^{-3}, 10^{-4}, and 10^{-5}) using AFS and spread on media mentioned above. To avoid contamination, 50 µg/ml cycloheximide was mixed to the medium before pouring. The plates were incubated at 26 °C for almost 2 weeks and pure colonies were picked

and streaked further for bioassay. For further use, the strains were subcultured and stored in 15% (v/v) glycerol stock of media at − 80 °C.

Detection of antagonistic activity against fungal pathogens

Antagonistic assay of bacteria isolated from soil, roots, and leaves was determined by checking fungal pathogens' growth inhibition using a confrontation bioassay. The plant pathogenic fungi, *Pythium ultimum*, *Phytophthora capsici*, *pyricularia oryzae,* and *Rhizoctonia solani,* were obtained in this laboratory. Antagonistic assays against pathogenic fungi were performed using half-strength (½) R2A and ½ PDA in 50% filtered seawater. All the isolated bacteria were streaked at equidistant places in the plates and mycelial disc of fungal pathogens was placed in the centre of the plate and incubated at 28 °C for 2–4 days. All isolated strains were checked for antagonistic activity in two independent replicates. The antagonistic activity was then checked by measuring the fungal mycelial inhibition area around the bacterial colony.

Extraction of bacterial DNA and 16S rRNA gene sequencing

The 19 antagonistic strains were further used for genomic DNA extraction using DNA extraction kit (Thermo Scientific, Waltham, USA) for 16S rRNA gene sequencing to identify bacterial strains. The 16S rRNA gene fragment was amplified using bacterial universal primers 27F (5′-AGA GTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTAC CTTGTTACGACTT-3′). Amplifications were performed as described previously (Bibi et al. 2012). The PCR products were purified using PCR purification kit (Thermo Scientific, Waltham, USA) and were sequenced by Macrogen (Seoul, Korea). 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 Center for Biotechnology Information (NCBI). For phylogenetic analysis, multiple alignments of the sequences were performed using CLUSTALX (Thompson et al. 1997), and using the BioEdit software (Hall 1999), gaps were edited. The phylogenetic tree based on 16S rRNA gene sequences was constructed using the neighbor-joining method in a MEGA6 Program with bootstrap values based on 1000 replications (Tamura et al. 2013).

Production of hydrolyzing enzymes

From these antagonistic bacteria, production of fungal cell wall hydrolysis enzymes was measured. Amylase activity was determined according to described method (Kumar et al.

2012). To determine cellulase activity carboxymethylcellulase (CMC), agar media were used as described previously (Hendricks et al. 1995). Clear zone around bacterial colonies on CMC agar indicates positive activity. Antagonistic bacteria were further screened for proteolytic activity using skim milk ½ R2A agar plates containing 10.0 gl⁻¹ of skim milk. The isolates showing zones of skim milk clearance are designated as protease producing bacteria. Lipase activity of the antagonistic isolates was detected on tributyrin ½ R2A agar plates containing 1% (v/v) of tributyrin. Lipase-producing bacteria make clear zones by hydrolysis of tributyrin.

Nucleotide sequence numbers

In this study, nucleotide sequences of 19 antagonistic strains have been deposited in the GenBank database under accession numbers KT989842–KT989860.

Results

Isolation of rhizo and endophytic bacteria

To determine the rhizo and endophytic bacterial population in marine grass, *H. uninervis*, and soil attached with soil, roots, and leaves samples, have been used for isolation of bacteria. For isolation, different culturing media were used in different concentrations to increase recovery of bacterial population from different parts of plants. A total of 162 morphologically distinct bacterial strains were isolated from rhizo and endosphere of *H. uninervis*, growing in the coastal area of Jeddah during Nov 2015. Majority of the bacterial strains (40.7%) were isolated from rhizosphere. While from root, 38.2 and 22.2% were obtained from leaf using different culturing media.

