The Absorption Characteristics and the Genetic Response of *Beauveria Bassiana* Z1 under Cd^{2+} Stress

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Research Article

Keywords: Beauveria bassiana Z1, Cd2+, absorption characteristics, transcriptome sequencing, cytochrome P450

Posted Date: September 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-797923/v1

Abstract

Cadmium (Cd^{2+}) has carcinogenic and teratogenic toxicity, which can be accumulated in the human body through the food chain, endangering human health and life. In this study, a fungus named *Beauveria bassiana* Z1 was isolated and identified to absorb Cd^{2+}, and its Cd^{2+} absorption rate was 85.1 % when the Cd^{2+} concentration was 1 mM. Furthermore, the absorption rate of activated strain was significantly higher than that of inactivated, especially with a high Cd^{2+} concentration. The genetic response results show that the expression of Reactive Oxygen Species (ROS) scavenging enzyme gene and the stress resistance genes (p-type ATP transferase, heavy metal tolerance protein, cytochrome P450) was up-regulated, which was conducive to the ROS removal and heavy metal modification, thereby improving the tolerance of strain Z1 to Cd^{2+}. The research results are of great significance for elucidating the mechanism of fungi for heavy metal, and provide theoretical support for bioremediation of heavy metal pollution.

1 Introduction

Cd^{2+} resources are widely used in the industrialization in modern society, Cd^{2+} has a cumulative effect and is gradually enriched in water, soil and various organisms (Afzal et al., 2017). Cd^{2+} can bind to the hydroxyl, amino, and sulfhydryl groups of proteins, thereby inhibiting the biological activity of enzymes, affecting the expression of genes related to cell apoptosis and proliferation, and bringing serious harm to physical and mental health (Cuypers et al., 2010). Consequently, efforts have been made to effectively remove Cd^{2+} from contaminated environments. For example, Wang et al. and Yang et al. designed and obtained a variety of metal-free nanomaterials which could effectively remove and reduce various organic pollutants and heavy mental Cr(VI) (Yang et al, 2020, Wang et al,2020). Currently, many strategies have been proposed to remove Cd^{2+} from the environment, including chemical precipitation (Agwaramgbo et al., 2013), electrolysis (Champault et al., 2014), ion exchange (Hosseini et al., 2020), membrane separation technology (Mohammed and Sahu, 2019), activated carbon adsorption (Vajihe et al., 2019), etc. However, these strategies are generally characterized by high operation cost, complicated operation, high energy consumption and possible secondary pollution (Kavita and Keharia, 2012; Simonescu and Ferdes, 2012). Microbial biodegradation is considered to be a promising strategy in dealing with heavy metal and recovering contaminated environments due to its low operation cost, easy operation, low energy consumption and lack of secondary pollution (Xu et al., 2020).

The microorganism absorption modes of fungi for heavy metals include the metabolism-independent binding the metals bind to the cell walls, and metabolism-dependent intracellular accumulation (Kirillova et al., 2017; Xu et al., 2014). In the metabolic independent mode, heavy metals were adsorbed by microorganisms through surface complexation, coordination, chelation, extracellular precipitation, and ion exchange (Paknikar et al., 2003). The metabolism-dependent mode mainly includes the cell membrane efflux, vacuolar compartment detention, heavy metal chelation and oxidoreductase detoxification (Delalande et al., 2010). Generally, heavy metals are first chelated to the microbial cell wall

through surface bonds, and then enters the cell through endocytosis and are captured by the vacuole or interact with glutathione, metallothionein, citrate and phytochelatin (Jacquart et al., 2017). As one kind of heavy metals, Cd^{2+} is eventually deposited inside the cells, which may affect the growth and metabolism of microorganisms. Although the absorption process of Cd^{2+} by microorganisms has been reported (Feng et al., 2018), the genetic response of microbes to Cd^{2+} is still unclear. Studying the microbial gene response to Cd^{2+} stress is crucial for clarifying the tolerance mechanism of microorganisms to Cd^{2+} and providing a genetic basis for heavy metal bioremediation.

Beauveria bassiana is a classical entomogenous fungus used in the microbial control of pests, widely applied in the field of agriculture and forestry (Luo, 2016). *Beauveria bassiana* has attracted extensive attention due to its super stress resistance under the dual stress of external environmental factors, such as high temperature, sunlight, ultraviolet radiation, chemical pesticides, and the toxin in the host caused by the pests. It has become one of the model strains for studying the interaction between filamentous fungi and host as well as between fungi and environment (Zhang, 2016). Previous studies have shown that *Beauveria bassiana* has the potential to tolerate heavy metals. The maximum tolerance concentrations of Pb^{2+} and Cd^{2+} by *Beauveria bassiana* jb15 were 1200 mg/L and 200 mg/L respectively. Under the optimal absorption conditions, the absorption rates of Pb^{2+} and Cd^{2+} were 52.27% and 62.38% (Xie et al.,2020). In this study, a highly Cd^{2+}-tolerant fungus named *Beauveria bassiana* Z1 was isolated, and the absorption capacity and absorption characteristics of strain Z1 to Cd^{2+} were studied. Moreover, the transcriptome database of strain Z1 under Cd^{2+} stress was constructed and the gene response to Cd^{2+} stress was investigated. This research is of great significance for understanding the genetic response of microorganisms to Cd^{2+} and elucidating the mechanism of microorganisms against Cd^{2+} stress.

