Cyclic lipopeptide biosurfactant from *Bacillus tequilensis* exhibits multifarious activity

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Abstract

Bacillus tequilensis strain CH had been previously shown to produce a biosurfactant. In this study, chemical structure of the purified biosurfactant was determined by using high performance liquid chromatography and liquid chromatography-mass spectroscopy as a 10 amino acid cyclic lipopeptide (CL). The cyclic lipopeptide was found to be active against *Anopheles culicifacies* larvae with a LC₅₀ of 110 µg/ml in 2 days. 1 ppm cadmium (Cd) which had a profound mutagenic effect on the cell division of onion (*Allium cepa*) root tip cell resulting in abnormal metaphase, abnormal anaphase and nuclei elongation was partially reversed in the presence of 0.1 mg/ml of CL (52% cells dividing normally and 8% with abnormal division) and was comparable to control experiment where no Cd was present. Thus, the CL described in this report may have applications in eliminating larvae from water repository systems and in reversing the effects of cadmium pollution.

Keywords Biosurfactants · Lipopeptide · Larvicidal · Bioremediation

Introduction

Biosurfactants are amphiphilic compounds which contain both hydrophobic and hydrophilic moieties causing them to aggregate at interfaces between fluids with different polarities and thereby decrease interfacial surface tension. Biosurfactants are produced by many plants and microbes and they vary in their chemical structure, molecular size, and activity. Broadly, due to the nature of biosurfactants, they are used to increase the surface area of substrates, increase the bioavailability of substrates through solubilization/desorption and attachment/detachment of microbes from surfaces.

Due to their amphiphilic nature, they have potential in many applications such as detoxification of cadmium, induction of apoptosis in human kidney cells (Pradhan et al. 2014a, b), inhibition of biofilms (Kuiper et al. 2004; Pradhan et al. 2013, 2014a, b), and enhancing nanoparticle formation by forming liposome vesicles (Priyadarshini et al. 2016). Cadmium, lead, and mercury are the three most poisonous elements for human health present in soil, water and air. These heavy metals have no significant role on human metabolism. Cadmium has a more bio-persistent property and so it can remain in the organism's body for years (Wuana and Okieimen 2011). Total cadmium concentration increases in soil due to use of fertilizer, pesticides, sewage sludge and disposal of industrial wastes. Weggler et al. (2004) reported that plants can uptake a significant amount of cadmium from contaminated soil and water. Cd affects the activity of many enzymes like delta-aminolevulinic acid synthetase, arylsulfatase, alcohol dehydrogenase, and lipoamide dehydrogenase (Manahan 2003).

Cd can be removed from contaminated soil or water using chelating agents because chelating agents bind to metal ions. Prabhukumar et al. (2004) compared the efficiency of an elastin-like polypeptide (ELP) and EDTA for the removal of Cd from contaminated soil and water (Wen et al. 2009). Biosurfactants are also acting as good chelating agents for

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different metal ions. Juwarkar et al. (2007) reported that rhamnolipid had the capacity to remove 92% of Cd and 88% of Pb from contaminated soil after 36 h of leaching. In the present study, we have elucidated the chemical structure of a biosurfactant extracted from *Bacillus tequilensis* CH using high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectroscopy (LC–MS). The larvicidal effect of CL and potentiality to detoxify cadmium up taken by the plant were also studied.

Materials and methods

Culture and production of biosurfactant

Bacillus tequilensis strain CH isolated from a brackish water source (Chilika) in Odisha, India was shown to produce a biosurfactant (Pradhan et al. 2013). This bacterial strain was cultured in modified mineral salt media at 35 °C with a speed of 150 rpm in shaker incubator described as Pradhan et al. (2013). Then, the culture was centrifuged at 10,000 rpm to get cell-free supernatant after 7 days incubation. The cell-free supernatant was acidified with 3N HCl to bring the pH 2 which is required to precipitate biosurfactant. The acidified supernatant was kept in a refrigerator at 4 °C overnight. The next day, crude biosurfactant was found by solvent extraction process where chloroform and methanol were used at a ratio of 2:1. The crude biosurfactant was concentrated with the help of rotary evaporator (Priya and Usharani 2009; Saravanan and Vijayakumar 2012).

