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ORIGINAL ARTICLE

Biotechnological approaches in agriculture and environmental management - bacterium *Kocuria rhizophila* 14ASP as heavy metal and salt- tolerant plant growth- promoting strain

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Abstract

Plant growth-promoting bacteria (PGPB), possess multifarious beneficial traits and facilitate plant growth by both direct and indirect mechanisms under hostile conditions. The objective of the current study was to evaluate the potential application of 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) producing plant-growth promoting endophyte bacterium (PGPEB) *Kocuria rhizophila* strain 14ASP (National Center for Biotechnology Information; NCBI) Accession Number: LFIY00000000.1) heavy metals and salt resistance tolerance under hostile environment. The in vitro study was conducted to evaluate plant growth promotion characteristics, heavy metal and salt resistance of the *K. rhizophila* under normal and stressful environments. The strain 14ASP revealed the plant-growth promoting and of AAC deaminase activities under abiotic stress environment. *K. rhizophila* showed significant tolerance against different heavy metals on LB (Luria Ber-tani) agar plates and broth medium supplemented with cadmium (Cd), (50 mg L^{-1}), cooper (Cu), (50 mg L^{-1}), nickel (Ni), (50 mg L^{-1}), lead (Pb), (200 mg L^{-1}) and chromium (Cr) (500 mg L^{-1}), respectively. It also exhibited population density (OD = 0.31–0.45) at different concentrations of sodium chloride (NaCl) (0% 5%, 10%, and 15% *w/v*) in LB medium. The draft genome of strain 14ASP is 2.6 Mb and was assembled into 183 contigs with 2689,1 bp and a G + C content of 70% and 1,882,646 bp, encoded 2409 protein, 8 rRNAs and 46 tRNAs. Genome analysis identified genes, involved in hydrocarbon metabolism, heavy metal tolerance, biofilm formation, Indole Acetic Acid (IAA) and siderophore biosynthesis. All these properties were confirmed by in silico and draft genome analysis.

Keywords Biodegradation · Genomics · Heavy metal resistance · *Kocuria rhizopila* strain 14ASP · Plant growth promotion

Introduction

Heavy metal contamination and salinity stress are the major abiotic stresses that cause serious threats to the environment, agriculture and human health. Crop irrigation practices, increase salt concentration, overuses of chemical pesticides, and rapid industrialization are the major anthropogenic activities of soil salinity and heavy metals pollution in agricultural

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land (Kumar et al. 2016a, b; Kaur et al. 2018). These activities are responsible for unpredictable soil biological and chemical changes which directly affect plant germination, growth and food productivity (Jiang et al. 2019).

Soil salinity is one of the devastating abiotic stresses in agriculture foster growth retardation, physiological abnormalities, and lower production output of field crops throughout the world (El-Esawi et al. 2018; Aghai et al. 2019; Hayat et al. 2020a, b). Salt stress remains a major growth-limitation factor and has been estimated that up to 20% of cultivated areas are affected worldwide (Khan et al. 2021a, b). Salinity stress induces osmotic and ionic stress that leads to retarded growth in terms of both shoot and root length, fresh and dry weight reduced pigment content and hampers uptake of mineral elements (Alsaeedi et al. 2019; Najar et al. 2019). Excess of sodium (Na^+) and chlorine (Cl^-) may cause metabolic disturbances, limiting nutrient absorption (e.g., K^+ and Ca^{2+}) and disturbing the ionic balance. The nutrients imbalance adversely affects plants at cellular and sub-cellular levels through increasing Na and Cl, lower K^+ level and K^+/Na^+ ratio which disturbs ion homeostasis and causes cell death (Sellitto et al. 2019; Campos et al. 2019). Soil salinity limits crop productivity by impairing root growth, nutrient uptake, and metabolic processes (Isayenkov and Maathuis 2019). As general consequences, accumulation of NaCl in plant tissue negatively affect physiological, morphological, and biochemical processes, which decrease crop biomass and productivity (Khan et al. 2021a, b; Basu et al. 2017).

Many plant growth-promoting endophytes have been reported to improve plant performances under adverse drought conditions (Jayakumar et al. 2020). However, a few PGPEB are reported that alleviate salinity stress and improve plant growth under a saline environment (Shahzad et al. 2017). PGPEs facilitate plant growth, help in the uptake of essential minerals and improve plant adaptation in harsh conditions and proposed eco-friendly and cost- effective attractive alternatives to chemical-based agriculture (Khan et al. 2021a, b). PGPEs promote plant growth by expressing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves the immediate precursor of the plant hormone ethylene to produce α-ketobutyrate and ammonia (Kruasuwan and Thamchaipenet 2018). Several studies have reported the effectiveness of PGPEs for improving crop growth under abiotic stress, including salinity stress. Bacterial strains such as *Bacillus, Pseudomonas, Burkholdera*, and *Arthrobacter* have been identified as plant growth promoters under saline conditions in crop plants such as wheat, rice and maize (Lastochkina et al. 2017; Khan et al. 2021a, b; Kushwaha et al. 2020).

Besides soil salinity, contamination of agricultural land with heavy metals has become a serious concern and common threat to many agricultural sites. Heavy metal pollutants contaminate the environment through anthropogenic ways and can be deposited in soils, water bodies, or plant tissue. These non-biodegradable heavy metals easily enter the human body via the food chain and cause many serious health disorders (Kaur et al. 2018; Khanna et al. 2019; Rehman et al. 2018; Kumar et al. 2017).

To reinstate the heavy metals polluted land, bioremediation serves as a promising, eco-friendly, economically cheap and emerging approach to degrade, extract and immobilize the contaminants from soil sediments and groundwater (Alvarez et al. 2017; Kumar et al. 2016a, b). Applications of plant-growth-promoting bacteria (PGPB) have appeared as a promising approach in the mitigation of heavy metal from contaminated soil. The plant-associated bacteria assisted plant growth and show tolerance to heavy metals and reduce their uptake or translocation to aerial parts of plants by decreasing the metal bioavailability in the soils (Fan et al. 2018; Burges et al. 2017). In addition, certain bacteria can potentially alleviate phytotoxic effects by producing siderophores, organic acids, biosurfactants and extracellular polymeric substances (Asad et al. 2019, Mahapatra et al. 2020). Moreover, Bacteria can reduce the contents of heavy metals in soil or solution, mainly by adsorption (Wan et al. 2020), bioprecipitation (Chen et al. 2019), and methylation (Ogunlaja et al. 2020).

The objective of the current research was to investigate the heavy metal and salt resistance of plant growth-promoting endophyte *K. rhizophila* strain 14ASP previously isolated from the internal root tissues of the herbaceous medicinal plant, *Oxalis corniculata* L.

We also hypothesized and performed the draft genome sequence analysis to investigate whether *K. rhizophila* strain 14ASP genome possess the functional genes responsible for heavy metals and salt tolerance. The information from the genome will help us to understand the biotechnological importance of abiotic resistant plant growth-promoting bacteria in agriculture and environmental management sectors in the near future.

