Expression of SidD gene and physiological characterization of the rhizosphere plant growth-promoting yeasts

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A R T I C L E   I N F O

Keywords: Microbiology Siderophores Gene expression SidD gene Rhizosphere yeasts

A B S T R A C T

There is increasing evidence that rhizosphere microbes contribute to the stress mitigation process, but the mechanisms of this plant-microbe interaction are not yet understood. Siderophores-producing microorganisms have been considered important for enhancing metal tolerance in plants. In this study, rhizosphere yeasts were isolated from wheat (Triticum aestivum L.) and examined for siderophores production and heavy metal resistance. Out of thirty-five isolates, only eight yeast strains showed heavy metal-resistance and plant-growth promotion properties. The highest inorganic phosphate-solubilization was shown by Trichosporon ovoides IFM 63839 (2.98 mg ml⁻¹) and Saccharomyces cerevisiae FI25-1F (2.54 mg ml⁻¹). Two strains, namely YEAST-6 and YEAST-16 showed high siderophore production and heavy metal-resistance, were investigated for sidD gene expression under different levels of Cd²⁺ and Pb²⁺ toxicity stress. The heavy metal-resistant yeast strains were characterized and identified based on the phenotypic characteristics and their 18S rRNA genes sequence. SidD gene expression was induced by yeasts growing under iron-limiting conditions and excess of other heavy metal, suggesting that expression of sidD gene increases in the presence of 600–800 μM heavy metal but under iron limitation. Extensive studies of the microbe-plant micronutrient interactions will enrich our understanding of the rhizosphere role in the terms of plant growth promotion.

1. Introduction

The microbes’ siderophores form siderophore-complexes to deliver Fe to plants, simultaneously, the plant growth-promoting rhizomicrobes can also bind with other heavy metals for decreasing their bioavailability and toxicity (Khaksar et al., 2016). Metal-resistant and metal immobilizing plant growth-promoting rhizomicrobes have recently been studied to induce growth and reduce the heavy metals bioavailability and their accumulation within plants (Wang et al., 2013). Also, Khaksar et al. (2016) illustrated that micro-organisms might enhance heavy metal tolerant-proteins regulation, plant defense system, and phytohormones synthesis under Cd²⁺ stress. The observation that toxic metals induce the production of some siderophores proposes that these chelators may play an important role in bacterial heavy metals tolerance. Toxic metals enter the periplasm of Gram-negative bacteria mostly by diffusion across the porins (Khaksar et al., 2016). Thus, the binding of metals to siderophores in the extracellular medium reduces the free metal concentration, probably affecting the diffusion and therefore their toxicity (Sullivan and Gadd, 2019).

The main function of siderophore is to chelate Fe (III). Siderophores also form complex compounds with the other heavy metals, such as Fe³⁺, Cu²⁺, Zn²⁺, Ni²⁺ and Cd²⁺. Siderophores also form complexes with metals such as Cu²⁺, Zn²⁺ and Pb²⁺ and this leads to influence the metal mobility in the environment providing an important strategy to sequester toxic metals (Fu et al., 2016). Many studies reported that heavy-metal tolerant microbes can enhance directly the efficiency of siderophores production. It was reported that siderophores were interacting with other metals, therefore there is a competition for its binding sites between iron and other free metal ions such as Cd²⁺, Cu²⁺, Pb²⁺, and Zn²⁺ (Tóth et al., 2016).