Purification and characterization of biosurfactant

To determine amino acid nature and quantity in CL, it needs HPLC analysis. The biosurfactant was hydrolyzed with 6N hydrochloric acid at 105 °C for 25 h in a closed glass container. With diethyl ether solvent extraction method, fatty acid was extracted from hydrolysate. The aqueous phase containing amino acids of the peptide was heated to evaporate rest of the solvents. The amino acid mixture was analyzed by C18 reverse phase HPLC column (Eclipse Plus-C18, 4.6×50 mm, 1.8 µm Column) with the mobile phase consisting of acetonitrile and water in a ratio of 85:15. HPLC spectra at 250 nm were recorded.

Thin layer chromatography (TLC) purified biosurfactants were dissolved in CH₃OH/CH₃CN (50/50 v/v) for mass analysis. All mass spectrometry analysis was done on an LC/MS system (Waters Alliance System, USA). 15 µg/ml of sample was injected at a flow rate of 5 µl/min. Cone voltage and capillary voltages were 31 V and 3/41 kV, respectively. The flow rate of N₂ gas was 700 L/h and mass scan range was 100–2000 (*m/z*). Source temperature and de-solvation

temperature were set at 120 and 350 °C, respectively (Tran et al. 2008; Kaustuvmani et al. 2017).

Applications of purified biosurfactant

Larvicidal activity

Larvae of *Anopheles culicifacies* were obtained from the repository of Rearing and Colonization Unit of the Regional Medical Research Centre (RMRC), Bhubaneswar. To study the larvicidal activity, the larvae used were at third instar stage. 0.150 L of chlorine-free tap water was taken in disposable wax-coated paper cups (0.2 L) and 20 larvae of *A. culicifacies* were transferred to each cup. Experiments were conducted in duplicates with adequate controls. All these cups (experimental and control) were incubated at 28 ± 2 °C, 80–90% relative humidity with a photoperiod of 12 h light and 12 h dark. Concentrations of CL taken in this study were 85, 100, 110, 130 and 145 µg/ml. Dimethyl sulfoxide (DMSO) dissolved CL was used here.

Alleviation of toxic effects of cadmium

Freshly grown onion bulbs (*Allium cepa*) were selected as samples. The dry scales of bulbs were removed and cleaned. The bulbs were germinated in test tubes containing water with Cd and CL for 48 h at room temperature. These varying conditions were (1) distilled water (without Cd); (2) 1 ppm Cd in distilled water; (3) 1 ppm Cd and 0.1 mg/ml CL in distilled water, (4) 0.1 mg/ml CL in distilled water. The Cd was provided as cadmium chloride.

Roots were fixed with a mixture of 95% C₂H₅OH and 99.8% CH₃COOH (3:2) for 60 min and hydrolyzed in another mixture of 1 M HCl, 95% C₂H₅OH and 99.8% CH₃COOH (5:3:2) for 5 min at 60 °C. To observe chromosomal aberrations during mitosis root tips were squashed in Carbol Fuchsin solution (Maoxue 1989). Normal and abnormal dividing cells were calculated to detect the effect of Cd on the cell during mitosis (Fiskesjo 1985).

Results and discussion

Molecular characteristics

Surface tension (ST) of HPLC grade water was determined to be 71.59 mN/m with the help of Tensiometer. The decrease of ST was seen on the increment of the concentration of CL. ST of CL solution decreased from 69.08 mN/m to 29.4 mN/m when concentrations of CL were increased from 0.5 to 1 mg/L.

To determine the type of amino acids present in peptide moiety, HPLC analysis of peptide part of CL was carried

out. The CL peptide part was hydrolyzed and separated from the lipid part. The hydrolysate contains the mixture of amino acids which are separated and identified with the help of amino acid standards by reverse phase HPLC. The optimum separation of the individual peak (the amino acid mixture of CL) was achieved with a mobile phase that consists of 85:15 ratios of acetonitrile and water. The chromatogram is shown in Fig. 1. The peaks were identified as Asp, Glu, Ser, Pro, and Phe.

To determine the molecular weight and to get expected structure of CL molecule, LC/MS (Waters Alliance LC/MS system) analysis was done using Waters Alliance LC/MS system. The peaks describing the molecules and a probable structural appearance of the component of CL is illustrated in Fig. 2. Six major peaks were identified (Table 1) along with base peak (100% abundance). The maximum m/z value was 1400, which probably is the largest ion moving through the spectrometer, it was assumed that this ion corresponds to $[M + Na]^+$. The relative formula mass of the compound (M^+) was, therefore, estimated to be 1377 Da (1400 Da-molecular weight of Na). The m/z of base peak was 737 which represents $[M + Na - (Sc-Ser-Glu-Asp-Asp + H_2O)] + [M + Na - (Asp-Glu-Glu-Phe-Asp)]^+$ was represented by the peak of m/z 765. The fragment which was formed by the detachment of (Asp-Glu-Glu-Phe-Asp-Pro + H_2O) from $[M + Na]^+$ represented by peak, m/z 686. Peaks with m/z 437 and 309 were assigned to $[Sc-Ser-Glu-Na]^+$ and $[Sc-Ser-Na]^+$ fragments, respectively.