Materials and methods

Isolation, biochemical characterization and plant-growth promoting activities of *K. rhizophila*

Bacterial strain 14ASP was initially isolated from internal root tissues of *O. corniculata* L. (Family: Oxalidaceae), in (Plant-Microbe Interaction lab, Quaid-i-Azam University, Islamabad 45,320, Pakistan) in 2013. From the previous published data (Afridi et al. 2019) the biochemical characterization and plant-growth promoting activities (IAA, ammonia, siderophore, phosphate solubilization catalase) was performed of *K. rhizophila*. The strain was deposited to the National Center for Biotechnology Information (NCBI) with the accession number KF875448.

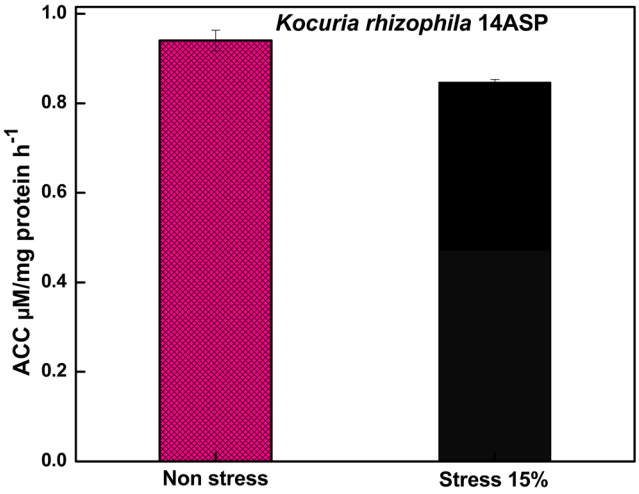

Fig. 1 ACC deaminase activity of *K. rhizophila* 14ASP under the non-stress (Control conditions and no NaCl) and salinity stress (NaCl 15%) using DF medium. Different lowercase letters above columns indicate a significant difference at $P < 0.05$ between isolates (Afridi et al. 2019)

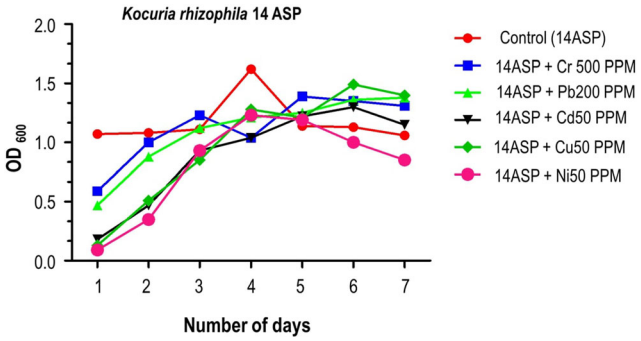

Fig. 2 Growth population density of heavy metal tolerant strain 14ASP at different heavy metals Cd, (50 mgL^{-1}) Cu, (50 mgL^{-1}) Ni, (50 mgL^{-1}), Pb, (200 mgL^{-1}) at day 1,4,6,7

ACC-deaminase activity and salt tolerance assay

The ACC-deaminase activity of strain 14ASP was proved and quantified in the previously published data Fig. 1 (Afridi et al. 2019). The concentration of ά-ketobutyrate (mmol) was measured using a spectrophotometer at 540 nm. The salt tolerance of *K. rhizophila* strain 14ASP was determined in LB medium supplemented with three different concentrations of NaCl such as 0% 5%, 10%, and 15% *w/v* based on estimation of population density. The conical flasks containing bacterial inoculum were kept in a shaker incubator at 220 rpm for 24 h. The optical density (OD) of bacterial culture was measured at $\lambda = 600$ nm using a spectrophotometer (Agilent 8453 UV–visible Spectroscopy System).

Evaluation of multiple heavy metal resistance assay

We used five heavy metals in the current study, comprising Cr, Pb, Cd, Cu and Ni (Table 1). The heavy metal salt solution was prepared by dissolving heavy metal salt in a specific amount of double distilled water (DSW) and filtered through (0.2 mm pore filters). The isolated plant growth promoting strain 14ASP was screened for heavy metals multiple tolerance against Cd^{2+}, Cu^{2+}, Ni, Pb, and Cr^{6+}. Each heavy metal was supplemented at 5 different concentrations (50, 50, 50, 200, 500 mg L^{-1}) to LB agar plates and broth medium (Peptone 10.00 g/L, yeast extract, 5.00 g/L, NaCl 5.00 g/L, and agar 30.00 g/L) respectively and adjusted the pH 7.2–7.4, incubated at 28 °C for 48 h. (Marzan et al. 2017). Finally, large colonies of strain 14ASP isolate were grown on the LB agar plates in the presence of heavy metals and the multiple resistance capacity to heavy metals was evaluated. The heavy metals added to LB agar medium in the following salt forms. $CdCl_2$, $CuSO_4.5H_2O$, $NiSO_4$ $(H_2O)_6$, $PbCl_2$, and $CrCl_3$. $6H_2O$.

Influence of heavy metals concentration on bacterial growth patterns/ population densities

To determine the optimum growth condition, bacterial cell concentration and the growth pattern of strain 14ASP were determined in LB broth supplemented by the heavy metals Cd^{2+}, Cu^{2+}, Ni, Pb, and Cr^{6+} at different concentrations (50, 50, 50, 200, 500 mg L^{-1}), respectively. A 1% (*v*/v) bacterial inoculum was inoculated into LB broth in the absence or presence of heavy metals incubated at 28 °C and 150 rpm for seven days. The influence of heavy metals concentration on bacterial population densities was evaluated by measuring the optical density (OD at λ =600 nm) using UV spectrophotometer after every 24 h up to seven days (Fig. 2) (Teng et al. 2019).

Table 1 Heavy metals, salts and concentrations used for tolerance experiments

Group	Metals	Salts	Heavy metal concentrations $mgL^{-}1$
Group I	Cadmium (Cd) Nickel (Ni)	Cadmium chloride, Nickel(II) sulfate	50, 100, 200,300,400,500
Group I I	Chromium (Cr) lead (Pb)	Chromium(III) chloride, Lead (II) chloride	50, 100, 200,300,400,500
Group I I I	Copper(Cu)	Copper(II) chloride dehydrate	50, 100, 200,300,400,500

Table 2 Project information

MIGS ID	Property	Term
MIGS -[Adam E]	Finishing quality	Improved-high-quality draft
MIGS-[Ahmad E]	Libraries used	400 bp Ion Xpress Plus enzymatically sheared, no PCR amplification
MIGS-[Patten CL]	Sequencing platforms	Ion Torrent PGM (Torrent Suite 4.4.3)
MIGS-[Adam E]	Fold coverage	106.0×
MIGS-[Goswami D]	Assemblers	SPAdes 3.1.0
MIGS-[Tamura K]	Gene calling method	Glimmer gene prediction, NCBI Prokaryotic, Genome Annotation Pipeline
	Locus tag	ACJ65
	Gen Bank ID	GCF_001038535.1
	Gen Bank Date of Release	June 23, 2015
	GOLD ID	Not registered
	Bio Project	PRJNA286912
	Project relevance	Agricultural/industrial

