



## Research article

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

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## ABSTRACT

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<sup>-1</sup>) and *Saccharomyces cerevisiae* FI25-1F (2.54 mg ml<sup>-1</sup>). 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 Cd<sup>2+</sup> and Pb<sup>2+</sup> 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 Cd<sup>2+</sup> 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 Fe<sup>3+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup> and Cd<sup>2+</sup>. Siderophores also form complexes with metals such as Cu<sup>2+</sup>, Zn<sup>2+</sup> and Pb<sup>2+</sup> 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 Cd<sup>2+</sup>, Cu<sup>2+</sup>, Pb<sup>2+</sup>, and Zn<sup>2+</sup> (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 chromosom. Unlike all siderophore genes, sidD is labeled as non-ribosomal peptide synthetase (NRPS) (Kozubal et al., 2019). Most of

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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 phosphates-solubilizing acids, 1-aminocyclopropane-1-carboxylate (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 Fe<sup>2+</sup> and Mn<sup>2+</sup> 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 elevating their photosynthetic efficiencies. The rhizomicrobes also result in the upregulation of metal transporter genes (ZIP genes) under Cd stress conditions that further lessens its accumulation in *Zea mays* (Wang 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 (Suksabye et al., 2016).

Microorganisms with the most effective siderophores mediated iron uptake have competitive advantages for using in biocontrol and as bio-fertilizers. 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, under Cd and Pb stress conditions, was also analyzed using qRT-PCR.

## 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 H<sub>2</sub>O (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'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-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](http://www.phylogeny.fr)), and visualization of the tree was achieved using TreeDyn.

### 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 fungi 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-sulfosalicylic acid 0.2 M) and was mixed carefully. The mixtures were kept for 10 min. The color development was determined by absorbance (A<sub>630</sub>) 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 [(A<sub>r</sub>-A<sub>s</sub>)/A<sub>r</sub>] 100 = % siderophores units; where A<sub>s</sub> is the absorbance value of the sample, and A<sub>r</sub> 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 Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> on Pikovskaya's agar medium contained L<sup>-1</sup>: Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, 5 g; glucose, 10 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g; yeast extract, 0.5 g; NaCl, 0.2 g; KCl, 0.2 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.002 g; and MnSO<sub>4</sub>·H<sub>2</sub>O, 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<sup>-1</sup>), as carbon source, instead of NaNO<sub>3</sub>. 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 agitated at 150 rpm. Following 7 days, each yeast culture was centrifuged for 10 min at 13,000 xg. Salkowski's reagent (10 mM FeCl<sub>3</sub> in 35% HClO<sub>4</sub>) 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  $\alpha$ -ketobutyrate (Bhagat et al., 2014). Minimal media containing ACC as a sole nitrogen source ( $\text{g L}^{-1}$ ;  $\text{Na}_2\text{HPO}_4$  2.13,  $\text{KH}_2\text{PO}_4$  1.36,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.7,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.2,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.04,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0.02,  $\text{H}_3\text{BO}_3$  0.003,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.02,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.007,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  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, 4-dinitrophenyl hydrazine (0.1%) was transferred to the mixture and left again at 30 °C for 30min. 2 ml of sodium hydroxide solution (2 N) was added and absorbance was measured using UV spectroscopy at 540 nm. One unit of the enzyme activity was defined as the enzyme amount able to liberate 1  $\mu\text{g}$  of  $\alpha$ -ketobutyrate/min.  $\alpha$ -ketobutyrate concentration in each sample was determined by comparison with the standard curve of  $\alpha$ -ketobutyrate (Sigma-Aldrich Co., K401) according to Kaur et al. (2018). The enzyme assays were performed in triplicates.