Screening of bacteria on the basis of antifungal activity

All isolated strains of bacteria were screened for their inhibitory activity against oomycetes pathogenic fungi, *Py. ultimum* and *P. capsici* in an in vitro assay and active one were further tested for other fungal pathogens, i.e., *P. oryzae* and *R. solani*. Of the 162 tested bacteria, only 19 (11.7%) exhibit inhibitory activity against fungal pathogens used. Activity of these bacteria against *Py. ultimum* was significant as compared to *P. capsici*. All active isolates also showed activity against two other fungi, where stronger inhibition was observed against *P. oryzae* (84.2%) as compared to *R. solani* (36.8%) (Table 1). From root, more number of antagonistic bacteria (52.6%) were isolated as compared to soil (36.8%) and leaves (10.5%). Among the 19 antagonistic bacteria, *Bacillus*

and *Staphylococcus* were dominant genera followed by species of *Vibrio* and *Kocuria*. The strong fungal inhibition was observed for rhizospheric strains EA25 and EA28 with 9–12 mm mycelial inhibition of oomycetes. Another endophytic strain EA44 showed same spectrum of fungal mycelia inhibition with 9–12 mm (Table 1). The antagonistic rhizobacteria EA27 and EA31 had weak inhibition activity (3 mm, +) against oomycetes pathogens and both were inactive against *P. oryzae* and *R. solani*. While rest of the strains showed moderate activity (4–6 mm). Endophytic isolates from roots and leaves also showed weak-to-moderate activity.

### Identification of antagonistic bacteria and their phylogenetic analysis

Antagonistic rhizo and endophytic bacteria were further identified by partial 16S rRNA gene sequence analysis. Thirteen different genera were encountered and further assigned to four major classes: Firmicutes (*n* = 8; 42.1%), Actinobacteria (*n* = 5; 26.3%), α-Proteobacteria (*n* = 3; 15.7%), and γ-Proteobacteria (*n* = 3; 15.7%) (Fig. 1).

Phylogenetic analysis was carried out and a tree was constructed from the data using the neighbor-joining method (Fig. 2). Phylogenetic tree based on 16S rRNA gene sequences obtained from this study and data retrieved from NCBI for closely related strains have shown significant branching patterns with high bootstrap values (100%) (Fig. 2). The antagonistic bacterial strains exhibited sequence similarity of 96.6–99.9% with the related-type strains. High bootstrap values have been observed for strains of all representative classes. The dominant phylum of antagonistic strains was Firmicutes (42.7%), where *Bacillus* was the dominant genus, and revealed their broad distribution among the host plant (Fig. 1). The antagonistic rhizo and endophytic bacteria from phylum Firmicutes were comprised of the 4 genera, *Bacillus* (*n* = 3; 37.5%), *Staphylococcus* (*n* = 3; 37.5%), *Jeotgalicoccus* (*n* = 1; 12.5%), and *Planococcus* (*n* = 1; 12.5%). Actinobacteria (*n* = 5; 26.3%) with the sequence similarity of 97.7–99.4% were accounted for different parts of the host plant. Actinobacteria comprised of four different genera, mainly *Kocuria* (*n* = 2; 40%), *Arthrobacter* (*n* = 1; 20%), *Ornithinimicrobium* (*n* = 1; 20%), and *Corynebacterium* spp. (*n* = 1; 20%). Actinobacteria were mainly recovered from roots of host plant. The antagonistic rhizo and endophytic bacteria from class Proteobacteria were belonged to α-Proteobacteria and γ-Proteobacteria. Both these subdivisions were comprised of six rhizo and entophytic bacteria with the similarity of 16S rRNA gene sequence 97.0–99.9%. The antagonistic isolates belonging to class Proteobacteria comprised of five genera (Table 1).

Table 1 Taxonomic identification, antagonistic activity, and enzyme production of rhizo and endophytic bacteria from sea grass