Classification and features

The bacterial strain 14ASP was isolated from internal root tissues of *O. corniculata* L. (Family: Oxalidaceae), in Islamabad, Pakistan in 2013 (Das et al. 2016). Strain 14ASP when grown on LB agar medium at 28 °C shows yellow shiny domed spherical colonies with approximately 3 to 3.2 mm in diameter after 24 h (Supplementary materials). Colonies were found in pairs, tetrads and packets after 72 h grown at the same temperature. Strain 14ASP showed optimum growth at 28 °C and growth rate gradually decreased as the temperature increased up to 45 °C in liquid and solid media at pH 7.0 after 48 h. Sequencing of the 16S rRNA gene was carried out and revealed that strain 14ASP (accession KF875448) showed 100% identity with other *K. rhizophila* strains deposited in public databases (Mufti et al. 2015). The phylogenetic relationships of *K. rhizophila* strain 14ASP with other species and related genera inferred by 16S rRNA gene sequences show its proximity with other *Kocuria* species and to genera such as *Arthrobacter* and *Micrococcus* (Supplementary materials). *K. rhizophila* strain 14ASP is a gram-positive, non-spore forming and non-motile, coccus bacteria belonging in the order *Micrococcales* and class *Actinobacteria*.

K. rhizophila strain14ASP characterized biochemically and showed positive response for β-galactosidase, and produced amylase, phosphatase, nitrate reeducates. The strain 14 ASP showed a negative response in the production of oxidase, urease, gelatinase, β-glucuronidase and acetoin (Voges–Proskauer reaction). The strain 14ASP showed resistance observed the population density at 15% NaCl supplemented media LB medium and NaCl is not mandatory for bacterial growth.

Genome project history of *K. rhizophila* 14ASP

The identification of *K. rhizophila* was performed by sequencing the 16S rRNA gene. (Supplementary materials). For genomic studies analysis, *K. rhizophila* was selected based on its, agriculture, environmental and adverse relevance problems which have proven the capacity and showed resistance to abiotic stress (salt) in the saline environment and tolerance to heavy metals t and promote plant growth in adverse conditions. The draft genome was prepared for an Ion Torrent (Personal Genome Machine) PGM using 400 bp chemistry, assembled using SPAdes 3.1.0, and the 183 contigs greater than 500 bp were annotated using the NCBI Prokaryotic Genome Annotation Pipeline and then re-annotated through Rapid Annotations using Subsystems Technology (RAST). After the draft genome was prepared, average nucleotide identity calculations (Federhen et al. 2016) with related genomes present in Gen Bank revealed that *K. rhizophila* strain 14ASP shares 99.60% similarity with the genomes of *K. rhizophila* strains with 95.4% area of the chromosome. The Gen Bank accession number for this draft genome is LFIY00000000.1 (Table 2).

Growth conditions and genomic DNA preparation

K. rhizophila inoculated in 100 mL conical flask containing 50 mL LB media and placed in shaking incubator at 28 °C for two days. The bacterial aliquots suspension (2 mL) was taken and performed for 5 min at 12,000g, rpm and RNA-free genomic DNA was extracted with an Invitrogen PureLink Genomic DNA Mini Kit (Life Technologies Inc., Burlington, ON). DNA was quantified using a Qubit dsDNA HS Assay Kit (Life Technologies Inc., Burlington, ON) and DNA quality was evaluated on an agarose gel (Tilak et al. 2018; Zhu et al. 2020).

Genome sequencing and assembly

The draft genome of *K. rhizophila* strain 14ASP was generated and sequenced at the The Biological Sciences, Thompson

Rivers University, 900 McGill Road, Kamloops, BC V2C0C8, Canada, using Ion Torrent PGM sequencing technology. The Ion Xpress Plus Fragment Library Kit (Life Technologies Inc., Burlington, ON) was used for adaptor-ligated DNA preparation. The size was selected 480 bp on 2% agarose gel (E-Gel). Dilution factor of library was determined applying Ion Library Quantitation Kit. The Ion PGM Template OT2 400 kit on an Ion OneTouch 2 system were used for amplification prior the determination of library for dilution factor. Ion Sphere Quality Control Kit prior was used for quantification of the template ion sphere particles enrichment on an Ion OneTouch ES system and sequencing on a 316v2 chip with an Ion PGM 400 Sequencing Kit on an Ion Torrent PGM (Life Technologies Inc., Carlsbad, CA).

The average length was measured 262 bases total of 1.87 million reads which 2.6981 Mb data was generated (> 422 M Q20 bases) in Torrent Suite 4.4.3 and assembled by SPAdes 3.1.0 (Bankevich et al. 2012; Gurevich et al. 2013) (uniform coverage mode; kmers 21, 33, 55, 77, 99), into 183 contigs greater than 500 bp with a total length of 2,698,103 bp (N50 30,277 bp; largest contig 86,883 bp) at a mean coverage of 106.0× and G + C content of 70.80% of the genome.

Genome annotation

Annotation was initially done through NCBI prokaryotic annotation tools and then was re-annotated using RAST version 2.0 by utilizing its integrated gene calling FIG fam version release 70 (Berrios and Ely 2018; Huerta-Cepas et al. 2017). In addition, for predicting and assigning Clusters of Orthologous Groups (COGs), Blast2Go and WebMGA were used (Zhao et al. 2020). RAST and NCBI Prokaryotic Genome Annotation Pipelines were used for the published version.

Statistical analysis

The growth rate and duration of the lag phase of bacteria, optical density data were analyzed using the R software and optical density observed fitted (Team 2013). The differences between the treatments were tested at 5% level significance by performing One-way ANOVA. Tukey's unequal honest significant difference was used for separation of significant means.

Results

Isolation, biochemical characterization and PGP traits of 14ASP

K. rhizophila was isolated previously from root tissue of *Oxalis corniculata* L. (Family: Oxalidaceae), in the Plant-Microbe Interaction lab, Quaid-i-Azam University, Islamabad (Mufti et al. 2015). *K. rhizophila* portrayed positive response for various biochemical test by analyzing microbial identification kits QTS-24. The strain 14ASP showed potential for production of IAA (0.36 μg mL^{-1}) ammonia (36 μg mL^{-1}) and siderophore (the orange zones around the colonies determine the siderophore production) and solubilize inorganic phosphate to phosphate (zones from 16 to 17 mm), and also showed catalase activity and declared as a plant growth promoting bacteria (Afridi et al. 2019).

Evaluation of ACC-deaminase activity and salt tolerance assay

The bacterial population densities were measured at 600 nm wavelength OD at 24 h intervals. The growth patterns turned into the declined stage after 88 h at three salt stress levels. The results revealed that for the sole nitrogen source, strain 14ASP used ACC in in vitro plate experiment. The strain was grown in plates containing DF medium and showed maximum growth with or without a nitrogen source and the ACC deaminase activity exhibited positively. Anyway, strain 14ASP showed lesser growth in lack of nitrogen source DF medium comparatively with the plates containing ACC and positive control. The quantification of ACC deaminase production activity was assessed for *K. rhizophila*. The ACC deaminase activity measured by alpha ketobutyric acid production when the ACC cleavage by ACC deaminase. The ACC deaminase activity compared in both stressed (15% NaCl) and no stressed (0% NaCl) conditions. *K. rhizophila* strain 14ASP has the potential to use ACC deaminase enzyme for the cleavage of ACC and consequently use it as a nitrogen source. The ACC deaminase enzyme activity was observed higher in non-stress conditions while lesser in salt treated conditions once strain 14ASP inoculated. Figure 1).