## 2.7. Minimum inhibitory concentrations (MICs)

Yeast isolates were sub-cultured using (YPD) Broth ( $\text{g L}^{-1}$ ; 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  $\text{CdCl}_2$  and  $\text{Pb}(\text{NO}_3)_2$ , separately, were prepared in YDP 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  $\text{CdCl}_2$  and  $\text{Pb}(\text{NO}_3)_2$  was investigated using chromo azurol S (CAS) plate assay (Schwyn and Neilands, 1987). CAS-agar solution with different concentrations of  $\text{CdCl}_2$  and  $\text{Pb}(\text{NO}_3)_2$  (0, 250, 500, 750 and 1000  $\mu\text{M}$ ) 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 orange halos were detected. Quantitative real-time PCR (qRT-PCR) using *sidD*-forward (5-ACGCAACCGACTGGTTGTT-3) and *sidD*-reverse (5-ATTTCGTGCGAGACTCGGAT-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 TRIzol™ 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  $\mu\text{l}$  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/ $\mu\text{L}$ ) at constant volume (15  $\mu\text{L}$ ) 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  $2^{-\Delta\Delta\text{Cq}}$  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 (Soltanloo 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* PAO1. 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 juncea* 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  $\text{CdCl}_2$  and  $\text{Pb}(\text{NO}_3)_2$  (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 significantly 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 gene sequence.

### 3.1. Phylogenetic affiliation of the analyzed yeasts

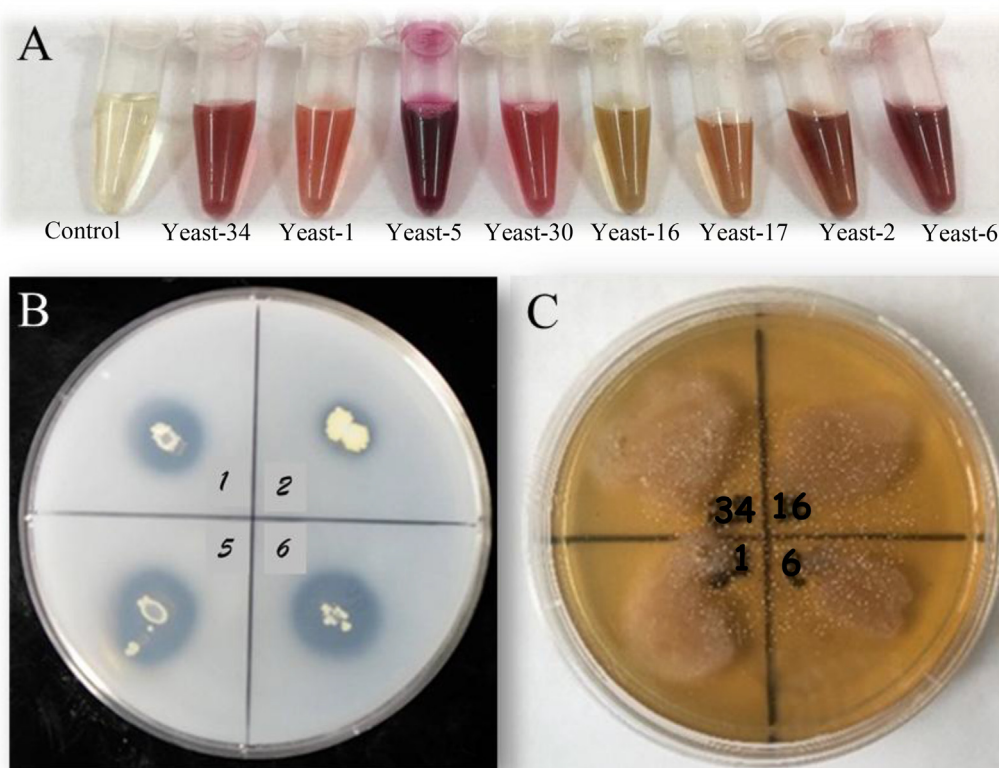
Yeast strains were isolated according to their different morphological features. The parameters considered were colony characteristics, color, shape, size, and spores. The fractional 18S rRNA gene sequences obtained were compared to the succession accessible in the GenBank database using the BLAST browser (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 rDNA showed that the strains YEAST-1 and YEAST-16