Strain lab no	Accession numbers	Closely related-type strain	Identity (%)[a]	Antibacterial activity against[b]				Enzymatic activities[c]			
				Py. ultimum	P. capsici	P. oryzae	R. solani	Amylase	Cellulase	Protease	Lipase
Soil											
EA25	KT989842	Sulfitobacter dubius KMM 3554^T	97	++++	+++	+++	−	−	−	−	−
EA26	KT989843	Moraxella osloensis NCTC 10465^T	99.30	++	+	+	+	−	−	−	−
EA27	KT989844	Vibrio neocaledonicus NC470^T	99.90	+	+	−	−	+	−	+++	+
EA28	KT989845	Staphylococcus epidermidis ATCC 14990^T	99.40	+++	+++	+++	+++	−	−	++	−
EA29	KT989846	Arthrobacter flavus JCM 11496^T	98.90	++	−	+	−	−	−	−	−
EA30	KT989847	Planococcus donghaensis JH 1^T	96.60	++	+	++	−	−	−	+	−
EA31	KT989848	Bacillus subtilis subsp. inaquosorum KCTC 13429^T	99.50	+	+	−	−	−	+	−	−
Root											
EA32	KT989849	Roseivivax lentus S5-5^T	98.80	++	−	++	−	−	−	−	−
EA33	KT989850	Kocuria palustris DSM 11925^T	97.70	w	+	+	+	+	−	−	−
EA34	KT989851	Bacillus sonorensis NBRC 101234^T	98.60	+	++	+	+	−	−	+	−
EA35	KT989852	Bacillus safensis FO-36b^T	97.10	+	++	++	++	+	+	++	+
EA36	KT989853	Vibrio neocaledonicus NC470^T	99.90	+	+	++	−	−	−	+	−
EA37	KT989854	Ornithinimicrobium humiphilum DSM 12362^T	98.60	+	+	+	−	−	−	+	−
EA38	KT989855	Corynebacterium afermentans subsp. afermentans CIP 103499^T	99.40	+	++	++	−	−	−	−	−
EA39	KT989856	Kocuria atrinae P30^T	97.70	++	++	++	++	−	−	−	−
EA40	KT989857	Staphylococcus haemolyticus ATCC 29970^T	98.90	++	+	−	+	−	+	++	++
EA41	KT989858	Staphylococcus hominis subsp. novobiosepticus GTC 1228^T	99.10	++	+++	w	+++	−	−	++	+
Leaf											
EA 42	KT989859	Ruegeria mobilis NBRC 101030^T	98.30	+	w	−	−	−	−	++	−
EA 43	KT989860	Jeotgalicoccus aerolatus MPA-33^T	99.90	+++	+++	++++	−	−	−	−	−

[a] Identification based on partial 16S rRNA gene sequence analyses of all antagonistic bacteria

[b] Antagonistic activity of all bacteria isolated in this study. The activity was measured after 2–4 day incubation at 28 °C by measuring the clear zone of mycelial growth inhibition: +, 3 mm; ++, between 5 and 6 mm; +++, between 8 and 9 mm; ++++, between 11 and 12

[c] Production of amylase, cellulase, protease, and lipase was determined by plate assay. Enzymatic activity was estimated as zone of halo formed around bacterial colonies: −, Negative; +, 3 mm; ++, between 4 and 5 mm; +++, between 6 and 7 mm
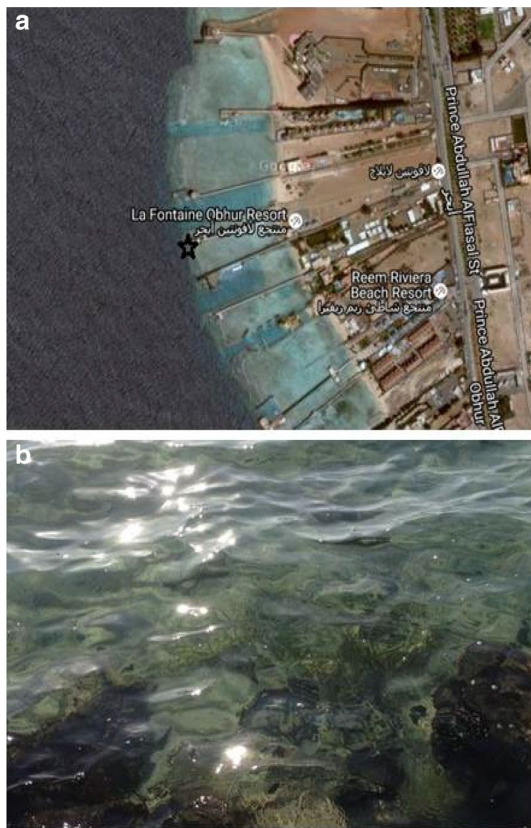
Fig. 1 Map and location of plant collection area from coastal are. **a** Location of coastal area of Jeddah, Saudi Arabia, used for plant collection marked with asterisk. **b** Photographic image of site from where plant specimen was collected. Map of the Obhur region was generated from maphill (http://www.maphill.com)
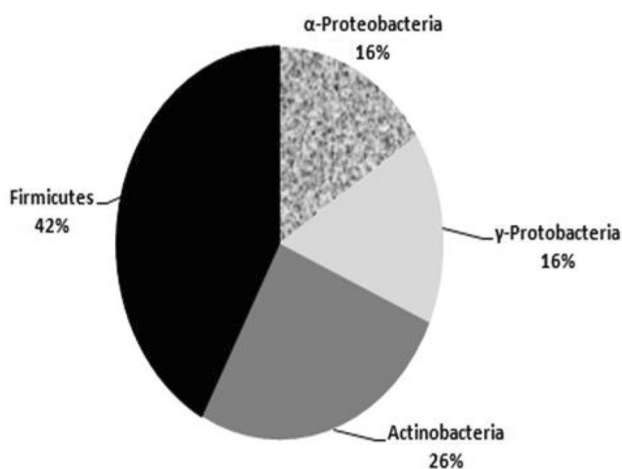
Fig. 2 Percentage composition of different phyla of antagonistic rhizo and endophytic bacteria isolated from marine grass on the basis of 16S rRNA gene sequence similarity