2 Materials And Methods

2.1 Strain

The bacterial strain was isolated from the Minfeng Chemical Plant (E 105°87', N30°22') in Chongqing, China. The selection medium for microorganisms was LB medium (5 g/L Yeast extract, 10 g/L Peptone, 5g/L NaCl) with 4 mM $CdCl_2$. Agar plates were made by adding 15 g/L of agar powder to medium. The single colonies were isolated by plate scribing method, and then the isolated single colonies were identified by species identification.

2.2 Phylogenetic analysis

Total genomic DNA was extracted using the Invitrogen™ genomic DNA extraction kit (Thermo Fisher, USA). Purified genomic DNA was assessed by Nano Drop 2000 (Thermo Fisher, USA) and the high quality DNA ($OD_{260/280}$ = 1.8-2.0, > 20 μg) was used as template for ITS rRNA gene amplification. The amplification primers were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-

TCCTCCGCTTATTGATATGC- 3'). PCR reactions were performed in a 20 μL volume containing 2 μL 10× Ex Taq buffer, 0.2 μL Ex Taq (5 U/μL), 1.6 μL dNTP Mix (2.5 mmol/L), 1 μL primer ITS1 (5 μmol/L), 1 μL primer ITS4 (5 μmol/L), 0.5 μL genomic DNA, and 13.7 μL ddH_2O. PCR amplification conditions were as follows: 1 × 95 °C 5 min; 25 × 95 °C 30 s, 56 °C 30 s, 72 °C 60 s; 1 × 72 °C 10 min; and holding at 10 °C. The obtained sequences were compared with other sequences in GenBank database using BLAST method. The phylogenetic tree was constructed based on ITS rRNA gene sequences using neighbor-joining method.

2.3 Experimental conditions

To investigate the absorption characteristics and mechanism of *Beauveria bassiana* Z1 to Cd^{2+}, the culture time of strain Z1 was different for different experimental purposes. The optimum growth temperature for strain Z1 was 25 ℃, and the rotating speed was 170 rpm. The Cd^{2+} removal efficiency of strain Z1 was evaluated at the different Cd^{2+} concentrations (1 mM,2 mM,4 mM,6 mM,8 mM,10 mM) by inoculating the LB medium with 3 mL *Beauveria bassiana* spore suspension (10^6 spores mL^{-1}) and being incubated at 25°C and 170 rpm for 5 d. Previous study shows that the absorption rate of living bacteria to Cd^{2+} reached the maximum after 5 d of culture. The bacterial suspensions were centrifuged at 7104 g for 5 min, and the cell-free supernatants were used for Cd^{2+} detection.

To study more absorption characteristics of strain Z1 to Cd^{2+}, the adsorption rates of Cd^{2+} by activated and inactivated strains were compared. The fungus liquid in logarithmic growth period and the fungus liquid after sterilization (at 121°C and 1.05 kg / cm^2 for 20 min) were added to the LB medium, separately. The Cd^{2+} concentration in the LB medium was set at 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM. After shaking for 24 hours, the extracellular absorption of Cd^{2+} reach the equilibrium (Gao et al.,2006; Galli et al., 2003). The bacterial suspensions were centrifuged at 7104 g for 5 min, and the cell-free supernatants were used for Cd^{2+} detection.

To investigate the effect of 450 μmol/L Taxifolin (cytochrome P450 inhibitors) on adsorption of strain Z1 to Cd^{2+}, the flask was inoculated with 3 ml spore suspension containing 100 mg/L Cd^{2+} medium, and the bacteria suspension was incubated at 25°C, 170rpm for 5 d. Then the bacterial suspensions were centrifuged at 7104 g for 5 min, and the cell-free supernatants were used for Cd^{2+} detection.

In the experiment of electron microscopy, the strain Z1 was cultured for 72h (reaching exponential period) because the morphology of exponential bacteria at that time was full and easy to be observed. In the transcriptome analysis, the strain Z1 was cultured for 48 hours because according to previous studies, the absorption rate of Cd^{2+} in high concentration cadmium solution significantly increased in 48h. Therefore, the transcriptome data measured were convincing.

2.4 Cd^{2+} analytical methods and calculation formula of absorption rate of Cd^{2+}

The Cd^{2+} removal efficiency of strain Z1 was evaluated at the different experiment conditions. By inoculating the LB medium with 3 mL *Beauveria bassiana* spore suspension (10^6 spores mL^{-1}) and being incubated at 25°C and 170 rpm for a certain time, the suspension incubations were obtained. Then for determination of the Cd^{2+} absorption rate, 2 mL suspension incubation was centrifuged at 7104 g for 5 min to isolate the cell-free supernatant. The Cd^{2+} concentration in the cell-free supernatant was measured by atomic absorption spectrometry (AA800, Perkin Elmer). Specifically, the concentration of the sample supernatant was diluted to the detection range of Cd^{2+} standard curve(0-5ug/L), and then the solution was measured by atomic absorption spectrometry (AA800, Perkin Elmer). Five samples were taken from each concentration, and each sample was measured repeatedly for three times.