Kocuria rhizophila strain 14ASP growth and colony formation on LB agar media show the tolerance ability to multiple heavy metals

The heavy metals assay conducted to evaluate the tolerance of multiple heavy metals on LB agar plates and broth medium respectively supplemented with Cd2+, Cu2+, Ni, Pb, and Cr6+ at different concentrations (50, 50, 50, 200, 500 mg L^{-1}), respectively over a period of 7 days. K. rhizophila strain 14ASP exhibited varied tolerance to heavy metal concentrations (Table 1, Fig. 2). *K. rhizophila* strain 14ASP demonstrated high metal tolerance to Cu (50 mg L^{-1}), following by Pb (200 mg L^{-1}), at 6th day of incubation period. The maximum growth for *K. rhizophila* strain 14ASP strain was observed on the seventh day of incubation on Pb (200 mg L^{-1}) when compared to control. (Fig. 2, Fig. S1).

Growth and population densities of *Kocuria rhizophila* 14ASP in LB liquid medium response to each heavy metal concentration

The growth rate (μ) of strain 14ASP was varied to each of the considered heavy metal-enriched media at different concentrations (Fig. 2). Initially the strain 14ASP came under stress when exposed to heavy metals stresses such as Cr, Pb,Cd, Cu, and Ni, in LB agar plates and broth medium respectively liquid medium, and significantly ($p < 0.05$) reduced the population densities as compare to control. The strain 14ASP showed resistance and kept maintained the population densities in all treatments and in control at day 5, 6 and 7 (Fig. 2).

To ascertain the heavy metals tolerance potential of strain 14ASP, the strain cultured in LB broth medium respectively liquid medium containing each heavy metals Cd^{2+}, Cu^{2+}, Ni, Pb, and Cr^{6+} at different concentrations (50, 50, 50, 200, 500 mg L^{-1}) and recording the bacterial populations densities based on the growth curve analysis and their resistance capacity was assessed after 24, 48, 72, 96, 120, 144, and 168 h. The strain 14ASP showed good tolerance capacity against different heavy metals and declined the growth after 144 h. Interestingly the strain 14ASP showed growth on Cr up to 500 mg L^{-1} and on Pb up to 200 mg L^{-1} while on Cd^{2+}, Cu, and Ni it was up to 50 mg L^{-1}. The introduction of heavy metals Cr, Pb, Cd, and Cu in media enhanced growth rates of strain 14ASP resulting to significantly ($p < 0.05$) higher growth rates compared to the controls, and this pattern was observed at day 1, 4, 6 and 7 with Cr, Pb, Cd, and Cu, Ni supplemented media (Fig. 2) but there were no significant differences in growths recorded at day two, three and five.

Table 3 Genome statistics for *Kocuria rhizophila* strain 14ASP

Attribute	Value	% of total
Genome size (bp)	2689,486	100
DNA coding (bp)	1,882,646	70
DNA G+C (bp)	1,882,640	70
DNA contigs	182	–
Total genes	2538	100
Protein coding genes	1932	76.12
RNA genes	55	2.17
Pseudogenes	486	19.15
Genes in internal cluster	NA	NA
Genes with function prediction (SEED)	1943	76.12
Genes assigned to COGs	1943	76.12
Genes with Pfam domains	NA	NA
Genes with signal peptides	25	0.9
Genes with transmembrane helices	1	0.03
CRISPR repeats	5	0.1

The growth response of 14ASP to heavy metals concentrated media was Cr > Pb > Cd > Cu > Ni.

Kocuria rhizophila strain 14ASP genome properties

The draft genomic sequences of *K. rhizophla* strain 14ASP were assembled in a linear form of length 2,698,103 bp having 183 contigs with an average G + C content of 70.8% (Table 3). Strain 14 ASP showed close resemblance and similarity to the previously published genomes of *K. rhizophila*

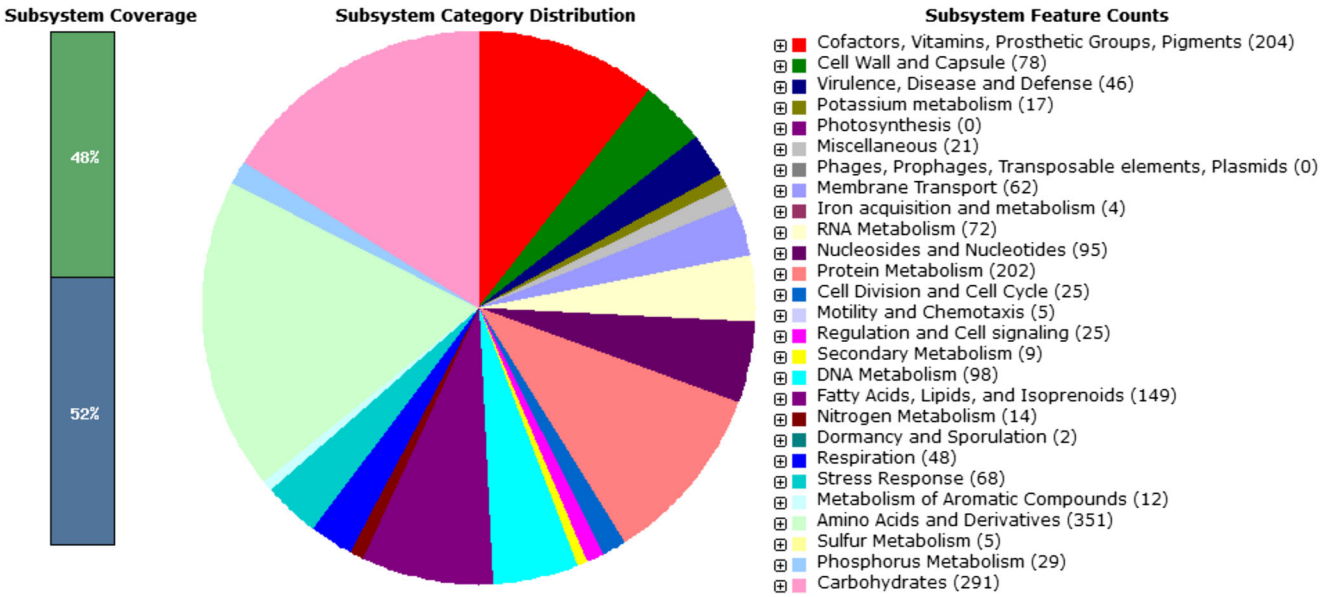

Fig. 3 Pie chart showing functional genome of *Kocuria rhizophila* strain 14ASP**.** The genes are predicted using FIGFams system integrated within RAST online server

P7–4 and *K. rhizophila* DC2201. Based on the RAST annotation, out of the 2538 predicted genes, 1932 (76.12%) were allocated predictive functions, and the remaining 606 (23.88%) were annotated as hypothetical proteins. Coding sequences were grouped into functional classes using the clusters of orthologous groups of functions (COG) (Galperin et al. 2021; Feldbauer et al. 2020) database (Table 4) based on WebMGA and Blast2Go (Zhao et al. 2020). In summary, the NCBI annotation showed that the genome has 182 contigs, contains 2538 genes, 1932 were coding sequences and had 486 pseudogenes, 46 tRNAs, 8 rRNAs and one Non-coding RNAs (ncRNAs) (Table.3).

