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Resistance and plant growth-promoting properties under Zn/Cd stress of Pseudomonas sp. ZnCd2003

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***Abstract*—*Pseudomonas* sp. PDMZnCd2003 is a plant growth-promoting bacteria (PGPB) that was isolated from Zn/Cd contaminated soil. This research aims to study the characteristics of the Zn/Cd resistance and plant growth promoting properties of *Pseudomonas* sp.PDMZnCd2003 under Zn/Cd stress. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods were carried out to evaluate the lowest concentrations of Zn and/or Cd that inhibited and killed the bacterium. The MIC concentrations for Zn, Cd, Zn plus Cd and fixed Cd plus Zn were 150 mg l^{-1}, 70 mg l^{-1}, 60/60 mg l^{-1} and 20/150 mg l^{-1}, respectively. The MBC concentrations for Cd were 300 mg l^{-1}, while the bacterial growth recovered from treatment with 400 mg l^{-1} of Zn, 100/100 mg l^{-1} of Zn plus Cd, and 20/2000 mg l^{-1} of fixed Cd plus Zn. The growth tended to decrease under Zn/Cd stress, however, the plant growth properties of IAA production, N_2 fixation and P solubilisation remained under Zn plus Cd at 20/20 mg l^{-1}. Due to the Zn-Cd resistance and plant growth promoting properties under heavy metal stresses, the results demonstrate that *Pseudomonas* sp. ZnCd2003 should be used as a biofertilizer to promote plant growth in a Zn/Cd phytoremediation process.**

Keywords—*Pseudomonas*, zinc, cadmium, plant growth promoting bacteria

Introduction

Heavy metals are enriched in the environment by human activity of different kinds: examples of such activities include mining and ore refinement. Results of these activities end up in outlets and wastes where heavy metals are transported to the environment. They persist and cannot be removed from the environment. [1, 2]. Metal wastes can exist as individual metals or, more often, as metal mixtures. Baker et al [3] reported that cadmium (Cd) never occurs in isolation in the natural environment but, rather, appears mostly as a guest metal in zinc (Zn) mineralization. The soil in the fields of Phatat Phadaeng sub-district, Mae Sot, Tak Province, Thailand, are a source of Zn mineralisation. High levels of Cd and Zn have been reported in sediment samples from the creek sand, paddy field areas and rice grains in the vicinity of mining [4, 5]. The health impacts of Cd overexposure on the Mae Sot population in 12 villages have been reported since 2007 [6, 7]. Therefore, the problem of Cd and Zn contamination in the Mae Sot area needs to be remedied.

Phytoremediation has been proposed as an alternative method to remove pollutants from contaminated areas or to render pollutants harmless. Phytoextraction, is one of the key processes of phytoremediation that involves the use of metal accumulating (hyperaccumulators) plants to remove metals from soil by concentrating them in harvestable parts of the plant. However, when it is not possible to remove the metals from the contaminated sites by phytoextraction, other viable options, such as *in situ* immobilization (e.g., phytostabilization) should be considered as an integral part of risk management [8]. The success of phytoremediation is dependent on the potential of the plants to yield high biomass and withstand the metal stress. In addition, microbial activities in the root/rhizosphere soils enhance the effectiveness of phytoremediation processes [9]. Much research has investigated the microorganisms in the rhizosphere of metal hyperaccumulative plants by isolating the bacteria that were tolerant to heavy metals and contained plant growth-promoting properties, such as production of 1-amino-1-cyclopropane carboxylic acid (ACC) deaminase and indole-3-acetic-acid (IAA), nitrogen fixation, phosphate solubilisation and production of allelochemicals that include metabolites, seiderophores, antibiotics, volatile metabolites, enzymes and others [10, 11]. However, the tolerance and responses of bacterial isolates to excess heavy metals should be investigated especially in the metabolite aspects [12-14].

Pseudomonas is a genus of Gram-negative aerobic bacteria, belonging to the family Pseudomonadaceae. A number of *Pseudomonas* strains have been reported with Zn and/or Cd tolerance. Under Zn and/or Cd stress, they promoted growth of dicot and cotyledon plants such as *Ricinus commmunis* [15], willow [16], pea [17], tomato [18], *Orychophragmus violaceus* [19], rice [20], sorghum [21], *Brassica napus* [22], pumpkin and mustard [23]. However, it is better to use indigenous microorganisms as they have already been adapted to survive in the polluted soil. *Pseudomonas* sp. PDMZnCd2003 was isolated from the rhisophere of *Gynura pseudochina* (L.) DC., a Zn/Cd hyperaccumulative plant, growing in a Zn mining [24]. It had Zn/Cd tolerance and multiple PGPB properties. This isolate produced seiderophore and extracellular polymeric substances (EPS) under Zn and/or Cd treatment [25].