Siderophore biosynthesis initiates from some precursors such as amino acids, citrate, and N5-acyl-N5-hydroxyornithine. Several genes for siderophore regulation have been identified in fungi that also have homologs in other microbial taxa (Ramos-Garza et al., 2015). In broadly studied fungi, siderophore operon includes sidA, sidC, sidD, sidG, sidF, and recently sidl gene, the locations of these genes are different on the fungal chromosomes. Unlike all siderophore genes, sidDis labeled as non-ribosomal peptide synthetase (NRPS) (Kozubal et al., 2019). Most of
fungal siderophores are formed simply through condensation of the NRPSs with N5-acyl-N5-hydroxy-L-ornithine units encoded by the conserved sidD genes (Kozubal et al., 2019). Under iron-deficiency conditions, the increased expression of the gene encoding iron/siderophore sidG was reported (Sullivan and Gadd, 2019). Moreover, metal resistant and growth-promoting bacteria provide resistance to plants in metal-polluted sites by the production of phytohormones such as gibberellins, indole-3-acetic acid (IAA), etc. They also lead to the production of phytates-solubilizing acids, 1-aminoacyclopropane-1-carboxylic acid (ACC) – deaminase, and siderophores that promote the plant growth, defense properties, and reduce the translocation of heavy metals within the plant tissues (Ramos-Garza et al., 2015; Sun et al., 2014). Burkholderia sp. D54 significantly improved the growth of Sedum alfredii by oxidizing Fe²⁺ and Mn⁴⁺ that further leads to Fe precipitation and prevents the uptake of heavy metals on the root surfaces (Guo et al., 2013). Similar trends were also detected by Dong et al. (2017) in which Burkholderia sp. inoculation reduced Cd translocation in rice plants and metal immobilizer plant growth-promoting microbes is a crucial factor in rhizosphere soils of wheat (Suksabye et al., 2016). B. subtilis was reported as metal resistant bacteria that reduced the Cd accumulation and promoted the growth of Oryza sativa grown under Cd stress conditions (Sukasabye et al., 2016).

Microorganisms with the most effective siderophores mediated iron uptake have competitive advantages for using in biocontrol and as biofertilizers. Specific nutrients are required for siderophores discharge and might vary from one microbe to another contributing an active role in its secretion. However, iron-controlled conditions stay unchanged (Ferreira et al., 2019). A better understanding of characteristics of metal resistant and metal immobilizer plant growth-promoting microbes is a crucial demand for the establishment of eco-friendly and effective strategies for crop production in heavy metals polluted environment. Only a few studies investigated yeasts in terms of plant growth promotion, however, different yeast genera are vital components of soil. Very often they contain multiple mechanisms mediated plant growth promotion.

The objectives of this study were to explore the key traits of plant-growth promotion in yeast strains, isolated from rhizospheric soil, and detect their tolerance to heavy metals stress. The sidD gene expression, yeast growth promotion in yeast strains, isolated from rhizospheric soil, and metal immobilizer plant growth-promoting traits as gibberellins, indole-3-acetic acid (IAA), etc. They also lead to the production of phytohormones such as gibberellins, indole-3-acetic acid (IAA), etc. They also lead to the production of phytates-solubilizing acids, 1-aminoacyclopropane-1-carboxylic acid (ACC) – deaminase, and siderophores that promote the plant growth, defense properties, and reduce the translocation of heavy metals within the plant tissues (Ramos-Garza et al., 2015; Sun et al., 2014). Burkholderia sp. D54 significantly improved the growth of Sedum alfredii by oxidizing Fe²⁺ and Mn⁴⁺ that further leads to Fe precipitation and prevents the uptake of heavy metals on the root surfaces (Guo et al., 2013). Similar trends were also detected by Dong et al. (2017) in which Burkholderia sp. inoculation reduced Cd translocation in rice plants and metal immobilizer plant growth-promoting microbes is a crucial factor in rhizosphere soils of wheat (Suksabye et al., 2016). B. subtilis was reported as metal resistant bacteria that reduced the Cd accumulation and promoted the growth of Oryza sativa grown under Cd stress conditions (Sukasabye et al., 2016).

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2. Materials and methods

2.1. Micro-organisms and culture conditions

The plant growth-promoting fungi (PGPF) strains were isolated from saline rhizosphere soil of wheat (T. aestivum L.) New Vally, Egypt. The soil samples were specifically taken in zipper bags and kept at 4 °C. All fungi were isolated and purified on malt extract agar (MEA) using the dilution-plate technique. Stock cultures of fungi were well maintained and stored at 4 °C before identification and plant growth-promoting traits investigations.