The total is based on the total number of protein coding genes in the genome based on BASys gene prediction.

Insights from the genome sequence

K. rhizophila strain 14ASP clustered showed close relation to other kocuria species e.g. *K. rhizophila* DC2201and *K. rhizophila* P7–4 based on phylogenetic analysis. After the annotation, the *K. rhizophila* genome exhibits 37 and 77 genes responsible for the biosynthesis acetoin and butanediol and degrades alkane respectively.

The annotated genome also revealed the total annotated genes which are involved in various biochemical and physiological functions. The genome contains, 33 genes which involved for biofilm formation, 67 for chemotaxis, 61 for heavy metals resistance, 55 for indole acetic (IAA) synthesis, 310 for the degradation of polyaromatic hydrocarbon, 33 for phosphorus solubilization and uptake, 142 for the synthesis of siderophore and 19 genes involved for heat shock resistance.

According to the conducted research of Mufti et al. (2015) and Haq et al. (2016), *K. rhizophila* strain 14ASP has been showed resistant from 0.025 to 0.1 mg mL^{-1} to different heavy metals such as Pb, Cu, Cr, and Cu and absorbed metals particularly Cd 9.1 mg g^{-1} and Cr 14.4 mg g^{-1} respectively. The *Kocuria* genus contains various species and strains(such as *K. kristinae*, *K. rhizophila* and *K. marina)* that capable of degrading phenol compounds and could be shown resistance up to 10% NaCl that could be grown and withstand against these environmental stresses in supplemented growth media (Raghupathi 2018). The genome of *K. rhizophila* comprises genes that are involved for significant functions such as adsorption of heavy metals and degradation of hydrocarbon which crucially contributes in contaminated environment as a sweeper and underpin bioremediation. The most important functional regulated genes were also deducted into the *K. rhizophila* genome that they contribute in very significant functions such as hydrocarbon degradation, heavy metal resistance siderophore biosynthesis, alkane degradation, polyaromatic hydrocarbons and heat shock resistance (Table.5).

Table 4 Number of genes associated with general COG functional categories

Code	Value	%	Description
J	131	6.78	Translation, ribosomal structure and biogenesis
A	56	2.89	RNA processing and modification
K	16	0.82	Transcription
L	76	0.93	Replication, recombination and repair
B	18	0.93	Chromatin structure and dynamics
D	25	1.29	Cell cycle control, Cell division, chromosome Partitioning
V	28	1.44	Defense mechanisms
T	25	1.29	Signal transduction mechanisms
M	27	1.39	Cell wall membrane biogenesis
N	0	0	Cell motility
U	27	1.39	Intracellular trafficking and secretion
O	20	1.03	Posttranslational modification, protein turnover, chaperones
C	48	2.48	Energy production and conversion
G	291	15.06	Carbohydrate transport and metabolism
E	351	18.16	Amino acid transport and metabolism
F	95	4.91	Nucleotide transport and metabolism
H	15	0.77	Coenzyme transport and metabolism
I	149	7.71	Lipid transport a metabolism
P	33	1.70	Inorganic ion transport and metabolism
Q	9	0.44	Secondary metabolites biosynthesis, transport and catabolism
R	321	16.61	General function prediction only
S	152	7.86	Function unknown
–	30	1.55	Not in COGs

Phylogenetic analysis and tree construction

A phylogenetic tree was constructed to show the current position of *K. rhizophila* strain 14ASP (arrow) in relation to other species and genera in the family Micrococaceae (Fig. 4). The tree was based on 884-bp nucleotides of the *K. rhizophila* strain 14ASP 16S rRNA gene aligned in the program Multiple Alignment using Fast Fourier Transform (MAFFT). The reconstruction of the tree was done in Mega7 (Russo and Selvatti 2018) with the Maximum likelihood method and substitution model TN93 + I. Accession numbers followed by species names and strain codes are shown on the tree. Bootstrap values higher than 70% are shown in the appropriate branching points. The scale indicates the number of substitutions per site. The K. rhizophila strain 14ASP shares currently a common ancestor with *K. rhizophila* strain S5 with 99% homology and *Kocuria marina* strain 3710 with 73% homology.

Table 5 Open reading frames (ORFs) corresponding to selected gene clusters of *Koccuria rhizophila* **strain** 14ASP. The table presents ORF of some important genes discussed in the text

Strain14ASP_155_	Length (aa)	Deduced function number	Protein homolog	Identity/similarity (%)	Accession (origin)
ACJ65_RS01295	309	Cobalt-zinc-cadmium resistance protein CzcD	*Acarvochloris marina* MBIC11017	84	NZ_LFIY0100004.1
ACJ65_RS01270	365	Cobalt-zinc-cadmium resistance protein CzcD	*Acarvochloris marina* MBIC11017	93	NZ_LFIY0100004.1
ACJ65_RS03315	341	3-oxoacyl-ACP synthase III	*Bacteriovorax marinus SJ*	82	NZ_ LFIY01000012.1
ACJ65_RS03300	507	NAD(P)H steroid dehydrogenase-like protein in alkane synthesis cluster	*Bacteriovorax marinus SJ*	84	NZ_ LFIY01000011.1
ACJ65_RS03320	405	AMP-dependent synthetase/ligase in alkane synthesis cluster	*Bacteriovorax marinus SJ*	93	NZ_ LFIY01000012.1
ACJ65_RS03235	166	Haloalkane dehalogenase-like protein	*Bacteriovorax marinus SJ:*	86	NZ_ LFIY01000012.1
ACJ65_RS03455	140	heat-shock protein HtpX	*Bacteriovorax marinus SJ:*	90	NZ_ LFIY01000012.1
ACJ65_RS03620	336	heat-inducible transcriptional repressor HrcA	*Bacteriovorax marinus SJ:*	93	NZ_ LFIY01000013.1
ACJ65_RS05960	168	heat-shock protein	*Bacteriovorax marinus SJ:*	80	NZ_ LFIY01000029.1
ACJ65_RS00750	344	siderophore-interacting protein	*Mycobacterium smegmatis* str. MC2 155 (246196.1)	86	NZ_ LFIY01000003.1
ACJ65_RS00860	256	siderophore-interacting protein	*Mycobacterium smegmatis* str. MC2 155 (246196.1)	93	NZ_ LFIY01000003.1
ACJ65_RS00050	284	siderophore-interacting protein	*Mycobacterium smegmatis* str. MC2 155 (246,196.1)	86	NZ_ LFIY01000001.1

Kocuria rhizophila strain 14ASP. Environmental sweeper and sustainable remedy in stressful agroecosystems regime