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Therefore, this research aims to study the characteristic of *Pseudomonas* sp. PDMZnCd2003 on Zn/Cd resistance. We determined the MICs (minimal inhibitory concentrations) and MBCs (minimum bactericidal concentrations) of the heavy metals, and investigated the effect of Zn/Cd stress on growth promotion abilities for IAA production, N_2-fixation and phosphate solubilisation. Our findings suggest that this bacterial isolate may assist the phytoremediation of Zn and Cd in the field.

Materials and Methods

A. Microorganisms

Pseudomonas sp. PDMZnCd2003 (accession number of 16S rDNA nucleotide sequence: JX193586) preserved in 50% (v/v) glycerol at –20°C was re-cultivated in nutrient broth (NB) or nutrient agar (NA) at room temperature (30±2°C) for 18-24 h. Morphological and biochemical characteristics of the bacterium are shown in Table I.

B. Assessment of metal resistance

The refreshed bacterium was aerobically cultured and shaken at 150 rpm, room temperature (30±2°C) for 18-24 hours to obtain the optical density at 600 nm (OD_{600}) of 0.5. To determine metal tolerance, 10 µl of bacterial suspension was inoculated into a nutrient agar plate (NA) containing Zn and/or Cd by the drop plate technique at 35±2°C for 24-48 h. The various Zn and/or Cd concentrations ranged from 5 to 200 mg l^{-1}, Cd plus Zn ranged from 5/5 to 200/200 mg l^{-1}. Zn and Cd solutions were prepared from $ZnSO_4.7H_2O$ (Ajax Finechem, Australia) and $3CdSO_4.8H_2O$ (Ajax Finechem, Australia), respectively.

The MICs of Zn and Cd were evaluated following a modified microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth [26]. Each well contained (1) 50 µl of Mueller Hinton broth, (2) 50 µl of Zn/Cd stock solution or sterile water (control), (3) 10 µl of resazurin solution and (4) 10 µl of bacterial suspension with the OD_{600} of 0.132 (McFarland No. 0.5, approximate cell density of 1.5 x 10^8 CFC/ml). The various Zn and/or Cd concentrations ranged from 5 to 400 mg l^{-1}, Cd plus Zn ranged from 5/5 to 100/100 mg l^{-1} and fixed Cd 20 mg l^{-1} plus Zn concentrations ranged from 20 to 2000 mg l^{-1}. Plates were prepared under aseptic conditions and incubated at 30±2°C for 24-48 h. The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration of the metals inhibiting growth was determined as when the colour changed from dark purple to purple and this was taken as the MIC value. The minimum bactericidal concentration (MBC) of Zn and Cd were determined by streak microbial suspensions that changed resazurin's colour from purple to dark purple, on Mueller Hinton agar plates. The lowest Zn and Cd concentrations with no growth of the bacteria on the agar plate was taken as the MBC value.

C. Quantitative monitoring of plant growth promoting properties

Indole-3-acetic acid production: The bacterial isolate was inoculated in trypticase soy broth (TSB) containing 0.2% w/v tryptophan in the absence or presence of Zn plus Cd (20/20 mg l^{-1}), and incubated at 30±2°C at 150 rpm in the dark. The supernatants were mixed with Salkowski's reagent (ratio 2:1) in the dark for 20 minutes. The optical density was measured at an absorbance of 530 nm [27]. The IAA concentration was determined using a standard curve of authentic IAA (Sigma-Aldrich, St. Louis, MO, USA).

Nitrogen fixation: The bacteria were cultured in N-free malate medium in the absence or presence of Zn plus Cd (20/20 mg l^{-1}), and incubated at 30±2°C at 150 rpm. Ammonia nitrogen (NH_3-N), an inorganic dissolved form of nitrogen in the supernatant was quantitatively analysed with Nessler's reagent as described by Cappuccino and Sherman [28]. The amount of NH_3-H was measured against a standard curve of ammonium chloride (NH_4Cl) (Ajax Finechem Pty Ltd, Australia).

Phosphate solubilisation: The bacteria were cultivated in the National Botanical Research Institute's phosphate growth (NBRIP) medium containing 0.5% (w/v) tricalcium phosphate, in the absence or presence of Zn plus Cd (20/20 mg l^{-1}) and incubated at 30±2°C at 150 rpm. Soluble phosphate in the supernatant was measured by the modified ascorbic acid method of Clesceri et al [29]. The concentration of the soluble phosphate was determined against a standard curve of potassium dihydrogen phosphates (KH_2PO_4) (Ajax Finechem Pty Ltd, Australia).