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

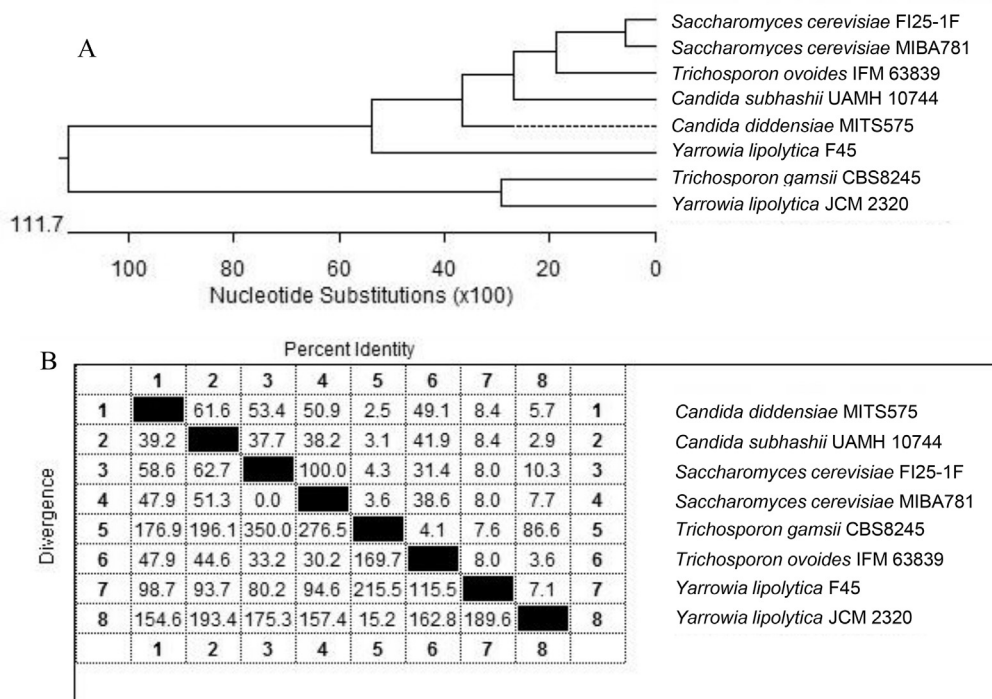
Strains	Cd <sup>2+</sup> MIC (mM)	Pb <sup>2+</sup> MIC (mM)	Max identity (%)	Strain of closest identity	Identification
YEAST-1	2.00	1.50	100%	<i>Yarrowia lipolytica</i> F45	<i>Yarrowia lipolytica</i> YEAST-1
YEAST-2	1.75	1.75	98%	<i>Candida diddensiae</i> MITS575	<i>Candida diddensiae</i> YEAST-2
YEAST-5	1.50	1.75	100%	<i>Trichosporon gamsii</i> CBS8245	<i>Trichosporon gamsii</i> YEAST-5
YEAST-6	1.75	1.75	100%	<i>Trichosporon ovoides</i> IFM 63839	<i>Trichosporon ovoides</i> YEAST-6
YEAST-16	1.75	2.00	99%	<i>Yarrowia lipolytica</i> JCM 2320	<i>Yarrowia lipolytica</i> YEAST-16
YEAST-17	1.50	1.50	100%	<i>Candida subhashii</i> UAMH 10744	<i>Candida subhashii</i> YEAST-17
YEAST-30	1.75	1.50	100%	<i>Saccharomyces cerevisiae</i> FI25-1F	<i>Saccharomyces cerevisiae</i> YEAST-30
YEAST-34	1.75	1.50	99%	<i>Saccharomyces cerevisiae</i> MIBA781	<i>Saccharomyces cerevisiae</i> YEAST-34