Heavy metal resistant PGPB mediated bioremediation, phytostimulation and stress alleviation is an eco-friendly method for sustainable agriculture in the metal contaminated and saline soil. Anthropogenic activities, industrialization, and the Inundate use of chemical pesticides contaminated natural and agricultural soil led to health problems and notoriously affect all bio eco-systems. The remediation of heavy metals using plant growth promoting bacteria (PGPB) is an innovative biotechnological, cost-effective and eco-friendly strategy using in heavy metals and saline affected agriculture land. The PGPB tailored various biochemical and physiological process such as mobilization (soil acidification, biosurfactant solubilization, chelation, or redox transformation) immobilization (sorption, precipitation, sequestration, and volatilization, siderophores, organic acids, biosurfactants, biomethylation) (Bhat et al. 2020; Pramanik et al. 2018). *B.* xiamenensis, *B. gibsonii* and *B. vietnamiensis* are plant growth promoting bacteria which potentially amended the heavy metals toxicity and restored the polluted soil regimes which might support the biotechnological and ecofriendly programs in the near future (Zainab et al. 2020; Ali et al. 2018). Strain 14ASP exposed to various heavy metals stress in vitro conditions and showed potential for bioremediation of the heavy metals contaminated and acidic soil. (Fig. 5). (Hussain et al. 2019) also stated that 14ASP assisted plant in phytoremediation when inoculated with *Glycine max* L plant in multi metal contaminated soil. Haq et al. conducted a study on 14ASP and reported that it cleans the cadmium and chromium contaminated water employing biosorption process (Haq et al. 2016).

Salinity stress is one of the major abiotic stresses directly responsible for stunted plant growth that eventually lead to reduced crop production. PGPB reported the safest and sustainable tool for the alleviation of soil salinity in agriculture sectors (Numan et al. 2018). These bacteria assist plant growth directly via nitrogen fixation, phytohormones, solubilize phosphate and sequester iron by the production of siderophore. Strain 14ASP, a plant growth promoting bacteria produce IAA, Siderophores and NH_3 has been reported alleviating salt stress in wheat two wheat verities Pasban 90 and Khiram. Strain 14ASP tailored numerous mechanisms, like root colonization, production of various enzymes (ACC deaminase, Superoxide dismutase (SOD), Peroxidase dismutase (POD), catalase) and apply osmotolerance mechanisms to cope with salt stress (Fig. 1) (Afridi et al. 2019). Therefor, plant growth promoting bacteria could be used as a sustainable and eco-friendly biotechnological tool for the bioremediation of heavy metals contaminated and acidic soil in the near future.

Discussion

During the last few decades, the plant beneficial microbes have been studied extensively with different aspects and have reported as a plant growth promoter, biological control antagonists, bio-stimulants, sweepers of contaminated environments and well-known assailants of their host in harsh conditions. The current study evaluated the multifarious functions of plant growth-promoting endophyte *K. rhizophila* strain 14ASP on the basis of a genomic and in vitro study in normal and stressful environments. The strain showed plant growth promoting ability by producing IAA, ammonia, siderophore, catalase and solubilize the inorganic phosphate to phosphate ions to available for plants (Afridi et al. 2019). The PGPB assist plant by tailoring direct and indirect mechanisms. They solubilize the unavailable nutrients and make them available to plants, and helping plant to produce the enzyme that helps break down potentially harmful oxygen molecules in cells) to ensure their survival in hostile (salt stress and heavy metals stress) environment.

The strain14ASP portrays the survival potential characteristics under the adverse (salt stress and heavy metals stress) regimes. The strain 14ASP has the ability to tackle these adverse conditions by producing ACC deaminase, antioxidant enzymes and growth hormones which revise, promote plant growth and maintain the sodium and potassium ions concentration balance in plant tissue (Fig. 5) (Afridi et al. 2019). Enhanced plant growth in wheat, rice, tomato, soybean, okra, and coping with adverse conditions by producing the ACC deaminase and antioxidant enzymes have been reported which could be suggested to use PGP bacteria as a plant growth promoter, and bio-fertilizers (Das et al. 2016; El-Esawi et al. 2018; Afridi et al. 2019; Bibi et al. 2019). ACC deaminase and antioxidant enzymes play key roles in mitigating stress by cleaving ACC to α-ketobutyrate and ammonia and scavenges the ROS to protect the cellular organelles and plasma membrane and reduce the drastic effect of stress ethylene in a stressful environment (Sarkar et al. 2018; Win et al. 2018; Zerrouk et al. 2019). The *K. rhizophila* bacteria produce ACC deaminase and antioxidant enzymes which assist plant growth in normal and stressful regimes. ACC deaminase producing plant growth promoting bacteria (*Burkholderia* sp. MTCC 12259, *Curtobacterium albidum* strain SRV4, *Achromobacter xylosoxidans* Bac5, *Serratia ureilytica* Oci9, *Herbaspirillum seropedicae*, *Enterobacter cloacae* HSNJ4 Oci13) inoculated in plants, e.g., *Oryza sativa*, *Ocimum sanctum*, *Medicago sativa* L., *Pisum sativum*, *Brassica napus* L., respectively) have been reported to promote plant growth and alleviate abiotic stress in the adverse environments (Noori et al. 2018; Sarkar et al. 2018; Vimal et al. 2019).

Soil salinity is one of the most devastating abiotic constraints in the agriculture sector which directly influences plant health, productivity and quality. Plant growth-