The percentage of metal absorbed (Y, %) was:

$$Y = \frac{C_0 - C_1}{C_0} \times 100\%$$

where C_0: is initial metal ion concentration in the cell-free supernatant before culture (mM); C_1: is final metal ion concentration in the cell-free supernatant after a certain period of culture (mM).

2.5 Electron microscopic observation

The morphological features of strain Z1 was examined by scanning electron microscope (SEM). The bacteria were cultured in the LB medium containing 10 mM Cd^{2+} and the LB medium without Cd^{2+}. After *Beauveria bassiana* Z1 was cultured to the exponential stage, 1 mL of the bacterial solution was centrifugated at 7104 g for 5 min. The supernatant was removed, and the cell pellet was fixed with 2.5 % glutaraldehyde solution for 3 h. Subsequently, these cells were washed three times with 0.2 mol/L phosphate buffer ($Na_2HPO_4.12H_2O$ 16.71 g/L, $KH_2PO_4$2.72 g/L), and were dehydrated in gradients with 30 %, 50 %, 70 %, 85 %, 95 %, 100 % ethanol solutions with each dehydration time of 15–20 min. After being dried in a critical point desiccator, the cell morphology was observed by scanning electron microscope (15.0 kv SEI, Zeiss, Germany).

2.6 Transcriptome analysis

The logarithmic growth cells of strain Z1 cultured in LB medium containing 10 mM Cd^{2+} were used for transcriptome analysis, and the experimental group without Cd^{2+} was used as a control. After culture for 48 h, 2 mL bacterial solution was centrifuged at 4°C and 7104 g for 5 min. The TransZol TM Reagent Kit (Thermo Fisher, USA) was used to extract the total RNA. The RNA concentration was measured by NanoDrop 2000 (Thermo Fisher, USA). The mRNA library was established according to the operation instructions of the TruSeq TM RNA Sample Prep Kit (Illumina). The library fragments were purified with magnetic frame (Thermo Fisher, USA). The libraries were sequenced on an Illumina Nova Seq 6000 platform (Illumina,USA). The low-quality adaptor sequences and sequence reads were removed, and the clean reads were mapped to the reference genome of (*Beauveria_bassiana* ASM28067v1) using HISAT2 software. GO enrichment analysis of the differential expression genes (DEGs) was performed by the GO

seq R package, and the statistical enrichment of DEGs in the KEGG pathway was determined by KOBAS software.

2.7 Accession number

The Illumina sequencing data were deposited into the NCBI Sequence Read Archive (SRA) database as the BioProject ID (PRJNA688063).

2.8 Statistical analysis

All experiments were carried out in triplicate and the data were presented as the mean value ± SD. The statistical analyses were conducted by Turkey's multiple range tests at $P < 0.05$ using OriginPro 2021.

3 Results And Discussion

3.1 Characterization of bacterial strain

The morphological and phenotypic characteristics of Z1 strain were identified. The fungus is divergent, white, folded in the middle, fluffy in texture, colorless or yellowish at the bottom of the colony. Under the scanning electron microscope, obvious spores and hyphae were observed. Spherical or oval sporophores were accumulated on the stem or mycelium of conidia. The spores were densely clustered on the top of the antler-shaped sporogenic cells, most of which were 0.5μm-1μm in diameter. After heavy metal treatment, the fugus of *Beauveria bassiana* can be observed that the number of spores decreased. Their mycelium mostly concentrated in the middle of the colony, and a large number of bright yellow substances were generated at the bottom of the colony. Furthermore, the number of conidia decreased, and their mycelium swelled and deformed, and there was precipitation on the surface of the mycelium.

Besides, the phylogenetic analysis was conducted by comparison of ITS rRNA gene sequences. As shown in Fig. 1, the strain Z1 was closely related to *Beauveria bassiana* isolate 08F04, *Beauveria bassiana* clone F19-N, and *Beauveria bassiana* isolate B4. Therefore, the isolated strain was designated as *Beauveria bassiana* Z1.

3.2 Adsorption of strain Z1 to Cd^{2+}

As shown in Fig. 2A, as initial concentration of heavy metal increased from 1 mM to 10 mM, the absorption capacity of strain Z1 to Cd^{2+} was always higher than 75.0 %. The Cd-resistant strain of *B. cereus sp.*S5 exhibited high Cd^{2+} resistance and effective Cd^{2+} removal from cadmium-polluted water, For Cd^{2+} concentrations of 0.97 mM, active spore biomass of the *B. cereus sp.*S5 provided good removal efficiency (> 80 %) (Wu et al., 2016). The adsorption capacity of *Beauveria bassiana* to various heavy metals, and the Cd^{2+} absorption rate was 63.4% when the initial Cd^{2+} concentration was 0.27 mM (Gola et al., 2016). SY-2 was found to tolerate maximum Cd^{2+} at 1.0 mM concentration. This strain also exhibited good absorption capacity (up to 35.7 %) of heavy metal at 0.5 mM concentration (Liaquat,

2020). Compared to the studies mentioned above, the selected strain Z1 has a high absorption capacity to Cd^{2+}.