2.2. Identification of strains

The isolated fungal strains were identified using direct microscopic examination and culture features according to Moubasher (1993). The extracted DNA pellet of selected fungal strains was washed in 70% ethanol (300 ml). The air-dried DNA pellet was dissolved in deionized H₂O (50 ml), then 1 ml of the washed DNA was used in 30–50 ml of PCR mixture. The DNA was extracted in duplicate for each sample. The primers ITS1F: 5′-TCCGTAAGGTGAACCTGCGG-3′ and ITS4: 5′-TCTTTGAGCTTATGATATGC-3′ were used for fungal amplification (Herlemann et al., 2011). PCR amplifications were performed in a thermal cycler for 30 cycles at 94 °C for 1 min for DNA denaturation, primer annealing at 56 °C for 30 s, and primer extension at 72 °C for 1 min. The PCR products were sequenced at Macrogen Inc. The partial gene sequences were matched with the full sequences presented in the GenBank database using the BLAST search (NCBI). The structure of the phylogenetic tree was produced by the online tool PhyML (www.phylogeny.fr), and visualization of the tree was achieved using TreeDynt.

2.3. Siderophore production

The siderophore-producing microorganisms were detected in the medium using chrome azurol S (CAS) agar plate assay. Due to the high toxicity of the CAS-agar blue medium, the CAS-agar half-plate technique was conducted according to Hussein and Joo (2014) using MEA media pH 5.6 for the qualitative detection of fungus production. The medium was inoculated and incubated at 27 °C for 7 days and the observed red zone was measured. The plates were checked daily for color development, the change in blue color indicates the siderophores activity. The CAS-liquid assay was carried out according to Schwyn and Neilands (1987) to estimate the production of the siderophore quantitatively. The pH was adjusted to pH 6.8 with a buffer of 0.1 M Pipes (Sigma, Prod. No. P1851). The cultures were agitated to the stationary phase in deferrated media. 0.5 ml CAS solution was added to 0.5 ml culture supernatant and 10 μl shuttle solution (5-sulfoisalicilic acid 0.2 M) and was mixed carefully. The mixtures were kept for 10 min. The color development was determined by absorbance (A630) for a lack of blue color using UV-VIS spectrophotometer (Agilent Co., USA). An uninoculated culture medium was served as blank and the uninoculated culture medium containing CAS and shuttle solutions were served as a reference. The siderophores production was calculated as [(As–Ac)/As] 100 = % siderophores units; where As is the absorbance value of the sample, and Ac is the absorbance value of the reference. The investigation was conducted in triplicate and average values were used.

2.4. Phosphate solubilization assay

Solubilization of the precipitated Ca₃(PO₄)₂ on Pikovskaya’s agar medium contained L⁻¹: Ca³⁺(PO₄)₂, 5 g; glucose, 10 g; (NH₄)₂SO₄, 0.5 g; yeast extract, 0.5 g; NaCl, 0.2 g; KCl, 0.2 g; MgSO₄·7H₂O, 0.1 g; Fe₂O₃·7H₂O, 0.002 g; and MnSO₄·H₂O, 0.002 g was used for isolation of phosphate-solubilizing fungi (Pikovskaya, 1948). The inoculated fungi were incubated at 27 °C for 7 days and the developed clear zone was measured (Pingale and Virkar, 2013; Yasmin and Bano, 2011). Phosphate solubilization was estimated quantitatively by inoculating 10 ml of Pikovskaya’s broth (initial pH was adjusted to 7.0) using an isolation loop full of spore suspension; cultures were incubated at 27 °C for 5 days and fungi at 27 °C for 7 days. To remove the color impurities, the broth was filtrated using Whatman No. 2 filter, and the supernatant was centrifuged at 10000 rpm for 10 min. In 96 well plate, an equal volume of supernatant and Barton’s reagent were added and left for 10 min, the intensity of the color was assessed using a colorimeter system (biotech nanodrop, USA) at wavelength 430nm, the phosphorus quantity was estimated using standard curve (Pingale and Virkar, 2017).