From the above HPLC and LC/MS data analysis, it was confirmed that CL was a cyclic lipopeptide with molecular weight 1377 Da. $(CH_3)_2CH(CH_2)_6CH(OH)CH_2CO-$ is the

side chain (β -OH fatty acid) with molecular weight 198 Da and Ser-Glu-Asp-Asp-Pro-Asp-Phe-Glu-Glu-Asp is the peptide part joins to side chain in a cyclic manner (links are: $-CO-Ser$ and $Asp-O-CH-$). Pecci et al. (2010) reported the production of surfactin and fengycin (lipopeptides) from *Bacillus licheniformis* V9T14 with molecular weight $[M + Na]^+$ 1030 and 1478 Da, respectively. The molecular weight of surfactin (C14/Leu7) produced by *Bacillus subtilis* was 1036 ($M + H$)⁺ (Faria et al. 2011). In the present study CL produced by *B. tequilensis* CH is having similar cyclic lipopeptide structure with a molecular mass of 1377 ($M + Na$)⁺ compared to reported surfactin and fengycin.

Applications (biological characterization)

Larvicidal effect of CL biosurfactant against *Anopheles culicifacies* larvae

Etiology of vector-borne diseases through mosquitoes is prevalent throughout the world. Many fatal diseases caused by mosquitoes belong to *Anopheles*, *Culex* and *Aedes*. This brings the necessity to search for economically viable and environmental friendly vector control strategies. Ghribi et al. (2012) reported the larvicidal activity of biosurfactant produced from *B. subtilis* SPB1 against *Ephestia kuehniella*. Surfactin produced from *B. subtilis* (VCRC B471) kills pupae of *Anopheles stephensi* (Geeta and Manomani 2010). Larvicidal activity of a cyclic lipopeptide was tested on larvae of *Culex quinquefasciatus* (Das and Mukherjee 2006). Our attempt was to test the effect of CL biosurfactant on *A. culicifacies* larvae responsible for causing malaria. It was

Fig. 1 HPLC chromatogram for amino acid mixture of CL

Fig. 2 Mass spectrum and chemical structure of lipopeptide produced by *B. tequilensis* CH

Table 1 Sodioted ions of CL in LC/MS system

Peptidic series product ions	$[M + Na]^+ (m/z)$
$[M + Na]^+$	1400
$[M + Na - (Asp-Glu-Glu-Phe-Asp)]^+$	765
$[M + Na - (Sc-Ser-Glu-Asp-Asp + H_2O)]^+$	737
$[M + Na - (Asp-Glu-Glu-Phe-Asp-Pro + H_2O)]^+$	686
$[Sc-Ser-Glu-Na]^+$	437
$[Sc-Ser-Na]^+$	309

(Sc: β -OH fatty acid side chain, Mr-198)

Table 2 Efficacy of different concentrations of CL against the larvae of *A. culicifacies* larvae

CL concentration	Treatment mortality (% of dead larva)				
	85 μ g/ml	100 μ g/ml	110 μ g/ml	130 μ g/ml	145 μ g/ml
24 h	10	10	40	40	40
48 h	20	40	50	60	60
72 h	30	80	80	80	80

observed that LC_{50} value of CL on *A. culicifacies* larvae is 110 μ g/ml in 2 days (Table 2).

Due to the absence of respiratory siphons in *Anopheles* larvae, spiracles play a major role in breathing and hence must attach frequently to surface of the water to avail oxygen from air. For this reason, they keep their body parallel to the surface of the water for respiration. Due to the

addition of biosurfactants (CL), the surface tension of water decreases. Hence palmate hairs of the larva cannot adhere to the water surface and larva sinks down to the bottom of water body causing the death (due to asphyxia). Geetha and Manonmani (2010) determined the LC_{50} value of surfactin against freshly emerged pupae of *Anopheles stephensi* as 2.6 μ g/ml which is very low in comparison to our study ($LC_{50} = 110$ μ g/ml). From these studies, we can say that pupa of *Anopheles stephensi* is more sensitive to lipopeptide than that of the larva of *A. culicifacies* as observed in the present study. Das and Mukherjee (2006) reported the larvicidal activity of two cyclic lipopeptides (CLPs) secreted by two *B. subtilis* strains (DM-03 and DM-04) against the larvae of *C. quinquefasciatus*, with LC_{50} values of 120.0 and 300.0 μ g/ml, respectively, which are higher compared to present study.