In order to reveal more characteristics of the Cd^{2+} absorption by strain Z1, the absorption rates of Cd^{2+} by activated and inactivated strain Z1 were studied within 24 hours. As shown in Fig. 2B, the absorption rate of activated strain was significantly higher than that of inactivated, especially with a high Cd^{2+} concentration. The absorption rate of inactivated strain Z1 to low concentration of Cd^{2+} (0.2 mM) was 28.8 %, and the absorption rate decreased with the increase of Cd^{2+} concentration. The extracellular absorption of heavy metals by inactivated strains was mainly dependent on the complexation with polysaccharide, chitin and glucan on the cell wall (Li, 2008). However, extracellular absorption is limited, so the absorption rate decreases with the increase of Cd^{2+} concentration. Moreover, Cd^{2+} can still be absorbed by the inactivated strains without energy provided by cells, since the inactivated strains release functional groups on the cell wall or in the cell. When the concentration of Cd^{2+} ranged from 0.4 mM to 1.0 mM, the absorption rate of strain Z1 to Cd^{2+} decreased slightly, which may be due to the mutual repulsion and competition of adsorption sites between Cd^{2+} in the solution. Whereas, the adsorption rate of Cd^{2+} by activated bacteria (42.6%) was much higher than that of inactivated bacteria (9.6%) when the concentration of Cd^{2+} was 1.0mM. In order to explore the high tolerance and adsorption effect of activated strains to Cd^{2+}, transcriptome analysis was carried out.

3.3 The genetic response of strain Z1 to Cd^{2+}

3.3.1 Overview of transcriptome sequencing and annotation

Figure 3A shows the correlation coefficient among the six samples. High correlation coefficient was obtained under the same treatment condition, while low correlation coefficient was observed under the different treatment conditions. The results indicate that the biological replicates and the transcriptome data were reliable. Figure 3B shows that there were a total of 2022 genes with significant differences after treatment of Cd^{2+}. Among them, 1104 genes were up-regulated and 918 genes were down-regulated. Furthermore, the up-regulated genes were enriched and analyzed by GO database (Fig. 3C). The genes annotated by GO database were classified into biological process and molecular function. In biological process, they were involved in the process of cell oxidative detoxification, oxidation-reduction process, peroxidase reaction and response to oxidative stress; in molecular function, they were related to peroxidase activity, antioxidant activity, oxidoreductase activity and hydrogen peroxide activity and metal ion binding.

3.3.2 Differential expression genes under Cd^{2+} stress

Heavy metals can bind to biological macromolecules in microbial cells, or induce increased intracellular reactive oxygen species (ROS) concentration, thus cause cells to be toxicity (Sharma et al., 2015). Gene enrichment analysis revealed that detoxification genes are mainly related to the removal of ROS, and the combination, transportation and effluxion of Cd^{2+}.Table shows that the CAT-related gene (gene6185) and

the GST-related genes (gene3338, gene2138) were significantly up-regulated. Moreover, the P-type ATP transferase-related genes (gene547, gene8035) were significantly up-regulated. The P-type ATP transferase is mainly related to the efflux process of heavy metal in cell, indicating that the up-regulated P-type ATP transferase genes may be related to Cd^{2+} binding and effluxion. In addition, gene1615 (ABC-2 type transporter), gene1765 (ABC transporter), gene1883 (heavy metal transporter), and gene7589 (presumed MFS drug efflux transporter) were significantly up-regulated. Gene7589 may be involved in the process of Cd^{2+} transport.

Fungi can defend against non-specific metal damage through a variety of ways, or adjust the metabolism of the whole cell to adapt to external environmental stress. There are also some metal-specific transcription regulators, oxidoreductases, molecular chaperones, etc. in *Beauveria bassiana* Z1 that are induced by Cd^{2+} (Table). These genes are usually related to the ability of the fungus to withstand stress. Actin-like proteins can enhance cell resistance to oxidative stress and participate in histone acetylation and DNA repair processes. For both the growth and development of eukaryotes and the tolerance of adversity stress, C_2H_2 zinc finger protein is important, Previous studies report that zinc finger proteins ZAT12 and ZAT7 played a central role in the signal transduction of ROS and abiotic stress, thus enhancing the ability to tolerate oxygen stress in Arabidopsis (Davletova and S., 2005;Rizhsky et al., 2004).