2.5. Indole acetic acid production

Seventeen different fungal isolates were quantitatively tested to produce indole-3-acetic acid IAA following Brick et al. (1991). Fungi were grown in Czapek-Dox broth modified with tryptophan (1000 μg ml⁻¹), as carbon source, instead of NaNO₃. Yeast isolates were inoculated with an isolation loop full of actively growing spores’ suspension in Sabouraud solution into 10 ml of liquid Czapek-Dox media, incubated at 30 °C, and agitation at 150 rpm. Following 7 days, each yeast culture was centrifuged for 10 min at 13,000 xg. Salkowski’s reagent (10 mM FeCl₃ in 35% HClO₄) was used to detect indole derivatives. One milliliter of each supernatant was mixed with the same volume of the reagent and incubated for 30 min in the dark. The optical density was measured with a spectrophotometer at 530 nm. The standard curve of IAA concentration...
was prepared and designed to calculate the equivalent concentration of IAA produced by each yeast strain in the bioassay media.

2.6. ACC deaminase activity

ACC deaminase activity was measured by the determination of α-ketobutyrate (Bhagat et al., 2014). Minimal media containing ACC as a sole nitrogen source (g L⁻¹: Na₂HPO₄ 2.13, KH₂PO₄ 1.36, CaCl₂·2H₂O 0.7, FeSO₄·7H₂O 0.2, MgSO₄·7H₂O 0.2, CuSO₄·5H₂O 0.04, MnSO₄·H₂O 0.02, H₃BO₃ 0.003, ZnSO₄·7H₂O 0.02, CoCl₂·6H₂O 0.007, Na₂MoO₄·2H₂O 0.004, 1-aminocyclopropane-1-carboxylic acid (ACC) 5 mM (Sigma-Aldrich Co., A3903), Glucose 10) was prepared and autoclaved. Yeast isolates were grown in 5 ml YPD medium at 27 °C until they reached the stationary phase. The cells were centrifuged, re-suspended in 2 ml minimal medium and incubated at 28 °C with shaking for 36–72 h. The yeast cells were collected by centrifugation for 5 min at 3000g. 0.2 ml supernatant (enzyme extract) and 0.2 ml (50 mM) ACC were added to 0.2 ml Tris-hydroxy methyl amino methane 0.1 M buffer (pH7.5), the mixture was shaken well and incubated at 30 °C for 30 min. 1.8 ml of 0.56 N hydrochloric acids (HCl) was added to stop the reaction. 0.3 ml of 2.7. Minimum inhibitory concentrations (MICs)

Yeast colonies were sub-cultured using (YPD) Broth (g L⁻¹: yeast extract 10, peptone 20, dextrose 20). 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mM of CdCl₂ and Pb (NO₃)₂, separately, were prepared in YPD broth. YPD solutions containing different concentrations of heavy metal were inoculated by the freshly growing yeast isolates. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the metal that inhibits the visible growth of the isolate. The cultures were incubated at 27 °C for 4 days, the experiment was conducted in triplicate. MIC for each strain was recorded.

2.8. Cd and Pb ions effect on siderophores biosynthesis

Siderophore production by potent yeast strains under different concentrations of CdCl₂ and Pb(NO₃)₂ was investigated using chromo azurul S (CAS) plate assay (Schwyn and Neilands, 1987). CAS-agar solution with different concentrations of CdCl₂ and Pb(NO₃)₂ (0, 500, 750 and 1000 μM) was prepared and autoclaved. After cooling, CAS-agar plates were inoculated with yeast strains and incubated at 27 °C for 4 days. Color development or orang halos were detected. Quantitative real-time PCR (qRT-PCR) using sidF-forward (5-ATTCGTGCGAGACTCGGAT-3) and sidF-reverse (5-ATTCGTGGACATCGGAT-3) primers were conducted to confirm the visual detection according to Pandey et al. (2014).

2.9. RNA extraction

50 mg of both stem and leaves were grounded in liquid nitrogen with pestle and mortar, put in a 1.5 ml Eppendorf containing 1 ml of TRIZolTM Reagent, and agitated vigorously for complete lysis of plant tissue (Li and Yao, 2005). RNA was isolated by the phenol/chloroform extraction according to Kay et al. (1987). The RNA was washed with RQ1 DNase (Promega, Spain) and purified by the NucleoSpin RNA Clean-up kit through a silica column (Hoerd, Macherey-Nagel, France) according to the manufacturer’s recommendations. The RNA pellets were air-dried for 15 min, redissolved in 50 ul RNase/DNase free water, and stored at −80 °C until use.