During cultivation in the media, bacterial cells were collected and separated from EPS by centrifugation and washed with 0.85% (w/v) NaCl and sterile-distilled water. The cell pellets were resuspened with sterile-distilled water, and the optical density of the bacterial growth at 660 nm was measured by Vis-Spectroscopy (Thermo Fisher scientific, Spectronic Genesys 215, USA). The pH changes in the culture media were determined with a pH meter (Denver Instrument Model 215, German).

TABLE I. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF *Pseudomonas* sp. PDMZnCd2003

Morphological characteristics	
Colony morphology	Blue-green, circular with curled margin and rough surface
Gram reaction	Gram negative, coccobacilli
Motility	Motile
Biochemical characteristics	
Casein hydrolysis[a]	1.45±0.03
Starch hydrolysis[a]	-
Cellulose hydrolysis[a]	2.29±0.35
Growth on triple sugar iron agar (TSI)[b]	K/NC, H_2S slant
Growth on peptone iron agar (PIA)[c]	+

[a]SI (solubilization index); mean values (n=2) ± standard deviation (SD)
[b]K/NC =alkaline slant/no change; no gas. Interpretation: peptone used aerobically, no fermentation
[c]H_2S; (+) indicates positive; (–) indicates negative

III. Results and Discussion

A. Zn and Cd resistance

Heavy metal resistance for bacterial strain present in various natural habitats such as soil, water, sediments soil. Therefore, *Pseudomonas* sp. PDMZnCd2003 was tested for its tolerance and resistance properties to Zn and/or Cd in both solid and liquid media. Table II shows the bacterial isolate's tolerance to NA (solid medium) contaminating with Zn, Cd and Zn plus Cd concentrations of 200/200 mg l^{-1}. In addition, the results of the MIC tests (liquid medium) of Zn, Cd, Zn plus Cd and fixed Cd plus Zn were 150 mg l^{-1}, 70 mg l^{-1}, 60/60 mg l^{-1} and 20/150 mg l^{-1}, respectively (Table III). The MBC occurred at 300 mg l^{-1} of Cd, while the bacterial growth recovered from treatment with 400 mg l^{-1} of Zn, 100/100 mg l^{-1} of Zn plus Cd and 20/2000 mg l^{-1} of fixed Cd plus Zn. To determine the MICs of heavy metals, most studies have used the medium that best supports the growth of the microorganism. Although metal-binding capacity of the microorganisms, chelation to various components of the media, and formation of complexes can cause a reduction in the activities of free metals [30], much research reported that the MICs of separate Zn and Cd against *Pseudomonas* strains were 13-130 mg l^{-1} and 16-281 mg l^{-1}, respectively [12, 16, 20, 22, 23, 31, 32]. They corresponded to the Zn/Cd tolerance properties of *Pseudomonas* sp. PDMZnCd2003. The tolerance properties occurred from the functional groups of thiol, carbonyl and amine in its structure, EPS and seiderphore [25].

B. Plant growth promoting properties under Zn/Cd stress

The growth of *Pseudomonas* sp. PDMZnCd2003 depended on the composition of the media. *Pseudomonas* sp. PDMZnCd2003 was isolated from Zn/Cd contaminated soil by nutrient broth (NB) containing 20 mg l^{-1} of Zn and Cd. In addition, the extractable amounts of Zn and Cd leached from the Zn/Cd contaminated soil in agricultural areas by diethylene triamine pentaacetic acid (DTPA) ranged from 30-120 mg kg^{-1} dry wt. and 1-10 mg kg^{-1} dry wt., respectively. Therefore, Zn plus Cd at the concentration of 20 mg l^{-1}, which had no Zn/Cd precipitation, was supplied to the media to study their effect on plant growth promoting properties.

A high cell density and EPS production was obtained when culturing in the complete medium of TSB. Zn plus Cd affected its growth in TSB and N-free malate medium as shown in Fig. 1(a, c). The decrease in the specific growth rate and prolonged lag-phase responding to high concentrations of Zn and Cd happened to *P. fluorescens* BA3d12 and *Pseudomonas* spp. strains KKU25000-4 to KKU2500-24 [12, 20].