In this study, gene7612 (Cytochrome P450 CYP684A2), gene4704 (CytochromeP450CYP5293A1), gene7335 (Cytochrome P450 CYP623C1) and gene7334 (Cytochrome P450 CYP655C1) were significantly up-regulated in strain Z1 under Cd^{2+}. Detoxification enzymes can catalyze metabolic detoxification when cells are poisoned by exogenous toxic substances. There are many kinds of detoxifying enzymes, among which monooxygenase is the most important. Monooxygenase is a multi-enzyme complex, among which cytochrome p450 is the terminal oxidase in the whole enzyme system, and plays a key role in the catalytic function (Sligar, 2010).Cytochrome P450 is responsible for activating oxygen molecules and binding to substrates. Cytochrome P450 oxidizes the substrate to hydroxyl compounds, superoxides and peroxides in the presence of oxygen, and hydroxylates the substrate in the absence of oxygen. For example, P450 plays an important role in the process of entomopathogenic fungi attacking insect hosts (Jackson et al., 2002).The outermost hydrocarbon of insects can be hydroxylated by microsomal P450 monooxygenase system, and then completely decomposed by peroxisome β oxidation reaction. Previous studies have found that heavy metals can induce the expression of cytochrome P450 gene (Liu et al., 2015). In order to further determine the relationship between cytochrome P450 and the response of strain Z1 to Cd^{2+}, Taxifolin, an inhibitor of cytochrome P450, was added to the experimental group. Compared with the control group, the adsorption rate of strain Z1 to Cd^{2+} significantly decreased, which further verified that cytochrome P450 gene played an important role in resistance to Cd^{2+} stress,as shown in Fig. 4.

4 Conclusion

In this study, the absorption characteristics and the genetic response of strain Z1 to Cd^{2+} were investigated. The results indicate that *Beauveria bassiana* Z1 was resistant to high concentration of $Cd^{2+}$and its Cd^{2+} absorption rate was 85.1 % when the Cd^{2+} concentration was 1 mM. Furthermore, the adsorption of Cd^{2+} by living bacteria was much higher than that of inactivated bacteria under the same conditions. It was found by transcriptome gene sequencing that the genes related to ROS scavenging enzymes and transport vectors were up-regulated under Cd^{2+} stress. Moreover, cytochrome p450 related genes were found to rise significantly under Cd^{2+} stress. This is the first time that cytochrome P450 was found to be involved in the defense mechanism of the organism against Cd^{2+} stress. This study suggests that cytochrome P450 may play an important role in heavy metal metabolism.

5 Declarations

Author Contribution

Tiantian Yu: Conceptualization, methodology, analysis and writing. Lijie Zhang: Conceptualization, methodology, analysis, funding, supervision and editing. Yanhui Gao Zhengfa Ma, Mei Zhang, Weiqun Tan, Lei Zhang and Yunru Zhang: Conceptualization, supervision and editing. Tiantao Zhao funding, supervision and editing.

Ethics approval and consent to participate

No conflicts, informed consent, or human or animal rights are applicable to this study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests in this paper.

Acknowledgements

The authors would like to thank Prof Tiantao Zhao for the great assistance, Chongqing University of Technology for providing the financial support for this study via the National Natural Science Foundation of China (No.51978117).

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7 Tables

Table Transcripts per million values of detoxification genes under cadmium stress

Gene ID	Description	control group	experimental group
1463	Actin-like protein	1.15	144.33
6120	C_2H_2 zinc finger Protein	0.28	74.01
7335	Cytochrome P450 CYP623C1	0.20	134.50
7334	Cytochrome P450 CYP655C1	1.16	1079.15
2612	Multiple copper oxidase	0.40	26.20
4704	CytochromeP450CYP5293A1	1.20	33.0
7612	CytochromeP450CYP684A2	1.45	1111.53
6185	Catalase	114.45	421.77
547	Calcium transfer P-type ATP transferase	4.10	11.20
8035	Calcium transfer P-type ATP transferase	68.40	213.90
1883	Heavy metal tolerance protein	8.20	20.20
7339	Monocarboxylic acid permease	0.25	1501.68
7589	Hypothetical MFS drug exosomes	3.07	1230.5
3338	glutathione S-transferase	100.29	494.4
2138	Glutathione S-transferase	24.62	123.75
1765	ABC transporter	21.39	31.42
1615	ABC-2 type transporter	34.42	50.17