2.10. cDNA synthesis

For the gene expression, complementary DNA (cDNA) was synthesized by adjusting constant concentration (1000ng/μL) at constant volume (15 μL) for each fungal RNA and synthesized into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Biosystems Applied™, US) according to the instructions of the supplier.

2.11. qRT-PCR analysis for RNAs

The quantitative real-time PCR (qRT-PCR) was achieved on the 7500 Real-Time PCR Instrument (Applied Biosystems™, USA) under the following conditions: start step at 95 °C for 7 min, initial denaturation for 20 s at 95 °C, annealing and extension for 60 s at 59 °C in 40 cycles. The relative expression data of total RNA was calculated according to the 2⁻ΔΔCq procedure and presented as fold change (Livak and Schmittgen, 2001). Each qRT-PCR experiment involved a multiplex reaction and GAPDH was used as an internal control (Soltanlou et al., 2010).

2.12. Statistical analysis

Statistical analysis was performed with SAS (2004) using Tukey’s test version 11.0 to compare the means (P > 0.05).

3. Results and discussion

Apart from their roles in plant growth promotion, siderophore-producing microorganisms have been also considered important for enhancing metal tolerance in plants. Shi et al. (2017) concluded that P. aeruginosa exhibited different tolerance to multiple heavy metals. Therefore, the ZGKD3 strain can act as a siderophore-producing microbe and can be applied to plants in Cd and Zn contaminated soils. Shi et al. (2017) indicated that 0.125 mM of Cd could induce siderophore production of P. aeruginosa PA01. Dimkpa et al. (2009) observed that Cd, Cu, and Ni stimulated siderophores production by Streptomyces sp. strains. Some studies demonstrated that siderophores could decrease or increase the toxicity of heavy metal in bacteria. The observation that heavy metals other than iron induce production of siderophores suggests that these chelators might play a role in microbial toxic metal tolerance (Hussein and Joo, 2017). Growth assays showed that bacterial strains capable of producing pyochelin and pyoverdine appeared to be more resistant to metal toxicity than the siderophores non-producing strains (Braud et al., 2010). Yu et al. (2017) revealed that Bacillus lincea could produce an excess of siderophore and might be possibly used to increase the phytoextraction of heavy metals from soil. In this study, a total of 35 rhizosphere yeast isolates were selected at random based on differences in the morphological features of the colonies and tested for heavy metal MIC using CdCl₂ and Pb(NO₃)₂ (Table 1). The strongest heavy metal resistant strains were subjected to siderophores, IAA, and ACCD production and phosphate solubilization activity (Figure 1). Eight of the yeast strains, namely YEAST-1, YEAST-2, YEAST-5, YEAST-6, YEAST-16, YEAST-17, YEAST-30, and YEAST-34, showed significant higher resistance to heavy metals when compared with other strains investigated in the current study. The rhizosphere heavy metal-resistant strains were identified and analyzed based on their 18S rRNA genes sequence.