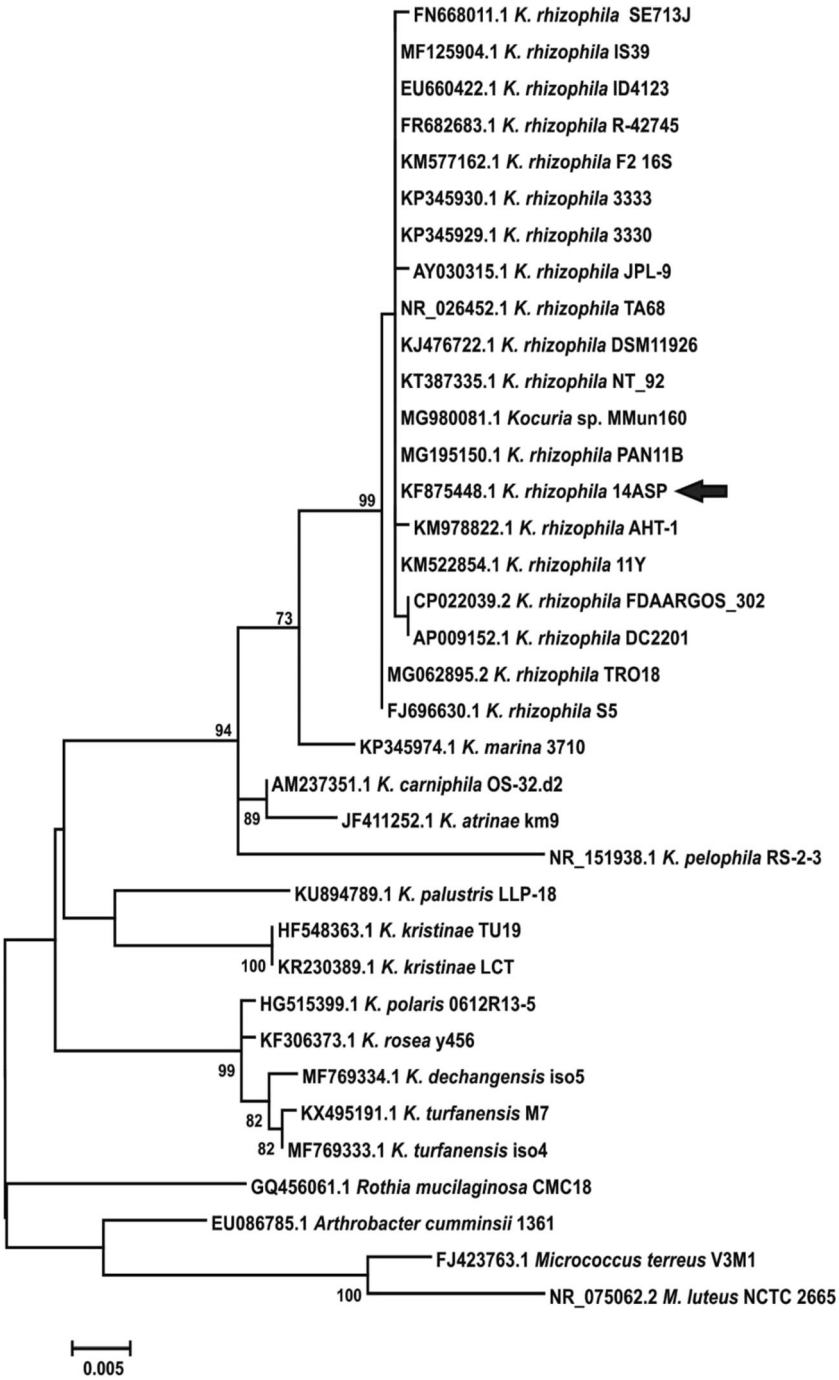

Fig. 4 Phylogenetic tree showing the position of *Kocuria rhizophila* strain 14ASP (arrow) in relation to other species and genera in the family Micrococcaceae. The tree was based on 884-bp nucleotides of the 16S rRNA gene aligned in the program MAFFT. The reconstruction was done in Mega7 (Tamura et al., 2013) with the Maximum likelihood method and substitution model TN93 + I. Accession numbers followed by species names and strain codes are shown on the tree. Bootstrap values higher than 70% are shown in the appropriate branching points. The scale indicates the number of substitutions per site

promoting bacteria have the capability to mitigate salt stress by promoting plant growth and underpin plant health by modulating biochemical, physiological and molecular mechanisms in plant, encountering saline environments. The strain 14 ASP screened at 5, 10 and 15% NaCl concentrations and grows well in both LB agar and LB liquid medium. The strain 14ASP multiplied in LB liquid medium normally at 5, 10 and 15% NaCl concentrations, when the optical density (OD at λ =600 nm) was measured at 12 h intervals. The strain 14ASP incubated and showed growth up to OD = 0.55–0.61, OD = 0.31–0.45, OD = 0.11–0.23 respectively at, 15% NaCl concentrations. Recently (Wu et al. 2020) reported that salt-tolerant bacteria tailor special mechanisms under high concentration of NaCl Such as increase osmotic pressure by prolonging the retardation period, slowing down the growth rate of the logarithmic phase, increasing spo0A gene expression, increasing biofilm formation, reducing Na^+ uptake, and changing the expression of metabolites and intracellular soluble proteins. Our findings are corroborated with the previously published data soybean inoculated with *Bacillus firmus*

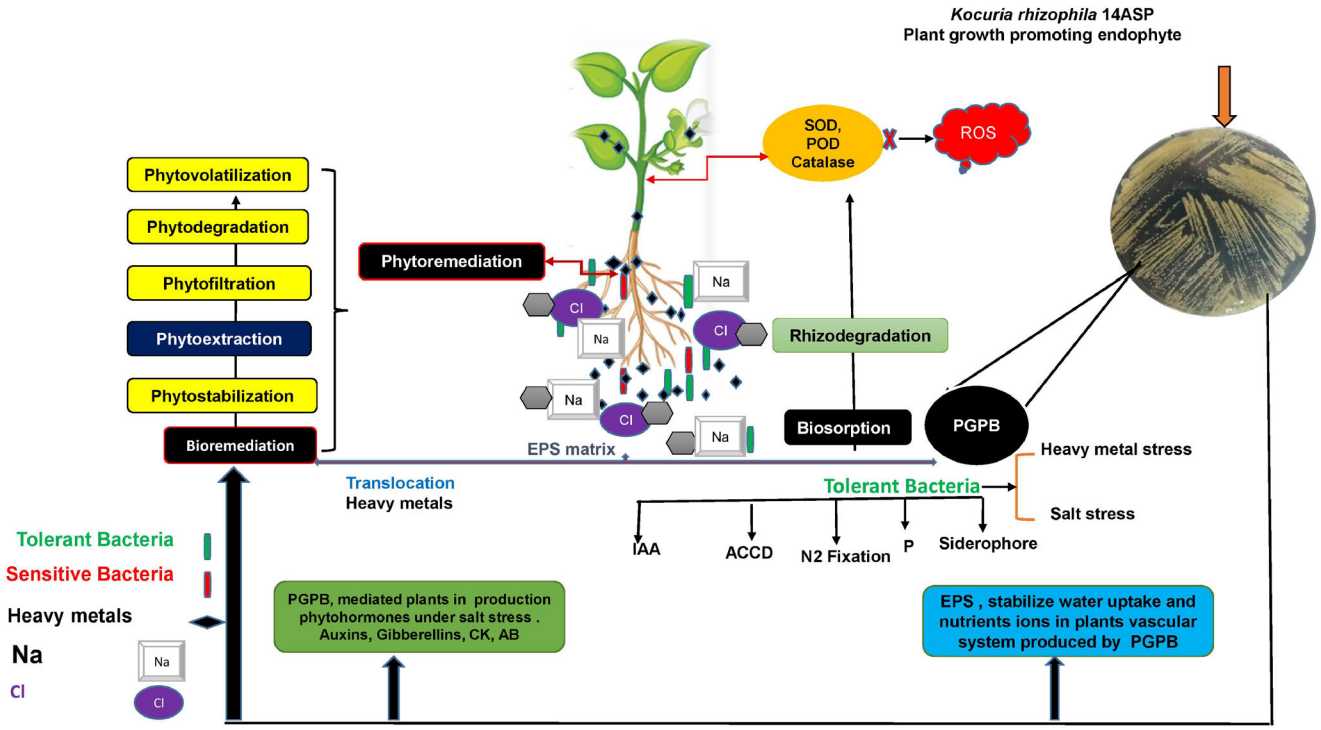

Fig. 5 Schematic diagram showing bioremediation biotechnological and environment friendly technique by the mutual assistance of plants and heavy metal resistant bacterium. The environmental stress tolerant and plant growth bacterium *Kocuria rhizophila* strain 14ASP not only promote plant growth but also assist plant in phytoremediation in hostile condition and sweep the heavy metals contaminated environment friendly via biotechnological and physiological processes

(SW5) (El-Esawi et al. 2018) and wheat inoculated with *Bacillus pumilus strain* FAB10 (Ansari et al. 2019).