Figures

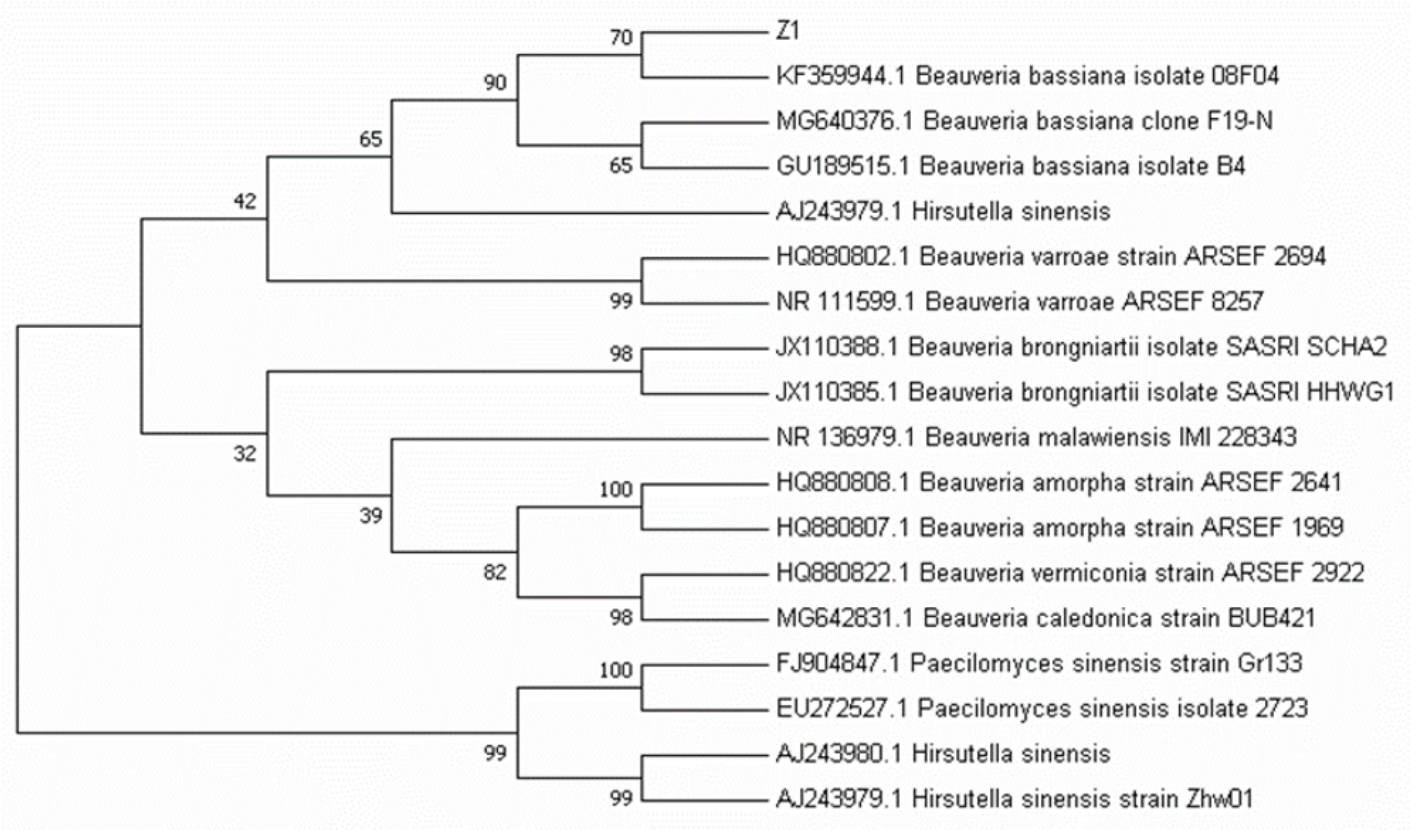

Figure 1

Phylogenetic tree of strain Z1 based on ITS rRNA gene sequences using neighbor-joining method.