3.1. Phylogenetic affiliation of the analyzed yeasts

Yeast strains were isolated according to their different morphological features. The parameters considered were colony characteristics, color, shape, size, and spores. The fractional 18S rRNA gene sequences obtained were compared to the accession available in the GenBank database using the BLAST browser (NCBI) to identify the isolated yeast strains (Table 1). The analyzed heavy metal-resistant yeast strains were phylogenetically affiliated by sequencing the 18S rRNA gene. Blast analysis of the complete 18S rRNA showed that the strains YEAST-1 and YEAST-16...
were affiliated with the genera *Yarrowia*, YEAST-2 and YEAST-17 were affiliated with the genus *Candida*, YEAST-5 and YEAST-6 were affiliated with the genus *Trichosporon*, and YEAST-30 and YEAST-30 were affiliated with the genus *Saccharomyces*, with sequence identities of 98–100% (Table 1). Figure 2 shows the phylogenetic tree for the eight isolated yeast strains from the wheat's rhizosphere soil based on comparisons of partial 18s rRNA sequences. The lengths of the upright lines are arbitrary; the lengths of the transversal lines are proportional to genetic distances. Bootstrap frequencies are given for multiple data set of 100 trials. Figure 3 shows the 18s rRNA gene sequence similarity for the isolated heavy metal-resistant yeast strains. It has been evidenced that the siderophores produced by microorganisms can protect them against heavy metals toxicity (Shi et al., 2017). Heavy metal species can bind accidentally to proteins and in this process influences the biological function of the chelator molecules. For example, the microbial inoculations and enzymes activity in the soil has been demonstrated insufficient performance when the metal levels of Cu and Zn were raised (Zannoni et al., 2008). Shi et al. (2017) mentioned that the effects of heavy metals on *P. aeruginosa* were different, the ability of siderophore production by *P. aeruginosa* varied with different heavy metals. The toxicity of heavy metals on bacteria is dependent on heavy metal accumulation in the cells. However, Cd$^{2+}$ and Zn$^{2+}$ significantly stimulated the siderophore production of the ZGKD3 strain. They supposed that more siderophore produced means more heavy metals absorbed by *P. aeruginosa* through the chelation of siderophore for heavy metals, which is known to be highly toxic to bacteria. Braud et al. (2010) demonstrated that the synthesis of siderophores by *P. aeruginosa* decreased the toxicity of multiple heavy metals. Khanna et al. (2019) revealed that Cd reduced root length, shoot length, fresh weight, and photosynthetic pigments of *Lycopersicon esculentum*. However, inoculations of *L. esculentum* seedlings by *P. aeruginosa* and *Burkholderia gladioli* mitigated the Cd stress along with enhancing the metal uptake in Cd-treated seedlings. Khanna et al. (2019) detected that metal resistant PGPR improved growth and photosynthetic pigments in *L. esculentum* under metal toxicity and enhanced expression

**Table 1. Heavy metal-resistant yeasts isolated from rhizosphere soils and their identification.**

| Strains     | Cd$^{2+}$ MIC (mM) | Pb$^{2+}$ MIC (mM) | Max identity (%) | Strain of closest identity | Identification       |
|------------|-------------------|-------------------|------------------|--------------------------|----------------------|
| YEAST-1    | 2.00              | 1.50              | 100%             | *Yarrowia lipolytica* F45 | *Yarrowia lipolytica* YEAST-1 |
| YEAST-2    | 1.75              | 1.75              | 98%              | *Candida diddensiae* MITS575 | *Candida diddensiae* YEAST-2 |
| YEAST-5    | 1.50              | 1.75              | 100%             | *Trichosporon gamsii* CBS8245 | *Trichosporon gamsii* YEAST-5 |
| YEAST-6    | 1.75              | 1.75              | 100%             | *Trichosporon ovoides* IFM 63859 | *Trichosporon ovoides* YEAST-6 |
| YEAST-16   | 1.75              | 2.00              | 99%              | *Yarrowia lipolytica* JCM 2320 | *Yarrowia lipolytica* YEAST-16 |
| YEAST-17   | 1.75              | 1.50              | 100%             | *Candida subhashii* UAMH 10744 | *Candida subhashii* YEAST-17 |
| YEAST-30   | 1.75              | 1.50              | 100%             | *Saccharomyces cerevisiae* FL25-1F | *Saccharomyces cerevisiae* YEAST-30 |
| YEAST-34   | 1.75              | 1.50              | 99%              | *Saccharomyces cerevisiae* MIBA/781 | *Saccharomyces cerevisiae* YEAST-34 |

**Figure 1.** Plant growth-promoting activities of the isolated rhizosphere yeasts; A) IAA production variation among the different heavy metal-resistant strains; B) the phosphate solubilization on Pikovskaya’s agar medium; C) the ACCD activity on minimal media-containing ACC as a sole nitrogen source by the different isolated yeast strains.
Figure 2. Phylogenetic analysis of the isolated heavy metal-resistant yeast strains; A) The phylogenetic tree showing the lengths of the upright lines are arbitrary; the lengths of the transversal lines are proportional to genetic distances. Bootstrap frequencies are given for a multiple data set of 100 trials; B) 18s rRNA gene sequence similarity for the isolated heavy metal-resistant yeast strains.