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.,

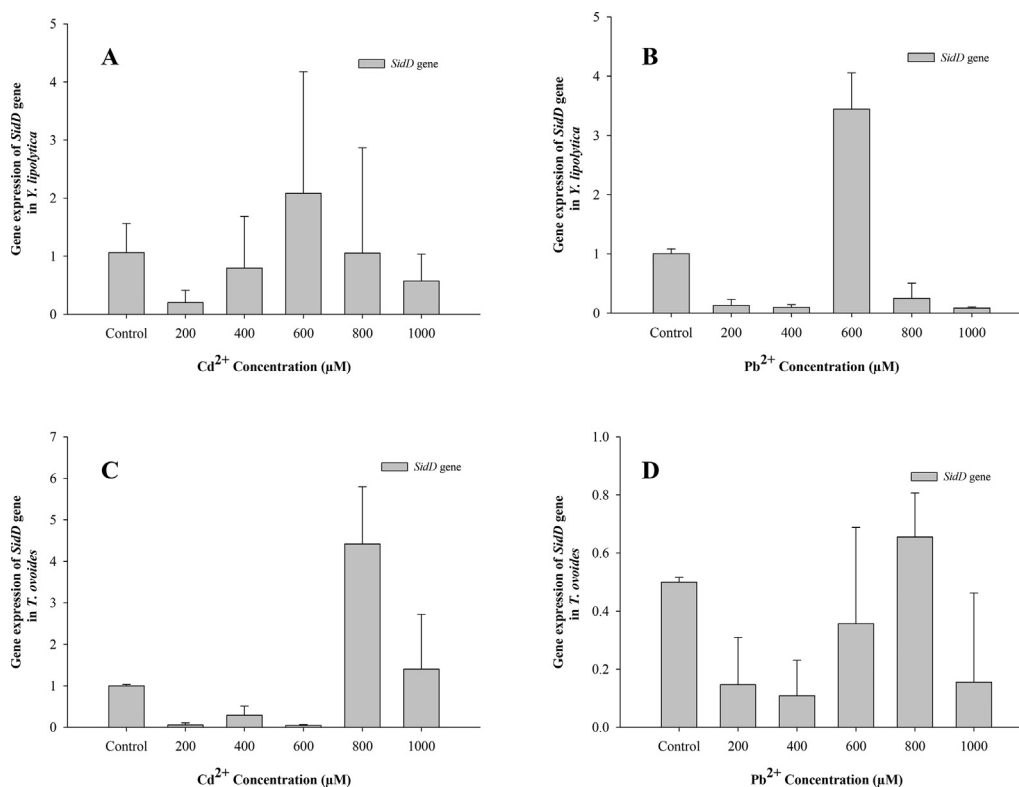
2008). Shi et al. (2017) mentioned that the effects of heavy metals on *P. aeruginosa* were different, the ability of siderophore production by *P. aeruginosa* varied with different heavy metals. The toxicity of heavy metals on bacteria is dependent on heavy metal accumulation in the cells. However, Cd<sup>2+</sup> and Zn<sup>2+</sup> 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



**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  $Pb^{2+}$ .

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

Yeast strains	Siderophores % (Unites)	IAA ( $\mu\text{g ml}^{-1}$ )	Phosphat sol. ( $\text{mg ml}^{-1}$ )	ACCD ( $\mu\text{mol ml}^{-1}$ )
<i>Yarrowia lipolytica</i> YEAST-1	57.11 $\pm$ 2.36bc	51.53 $\pm$ 2.53c	1.03 $\pm$ 0.07cd	39.36 $\pm$ 0.42b
<i>Candida diddensiae</i> YEAST-2	62.38 $\pm$ 0.87b	30.87 $\pm$ 1.79d	1.26 $\pm$ 0.07c	22.44 $\pm$ 1.21d
<i>Trichosporon gamsii</i> YEAST-5	63.04 $\pm$ 2.48b	82.63 $\pm$ 4.34a	1.32 $\pm$ 0.08c	29.37 $\pm$ 1.41c
<i>Trichosporon ovoides</i> YEAST-6	67.40 $\pm$ 0.33a	43.33 $\pm$ 3.04c	2.98 $\pm$ 0.09a	35.45 $\pm$ 1.60b
<i>Yarrowia lipolytica</i> YEAST-16	74.85 $\pm$ 0.78a	11.09 $\pm$ 2.71e	0.93 $\pm$ 0.09d	52.36 $\pm$ 0.72a
<i>Candida subhashii</i> YEAST-17	55.83 $\pm$ 3.37c	23.09 $\pm$ 1.88de	2.25 $\pm$ 0.04bc	21.55 $\pm$ 0.96d
<i>Saccharomyces cerevisiae</i> YEAST-30	8.16 $\pm$ 3.96e	65.26 $\pm$ 2.871b	2.54 $\pm$ 0.01b	20.49 $\pm$ 0.83d
<i>Saccharomyces cerevisiae</i> YEAST-34	24.70 $\pm$ 2.38d	72.03 $\pm$ 7.62ab	2.38 $\pm$ 0.05b	29.23 $\pm$ 1.40c