Heavy metals released from leather industries is a ruthless threat to terrestrial and agricultural environments, marine biodiversity and human population. Ample efforts have been made for effective remediation, however, productive remediation techniques including microbes based bioremediation approach - must still be developed and applied on field-scale. The utilization of heavy metal resistant microbes to restore contaminated environments are cost-effective, sustainable and eco-friendly technique. The strain 14ASP showed resistance to heavy metals (Cr^{6+}, Pb, Cd^{2+}, Cu, and Ni) and grows normally in the media supplemented with heavy metals at different concentrations (50, 100, 150, 200, 250, 300, 350, 400, 450, 500 mg L^{-1}). Several heavy metals resistant bacteria with plant-growth promoting activities have been reported such as *Cupriavidus taiwanensis* KKU2500–3, *Ochrobactrum* sp., *Bacillus* sp., *Pseudomonas* sp., *Pseudomonas jessenii*, *Delftia* sp. B9, *Azotobacter chroococcum*, *Pseudomonas putida* and *Bacillus pumilus* have been used in bioremediation. They facilitate bioremediation either by detoxification of heavy metals or increase the phytoremediation potential of plants under heavy metals stress regime (Cd, Pb, Ni, Cu) (Qiao et al, 2021; Zhang et al. 2020; Rahman 2020; Tiwari et al. 2020; Hayat et al. 2020a, b). *The most common mechanisms tailored by the heavy metals resistant microbes is the induction of the metal-binding specific proteins (metallothioneins - MTs) and melanin that chelates and facilitate the sequestration of heavy metals inside the cell.*

K. rhizophila strain 14ASP can biodegrade diesel fuel, and adsorbed heavy metals Cd and Cr (up to 9.1 and 14.4 mg g^{-1}, respectively) (Mufti et al. 2015; Haq et al. 2016).

The current study revealed some incongruent results since normally the microbes come under stress and reduce their growth rate and population densities in response to harsh conditions what we called inverse relationship comparatively with control under heavy metals concentration treatments (Mazumder et al. 2020; Oladipo et al. 2018) but when the strain 14ASP exposed to heavy metals stresses such as by Cr, Pb, Cd, Cu, and Ni, in LB broth medium, the population densities of strain 14ASP reported higher than control in Cr supplemented medium at day 3. Intriguingly, the strain 14ASP enhanced population densities at day 5, 6 and 7 when the control turned into the stationary stage in all heavy metals supplemented media compare to control. This growth pattern trend followed the previous studies where the bacteria inoculated with heavy metals concentration increased the growth and demonstrated that some of the tolerant bacteria use heavy metals for metabolisms and biological process for their growth and tolerance (Li et al. 2021; Manegabe et al. 2017; Oladipo et al. 2018).

The genome sequence of strain 14ASP also exhibited the responsible and involved genes for hydrocarbon metabolism and heavy metal tolerance. The strain 14ASP possess these genes showed by the RAST functional annotation in Fig. 3. The annotated genome of 14ASP displays heavy metals resistant and bio-degrading genes involved in alkane degradation. The genome also possesses genes for heavy metal tolerance and polyaromatic hydrocarbon degradation. Various genes associated with heavy metals resistance such as *Cu ATPase*, *multi copper oxidase*, *cadB*, *chrA*, *pbrA*, *MerA* and *NiCoT* have been reported in bacterial resistance and detoxification systems, respectively, against Cu, Cd, Cr, Pb, Hg, Ni, as well as in the involvement of transitional metal transport and *czc* family have been reported in bacteria, respectively. In the resistant gene taxonomic analysis, the most common bacterial phyla were *Proteobacteria* and *Actinobacteria* (Niestępski et al. 2020; Das et al. 2016; Xavier et al. 2019). The *Kocuria* genus contains various species and strains (such as *K. kristinae*, *K. rhizophila* and *K. marina)* that capable of degrading phenol compounds and could be show resistance up to 10% NaCl that could be grown and with- stand against these environmental stresses in supplemented growth media (Takarada et al. 2008; Sims et al. 2009; Raghupathi 2018).

The genome of *K. rhizophila* comprises genes that are involved for significant functions such as adsorption of heavy metals and degradation of hydrocarbon which crucially contributes in contaminated environment as a sweeper and underpin bioremediation. The most important functional regulated genes were also deducted into the *K. rhizophila* genome that they contribute in very significant functions such as hydrocarbon degradation, heavy metal resistance siderophore biosynthesis, alkane degradation, polyaromatic hydrocarbons and heat shock resistance (Fig. 5) (Wu et al. 2017; Xiao et al. 2018; Niu et al. 2018). Recently, Ducret et al. (2020), Idowu et al. (2020) and Hofmann et al. (2021) found that CzcR-CzcS, a two-component system is involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa.*

Based on genomic and in vitro studies analysis of *K. rhizophila*, we reported here for the first time a plant growth- promoting bacterium which shows resistance to salinity stress (15%) and various heavy metals (500 mg L^{-1}) and assisted plant growth in hostile conditions employing, physiological and molecular mechanisms. The genome sequence analysis study also have proven the up-regulation of the responsible genes under adverse conditions.

Conclusions

Kocuria rhizophila strain 14ASP exhibited tolerance to different heavy metal concentrations (50 mg L^{-1} to 500 mg L^{-1}) as well as salinity stress (15%). Genomic features of strain 14ASP were consistent with the plant growth-promoting activities that include phosphate solubilization, IAA production, hydrogen cyanide (HCN) and ammonia production. The genomic data of *K. rhizophila* revealed numerous functional genes involved in diesel and hydrocarbon degradation, heavy metal tolerance, ability for biofilm formation and heat shock resistance, IAA and siderophore biosynthesis. The current study, therefore, exhibits pieces of evidence that strain 14ASP is a promising candidate to improve plant growth under normal and adverse environmental conditions (e.g. heavy metals, salts). The plant growth-promoting microbes could be thus used in agricultural and environmental management sectors as bio-fertilizers and bio-remediators, which could prove a cost-effective, sustainable and eco-friendly tool for food production and environmental management in near future. Moreover, future comparative genomic studies will allow the scientific community to further explore the genomic characteristics that make strains of the genus Kocuria potentially applicable in agriculture, biotechnological and environmental management sectors.

Abbreviations ACC deaminase, 1-aminocyclopropane-1-carboxylate deaminase; CA, Citric acid; COG, Clusters of Orthologous Groups; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DSW, Double distilled water; HCN, Hydrogen Cyanide; IAA, Indole acetic acid; LB, Luria Ber-tani; MAFFT, Multiple Alignment using Fast Fourier Transform; MTs, Metallothioneins; NaCl, Sodium chloride; NCBI, National Center for Biotechnology Information; ncRNAs, Non-coding RNAs; OD, Optical density; ORF, Open Reading Frame; PGM, Personal Genome Machine; PGPB, Plant growth-promoting bacteria; PGPEB, Plant growth promoting endophyte bacterium; PGPR, Plant growth promoting rhizobacteria; POD, Peroxidase dismutase; RAST, Rapid Annotations using Subsystems Technology; ROS, Reactive Oxygen species; rRNA, ribosomal Ribonucleic acid; SOD, Superoxide dismutase

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Declarations

Competing interests The authors declare that there is no conflict of interest.

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