Production of hydrolytic enzymes by antagonistic bacteria

Furthermore, these antagonistic bacteria were considered for their production of hydrolyzing enzymes (amylase, cellulase, protease, and lipase) (Table 1). High proportion of antagonistic bacteria exhibited protease activity ($n = 9$; 47.3%) as compared to other enzymatic activities. Members of phylum Firmicutes such as EA27, EA28, EA35, EA, 40, and EA42 had stronger protease activity. The antagonistic strain EA28 displayed strong inhibitory activity against fungal pathogens and showed strong protease activity as well. Lipase production was observed at lower rate as compared to protease activity ($n = 4$; 21%). Three Firmicutes strains EA35, EA40, and EA42 and one γ-Proteobacteria strain EA27 showed lipase activity. Cellulase activity ($n = 3$; 15.7%) was observed only in strain EA27, EA33, and EA35, belonged to γ-Proteobacteria, Actinobacteria, and Firmicutes.

Discussion

Our objective was to isolate rhizo and endophytic bacteria from sea grass and to determine their antagonistic potential, taxonomic identity, and enzymatic activities. In this study, the nutrient media used for isolation of rhizo and endophytic bacteria effectively influenced the number of bacteria cultured. From soil, maximum numbers of bacteria were cultivable on ZMA, while more bacterial isolates were counted from roots and leaves on ½ R2A. Complex media contain more nutrients that allow fastidious bacteria to grow, while minimal media contain lower concentration of nutrients hence allow slow growing bacteria to grow (Hottes et al. 2004; Majzlik et al. 2011). While some slow growing bacteria need prolong period for growth on minimal media (Connon and Giovannoni 2002; Alain and Querellou 2009). In this study, the numbers of bacteria were highest on ½ R2A as compared to other two media used for culturing. Different rhizo and endophytic bacterial species can be cultivated from plants depending on the culturing media used for isolation, incubation conditions.

Sea grasses not only provide food for different marine organisms, but also harbour different bacterial communities as evident from the present study. In this study, more rhizospheric bacteria were isolated from sea grass, but they are less antagonistic than endophytic bacteria, where more antagonistic endophytic bacteria recover from root tissues of sea grass. Several previous studies also reported endophytic bacteria as potential candidates to defend host plant against invading pathogens (Podolich et al. 2015). Endophytes are more beneficial than rhizospheric antagonist-counterparts due to their ability to enter the host plant and trigger host defense system. Being inside host, they protect host against

different pathogens by production of active compounds (Thomas and Upreti 2014). In this study from sea grass, 162 bacteria were isolated. Their screening on the basis of antifungal activity results in 19 antagonistic bacteria. Bacterial identification on the basis of 16S rRNA gene sequence is a standard method for identification. These antagonistic

bacteria further were identified on the basis of 16S rRNA gene sequence and belong to Firmicutes, Actinobacteria, α-Proteobacteria, and γ-Proteobacteria.