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

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  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ , etc (Yu et al., 2017). Hussein and Joo (2017) demonstrated that  $\text{Zn}^{2+}$  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  $100 \mu\text{g mL}^{-1} \text{Zn}^{2+}$ . They also investigated the siderophores synthesis of a variety of rhizosphere fungal strains under  $\text{Zn}^{2+}$  ion stress. These strains were specifically isolated from the rhizosphere of *Panax ginseng*. The results indicated that  $\text{Zn}^{2+}$  ion increased the production of siderophore in iron-limited cultures, and the maximum siderophore production was detected at  $150 \mu\text{g/ml Zn}^{2+}$  ion for all strains except for *Mortierella* sp. which showed the highest siderophore production at  $200 \mu\text{g/ml Zn}^{2+}$  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* F45 (Table 1), whereas strain *Candida subhashii* UAMH 10744 showed relatively low tolerance to Cd (1.5 mM). For the remaining strains, Cd MICs ranged between 1.5 mM and 1.750 mM. *Y. lipolytica* JCM 2320 and *Trichosporon ovoides* IFM 63839 had Pb MICs of 2.0 mM and 1750  $\mu\text{M}$ , respectively. The Pb MIC of *C. diddensiae* MITS575 and *T. gamsii* CBS8245 was 1.75 mM. Pb resistance levels for *Trichosporon* and *Saccharomyces* species were 1.5 mM and 1.75 mM, respectively. Both of *Y. lipolytica* F45 and *Y. lipolytica* JCM 2320 tolerated 2 mM of Cd and Pb. Braud et al. (2010) found that pyochelin decreased the uptake of  $\text{Al}^{3+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Pb}^{2+}$ , and  $\text{Zn}^{2+}$  and pyoverdine decreased the uptake of  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Eu}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Tb}^{3+}$  and  $\text{Zn}^{2+}$ . 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 *Cyberlindnera 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 ( $52.36 \mu\text{mol ml}^{-1}$ ) (Table 2). Strains YEAST-1 and YEAST-16 (both *Y. lipolytica*) and YEAST-6 (*T. ovoides*) produced high ACCD, whereas all the other strains only produced less than  $30 \mu\text{mol ml}^{-1}$  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  $82.63 \mu\text{g ml}^{-1}$  and  $72.03 \mu\text{g ml}^{-1}$ , 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*, *sidF*, *sidH*, and *sidI* 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  $\mu\text{M}$  heavy metal but under iron-limiting conditions. Expression of the *sidD* gene was increased maximally by *Y. lipolytica* at 600  $\mu\text{M}$  Cd in the iron-free media. As shown in Figure 3, there was an approximate threefold expression of the *sidD* gene by adding 600  $\mu\text{M}$  Cd or Pb to the iron-free media. *SidD* gene expression was increased maximally by *T. ovoides* IFM 63839 at 800  $\mu\text{M}$  Cd in the iron-free media. However, the ions of Pb increased the *sidD* gene expression gradually from 200 to 800  $\mu\text{M}$ , 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  $\mu\text{M}$  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

(2019) illustrated the necessity to understand the biology and chemistry of siderophores before applying them in the field of agriculture. Moreover, they showed that the existence of adequate quantities of heavy metal might promote plant growth and development.

In conclusion, siderophores may have wider and more complex functions than previously thought. Their presence is beneficial for rhizosphere-associated microbes. Metal-resistant microorganisms able to produce siderophores have recently been suggested to improve growth and decrease the heavy metals accumulation within plants. In this study, eight yeast isolates were obtained from *Triticum sativum* rhizosphere soil affiliating with *Yarrowia*, *Candida*, *Trichosporon*, and *Saccharomyces*, which showed relatively high heavy metal tolerance, were analyzed for siderophores, IAA, and ACCD production, and phosphate solubilization. *Y. lipolytica* F45 and *Y. lipolytica* JCM 2320 tolerated 2 mM of Cd and Pb. For the remaining yeast strains, the inhibitory concentrations of these metals were between 1.5 mM and 1.75 mM. *SidD* gene is the most likely responsible for the biosynthesis of siderophores in fungi. The presence of 600–800 µM heavy metal, but under iron limitation, induced the *sidD* gene expression during the growth of *T. ovoides* IFM 63839 and *Y. lipolytica* JCM 2320 strains. The data currently available mention that siderophores are produced by yeasts not solely to scavenge for iron. This article considers the concept that siderophores play a significant role in protecting the rhizobial microbes against heavy metals toxicity and discusses the potential contribution in plant growth promotion.

## Declarations

### Author contribution statement

Saad S. El-Maraghy: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tohamy Anwar Tohamy: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Khalid Abdallah Hussein: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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