Figure 3. Effect of heavy metals stress on the gene expression of siderophores by yeast strains. (A) SidD gene expression in Y. lipolytica under different levels of Cd$^{2+}$ ions. (B) SidD gene expression in Y. lipolytica under different concentrations of Pb$^{2+}$ ions. (C) SidD gene expression in T. ovoides under different levels of Cd$^{2+}$ ions. (D) SidD gene expression in T. ovoides under different concentrations of Pb$^{2+}$.
of metal transporter genes. Therefore, they suggested that micro-organisms possess growth-promoting traits that can reduce metal toxicity in plants. It is still unclear how heavy metals other than iron ions stimulate siderophores production (Hussein and Joo, 2019). However, there are two possible hypotheses for the motivating impact of heavy metals on siderophores biosynthesis. First, heavy metal might be required in the siderophores biosynthesis pathway and their control (Rajkumar and Freitas, 2009). Second, the free siderophores amount might decline due to complexes formation with heavy metals ions. Thus, soluble iron is still scarce. Accordingly, more siderophore molecules would then be delivered (Hussein and Joo, 2019). In the current investigation, the CAS blue agar plate assay indicated that all the heavy metal-resistant yeast strains were able to produce siderophores (Table 2).

All siderophores, regardless of catecholate or hydroxamate type, have nitrogen or oxygen electron donors, which can chelate metals cations such as Al^{3+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, etc (Yu et al., 2017). Hussein and Joo (2017) demonstrated that Zn^{2+} alone was able to activate bacterial siderophores synthesis. Heavy metals affect the biosynthesis of the siderophore in microorganisms. Media-containing Zn ions showed a significant impact on pyoverdine biosynthesis of rhizosphere bacteria. The siderophores production of all tested strains was increased by 100 μg ml⁻¹ Zn^{2+}. They also investigated the siderophores synthesis of a variety of rhizosphere fungal strains under Zn^{2+} ion stress. These strains were specifically isolated from the rhizosphere of Panax ginseng. The results indicated that Zn^{2+} ion increased the production of siderophore in iron-limited cultures, and the maximum siderophore production was detected at 150 μg/ml Zn^{2+} ion stress (Hussein and Joo, 2019). Extremely high Cd resistance (the MIC was 2.0 mM of Cd in solid medium) was observed for Yarrowia lipolytica JCM 2320 and Trichosporon ovoides JCM 2320 tolerated 2 mM of Cd and Pb. Braud et al. (2010) found that pyochelin decreased the uptake of Al^{3+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}, and pyoverdine decreased the uptake of Al^{3+}, Cu^{2+}, Eu^{3+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}. Hence, the presence of the siderophores could reduce metal accumulation in microbes.

3.2. ACC deaminase and indole acetic acid (IAA)

ACC deaminase was firstly identified in the yeast species Hansenula saturnus currently re-classified as Cytherbinderina saturnus (Nascimento et al., 2014). Therefore, ACC deaminase- producing microbes may improve plant growth by declining the detrimental effect of stress exerted by ethylene. Fungi which express ACCD can mitigate the different stresses that affect plant crops and development (Brotman et al., 2013). The genes encoding ACC deaminase have been isolated from a variety of soil fungi (Viterbo et al., 2010). Y. lipolytica JCM 2320 showed the maximum ACCD production (52.36 μmol ml⁻¹) (Table 2). Strains YEAST-1 and YEAST-16 (both Y. lipolytica) produced high ACCD, whereas all the other strains only produced less than 30 μmol ml⁻¹ of ACCD. Trichosporon ovoides IFM 63839 and Yarrowia lipolytica JCM 2320 were potent in siderophores and ACCD production. An evaluation of the IAA production of the heavy metal-resistant yeast strains revealed that T. gamsii was the most potent producer of IAA among all the isolates tested (Table 2). Two strains, T. gamsii CBS8245 and S. cerevisiae MIBA781, showed the highest IAA biosynthesis 82.63 μg ml⁻¹ and 72.03 μg ml⁻¹, respectively.