The phylogenetic analysis using 16S rRNA gene sequence, group all rhizo and endophytic bacteria into four phyla, Firmicutes (*Bacillus*, *Staphylococcus*, *Jeotgalicoccus*,

Fig. 3 Phylogenetic distribution of antagonistic bacteria isolated from marine grass on the basis of 16S rRNA gene sequences obtained from rhizo and endophytic bacteria and closely related sequences of the type strains of other species. The phylogenetic relationships were inferred from the 16S rRNA gene 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 numbers for each sequence are shown in parentheses. Bar, 0.01 accumulated changes per nucleotide

and *Planococcus*), Actinobacteria (*Kocuria*, *Arthrobacter*, *Ornithinimicrobium,* and *Corynebacterium*), α-Proteobacteria (*Sulfitobacter*, *Roseivivax,* and *Ruegeria*), and γ-Proteobacteria (*Moraxella* and *Vibrio*) (Figs. 2, 3). Our results indicated that Firmicutes was the most dominant group. Bacteria belonging to genus *Bacillus* were more common in both rhizo and endophytic bacteria isolated in this study. Antagonistic properties of Firmicutes from marine environment are already reported (Thomas et al. 2010; Mondol et al. 2013). Species of *Bacillus* are easy to culture and can survive under harsh conditions. Bioactive metabolite and enzyme production is common feature of *Bacillus* species (Mondol et al. 2013; Sinimol et al. 2016). In addition to *Bacillus*, *Staphylococcus* is another representative of Firmicutes in this study, and is already known to produce antimicrobial metabolites (Barbieri et al. 2005; Mani et al. 2016). The second phylum of this study Actinobacteria was recovered from both soil and root of the sea grass. Actinobacteria both in terrestrial and marine environment are rich source of antibiotic production and are resistant to different fungal and bacterial pathogens (Manivasagan et al. 2014; Hamedi et al. 2015). In this study, most of the Actinobacteria were isolated from roots of the sea grass. There is diversity in *Actinobacteria* spp. of root as they belong to different genera and are different from Actinobacteria isolated from rhizosphere in this study.

In this study, 32% of the antagonistic bacteria belong to two groups of Proteobacteria, α-Proteobacteria, and γ-Proteobacteria. Species of different genera, *Sulfitobacter*, *Roseivivax,* and *Ruegeria* mainly belong to α-Proteobacteria. Strains belong to *Sulfitobacter* and *Ruegeria* and *vibrio* already reported for antimicrobial activities (Desriac et al. 2013; Blunt et al. 2015). While for *Roseivivax* and *Moraxella* spp., no such activity has been reported before.

Some antagonistic bacteria isolated in this study were also able to produce hydrolytic enzymes. These hydrolases producing bacteria mainly belong to Firmicutes. Most of the isolates exhibited proteolytic activity. Others showed cellulase, protease, and lipase activities. Production of these hydrolases indicates their potential to colonize root intracellularly by producing such hydrolytic enzymes. Strain EA35 belong to Firmicutes was positive for all enzymatic activities tested (Table 1). To the best of our knowledge, this is the first study ever presented from Saudi Arabia. Here, we isolated rhizo and endophytic bacteria from sea grass and checked their antifungal and enzymatic potential.

Conclusion

The objective of present study was to isolate and screen bacteria from sea grass for their antagonistic and enzymatic activities. Use of different media for culturing and further screening for antifungal activity results in diverse group of antagonistic rhizo and endophytic bacteria. Furthermore, enzymatic activities of these antagonistic bacteria confirm their potential role in defense of host plant against different pathogens. Overall, sea grass appears to be an important source of antagonistic bacteria that can be use for antibiotic production or as biocontrol agents. Our findings will provide a basis for further studies related to the microbiome of Red sea in Saudi Arabia.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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