TABLE III. METAL TOXICITY OF *Pseudomonas* sp. PDMZnCd2003

Metals (mg l^{-1})	Metal toxicity	
	MIC	MBC*
Zinc	150	-
Cadmium	70	300
Zinc+Cadmium	60/60	-
Fixed Cadmium+Zinc	20/150	-

*(–) indicates no MBC found in test condition

The pHs in TSB increased from 7.0 to 8.3-8.8 during the bacterial growth (Fig. 1(a)), the alkaline pHs might be due to protein utilized aerobically and also secretion of yellow-green compounds. The protein digestion to ammonia (NH_4^+) and hydrogen sulfide (H_2S) production under the aerobic condition were shown in biochemical testing of TSI and PIA (Table I). The secretion of pyoverdine, a yellow-green fluorescent siderophore, affected the alkaline pH in the culture medium as found in *Pseudomonas fluorescens* [33]. The secretion of yellow-green compounds was found only in cultivation in TSB medium. Therefore, the increase of alkaline pHs in N-free malate medium (Fig. 1(c)) was probably caused by nitrogen fixation into ammonium (NH_4^+) [34].

Pseudomonas sp. ZnCd2003 was able to produce IAA when supplemented with tryptophane. Fig. 1(b) shows IAA production under the absence and presence of Zn plus Cd (20/20 mg l^{-1}). In late log-phase after 13 h of incubation, the maximum IAA productions in the absence and presence of Zn plus Cd were 234.76±0.78 µg ml^{-1} and 233.56±4.78 µg ml^{-1}, respectively. The Zn plus Cd had negative effects on IAA production after the stationary phase. Although a negative effect of metal cations (Fe^{3+}, Al^{3+}, Cd^{2+}, Cu^{2+} and Ni^{2+}) on auxin production has been significantly demonstrated [13, 35], seiderophore may reduce the toxic effect of metal cations by chelation [35]. In addition, Ananthalakshmi et al [36] reported the formation of IAA-metal complexes possibility leaded to decreasing amounts of free IAA.

Although Zn plus Cd affected the bacterial growth in N-free malate medium as shown in Fig. 1(c), the NH_3-N production was slightly different. The maximum NH_3-N concentrations in the absence and presence of Zn plus Cd were 6.70±0.09 mg l^{-1} and 6.28±0.05 mg l^{-1}, respectively (Fig. 1(d)). The decrease in the NH_3-N concentration after 13 hours might be due to excess ammonium in the growth medium that results in immediate repression of the *nif* gene transcription [34].

Fig. 1(f) shows the presence of Zn plus Cd affected to phosphate solubilisation, even the bacterial growth in the control and treatment were similar (Fig. 1(e)). The maximum PO_4^{3-}-P concentrations in the absence and presence of Zn plus Cd were 366.06±0.98 mg l^{-1} at 17 hours and 301.85±0.78 mg l^{-1} at 47 hours, respectively.

TABLE II. METAL TOLERANCE OF *Pseudomonas* sp. PDMZnCd2003

Treatment	Zn/Cd Concentration (mg l^{-1})									
	5	10	15	20	30	40	50	70	100	200
Zinc	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Cadmium	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Zinc+Cadmium	+++	+++	+++	+++	+++	+++	+++	+++	+++	++

*(–) indicates no growth; (+) low growth; (++) moderate growth; (+++) high growth