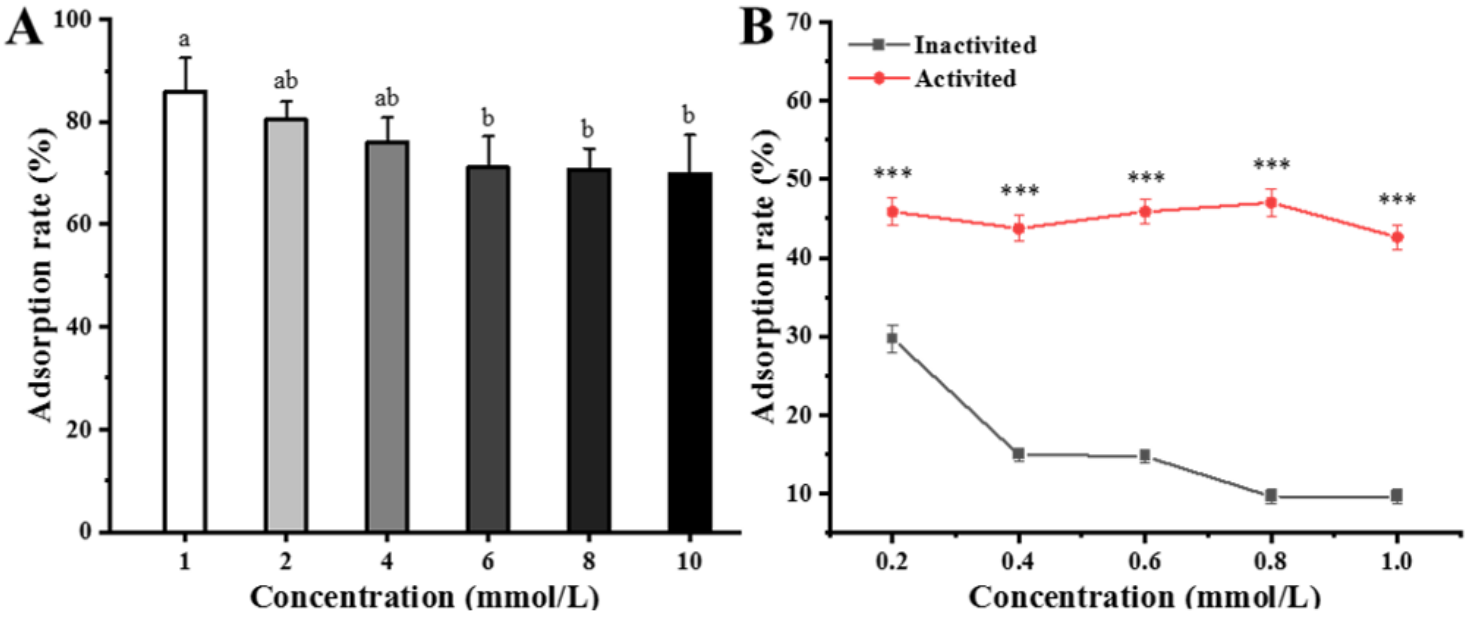

Figure 2

Absorption of Cd2+ by strain Z1. Different letters or asterisks on the error bars indicate statistical differences between groups according to Turkey's multiple range tests at $P < 0.05$. The asterisks in figure

2B represent statistical differences between the actived group and the inactivated group at the same concentration. Error bars represent standard deviation (SD) of the means.