3.3. Expression of the SidD gene

SidD gene is the most likely responsible for the biosynthesis of fungal siderophores. Besides the gene products known to be involved in the synthesis of the extracellular siderophores (e.g. sidD) which are an esterase-like protein, were induced under the same conditions, suggesting its role in extracellular siderophores biosynthesis. Haas (2014) illustrated that the genetic deletion of extracellular siderophores (sidD, sidI, sidH, and sidL mutants) decreases oxidative stress resistance, condensation, and growth during iron-deficiency but not during iron-adequacy, which enables restitution by other iron acquisition systems. In Aspergillus fumigatus, the genes required for the synthesis of the extracellular siderophores showed a significantly increased expression level in germinating spores. In this study, the sidD gene expression for siderophores production by yeast strains was detected under heavy metals stress. Expression of this gene was induced by growing under iron-limiting conditions and excess of other heavy metal, suggesting that expression of sidD gene increases in the presence of 600–800 μM heavy metal but under iron-limiting conditions. Expression of the sidD gene was increased maximally by Y. lipolytica at 600 μM Cd in the iron-free media. As shown in Figure 3, there was an approximate threefold expression of the sidD gene by adding 600 μM Cd or Pb to the iron-free media. SidD gene expression was increased maximally by T. ovoides IFM 63839 at 800 μM Cd in the iron-free media. However, the ions of Pb increased the sidD gene expression gradually from 200 to 800 μM, but still lower than control. In the case of T. ovoides IFM 63839, there was more than fourfold expression of sidD gene by adding 800 μM Cd to the iron-free media. Franken et al. (2014) analyzed the expression of the genes in A. niger using the available transcriptome data sets of Carvalho et al. (2012) and Nitsche et al. (2012) to check whether the siderophore biosynthesis genes show co-regulated expression. They concluded that most of the genes showed low expression under the cultivation conditions, even when using different carbon and nitrogen sources. Several of the identified genes displayed hardly expression under the tested conditions. Most of the siderophore metabolic genes are nearby each other in the genome, which facilitates their co-regulation and expression. Interestingly in this regard, in contrast to siderophores’ metabolic genes, expression of the SidL and PptA genes is not regulated by iron availability. Hussein and Joo

### Table 2. Plant growth promoting activities of the selected heavy metal-resistant yeast strains.

| Yeast strains          | Siderophores % (Units) | IAA (μg ml⁻¹) | Phosphat sol. (mg ml⁻¹) | ACCD (μmol ml⁻¹) |
|------------------------|------------------------|--------------|------------------------|------------------|
| Yarrowia lipolytica YEAST-1 | 57.11 ± 2.36bc | 51.53 ± 2.53c | 1.03 ± 0.07cd | 39.36 ± 0.42b |
| Candida diddensiae YEAST-2 | 62.38 ± 0.87b | 30.87 ± 1.79d | 1.26 ± 0.07c | 22.44 ± 1.21d |
| Trichosporon ovoides YEAST-5 | 63.04 ± 2.48b | 82.63 ± 4.34a | 1.32 ± 0.08c | 29.27 ± 1.41c |
| Trichosporon ovoides YEAST-6 | 67.40 ± 0.33a | 43.33 ± 3.04c | 2.98 ± 0.09a | 35.45 ± 1.60b |
| Yarrowia lipolytica YEAST-16 | 74.85 ± 0.78a | 11.09 ± 2.71e | 0.93 ± 0.09d | 52.36 ± 0.72a |
| Candida subhijadi YEAST-17 | 55.83 ± 3.37e | 23.09 ± 1.88de | 2.25 ± 0.04bc | 21.55 ± 0.96c |
| Saccharomyces cerevisiae YEAST-30 | 8.16 ± 3.96d | 65.26 ± 2.87b | 2.54 ± 0.01b | 20.49 ± 0.83d |
| Saccharomyces cerevisiae YEAST-34 | 24.70 ± 2.38d | 72.03 ± 7.62ab | 2.38 ± 0.05b | 29.23 ± 1.40c |

Same letters within a column are not significantly different at P < 0.05.
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