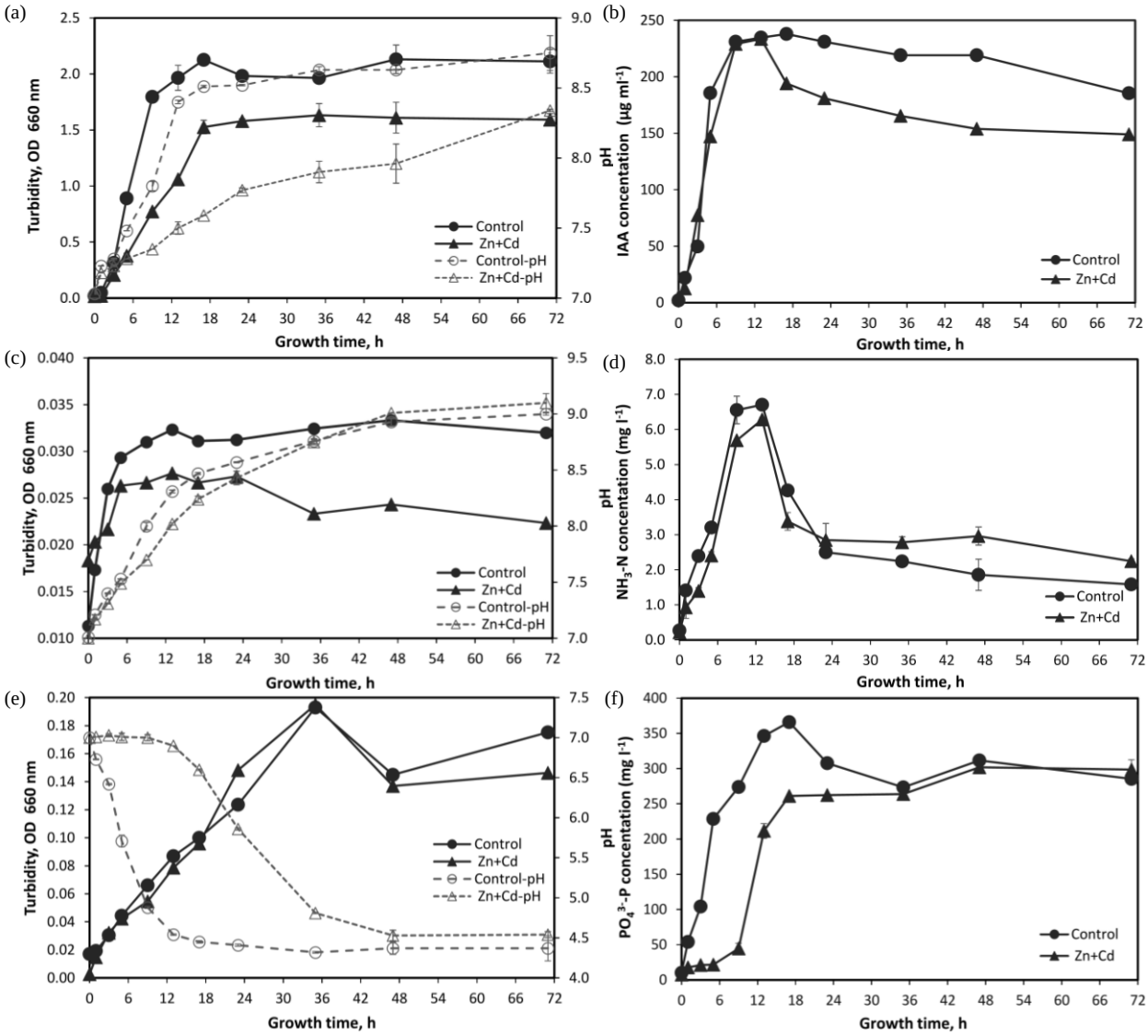

Figure 1. Growth, pH change, and properties of IAA production, N_2 fixation and P solubilisation of *Psudomonas* sp. PDMZnCd2003 under cultivation in the absence and presence of Zn plus Cd (20/20 mg l^{-1}). (a) Bacterial growth in TSB medium, (b) IAA production, (c) bacterial growth in N-free malate medium, (d) nitrogen fixation, (e) bacterial growth in NBRIP medium and (f) phosphate solubilisation.

The properties of the bacterium when changing the inorganic phosphate to the soluble form seemed to correspond with acid production as the pH change in Fig. 1(e) shows. The pHs of the NBRIP medium containing 0.5% (w/v) tricalcium phosphate with the absence and presence of Zn plus Cd gradually decreased from ~7.0 to ~4.5 at 17 hours and 47 hours of cultivation, respectively (Fig. 1(e)). The production of organic acids by phosphate solubilizing bacteria has been well documented. The hydroxyl and carboxyl groups of organic acids can chelate the cations bound to phosphate, thereby converting mineral phosphate into soluble forms [37, 38]. Among them, gluconic acid was reported as the principal organic acid produced by *Pseudomonas* sp. [39].

Conclusions

The beneficial effects of bacterial inoculants can be realized only if they survive competitively in the rhizosphere. In this study, *Pseudomonas* sp. ZnCd2003 was not only able to withstand high Zn and/or Cd concentrations, but also showed the properties of IAA production, N_2 fixation and P solubilization under the metals stress. The use of purified IAA and/or N, P fertilizer to enhance plant growth may be expensive, and therefore unsustainable for large-scale phytoremediation, especially in resource-poor countries. Therefore, we demonstrate that *Pseudomonas* sp. ZnCd2003 could serve as an efficient biofertilizer candidate for microbe assisted phytoremediation in Zn/Cd contaminated areas.

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This paper introduces a plant growth-promoting bacterium containing Zn/Cd resistance properties, which could be applied for bioremediation of Zn/Cd contaminated land.

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