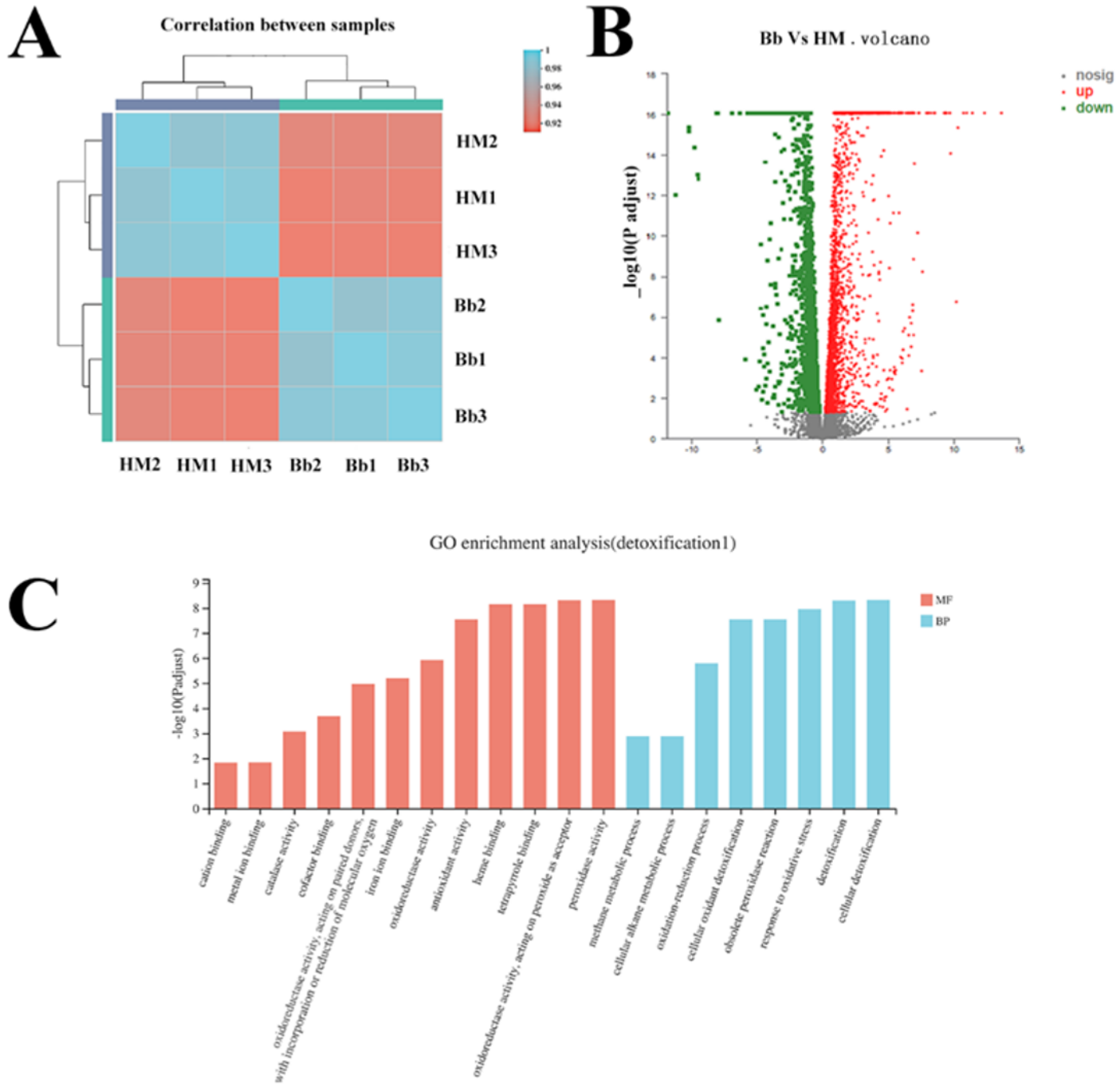

Figure 3

(A) The correlation plot between different samples. Control group (Bb1, Bb2 and Bb3), experimental group (HM1, HM2 and HM3). (B) Volcano distribution map of differentially expressed genes. The gray dots indicate genes with no significant difference, the red dots and the green dots indicate the up-regulated genes and the down-regulated genes, respectively. (C) GO enrichment map of genes related to heavy metal resistance. The ordinate represents the GO term, and the abscissa represents the significance level

of enrichment, corresponding to the height of the column. The smaller the FDR is and the greater the value of - log10 (padjust) is, the more significant the GO term is enriched. The three colors represent three categories: biological process (BP), cellular component (CC) and molecular function (MF).

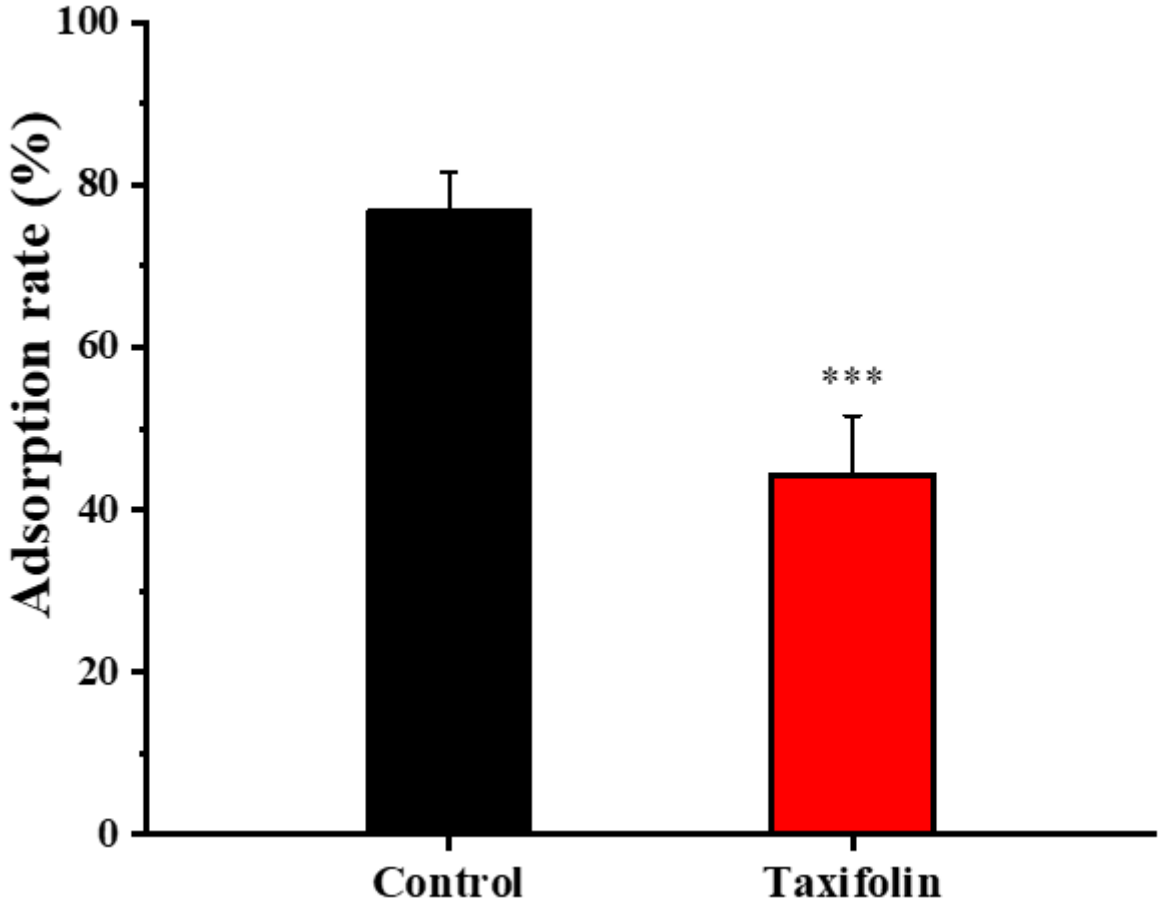

Figure 4

Effect of taxifolin on the adsorption of Cd2+ by Beauveria bassiana Z1. Asterisks on the bars indicate statistical differences between groups according to Tukey's multiple range tests at $P < 0.05$. Error bars represent standard deviation (SD) of the means.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.docx