Alleviation of Cd toxicity in onion cells

Cadmium ions in the water used for germination of onion root cells show some profound adverse effect on the growing cells (Fig. 3; Table 3). In absence of Cd ions, most of the onion root tip cells (which represent the actively growing cells) were in normal dividing stage (60%) with less than 4% with any visible abnormality. But, in presence of even 1 ppm of Cd, most of the onion root tip cells were in non-dividing stage (56%) and about 40% cells showed abnormal mitotic division (Table 3). This result indicates that Cd has a profound mutagenic effect on cell division of onion root tip cells. This effect can be related to the occurrence of

Fig. 3 Onion root cells division in **a** water, **b** presence of 1 ppm Cd, **c** 1 ppm Cd+0.1 mg/ml CL, **d** 0.1 mg/ml CL. *NM* normal metaphase, *AM* abnormal metaphase, *NA* normal anaphase, *AA* abnormal anaphase, *NT* normal telophase, *NC* normal cytokinesis, *EN* elongated nucleus

Table 3 Effects of Cd on cell division on the root tip cells of *Allium cepa*

Expt. no.	Condition	Normal division (%)	Abnormal division (%)	Non-dividing cells (%)	Remarks
1	Only in water	60	4	36	Normal mitosis
2	With 1 ppm Cd	4	40	56*	Abnormal mitosis *Nuclei elongated and delocalized
3	1 ppm Cd + 0.1 mg/ml CL	52	8	40	Normal mitosis
4	With 0.1 mg/ml CL	76	44	20	Huge dividing cells Normal mitosis

abnormal metaphase, abnormal anaphase and nuclei elongation, etc., in the root cells of *Allium cepa* or the onion. A report suggests that Cd may be interacting with calmodulin (CaM) present in mitotic spindle which causes the abnormality in cell division (Liu and Jiang 2003). The interaction leads to thus deficit uptake of Ca^{2+} resulting in the chromosomal aberrations. In presence of 1 ppm Cd along with 0.1 mg/ml of CL, the adverse effect of Cd became nullified as 52% cell division appeared normal with only 8% abnormal division which was comparable to control experiment where no Cd was present in the water. Moreover, only in presence of 0.1 mg/ml of CL, dividing cells do not show any adverse effect thus non-toxic to the onion root cells.

As biosurfactants are amphoteric in nature, the presence of polar head helps them to form micelles with available metal ions in the environment. Anionic biosurfactants like rhamnolipid, sophorolipid, and surfactin chelate cationic metal ions making them bio-unavailable for microflora or plants in soil or water. As CL is an anionic biosurfactant

(confirmed from cetyltrimethylammonium bromide agar assay or called as CTAB agar assay), it forms complex with Cd^{2+} ions and makes them unavailable for plants. Here CL acted as a chelator for Cd. Thus, CL is capable of alleviating the toxic mutagenic effect of Cd to plants cells. *Pseudomonas fluorescens* strain isolated from sugar beet rhizosphere produces cyclic lipopeptide and CL shows antibiotic activity (Nielsen et al. 2002). *Bacillus subtilis* SPB1 produces lipopeptide types of biosurfactants and at optimized condition it produces 4.92 g/L biosurfactants (Ghribi and Chaabouni 2011). Mukherjee (2007) isolated CL from *B. subtilis* and used it for laundry detergent formulations.

Conclusion

Vector-borne diseases such as malaria, dengue, and chikungunya are a fatal scourge of humanity inflicting mortality and morbidity. Due to global warming and other risk

factors, there has been a resurgence and reoccurrence of vector-borne diseases particularly in India (Panneerselvam et al. 2013). As the ecology and climate are suited to the proliferation of the vectors (Nabar and Lokegaonkar 2015), *A. culicifacies* is thought to be a major vector for the transmission of malaria in India, but because of the complexities in vector species, discrimination, and identification of the particular species causing malaria has remained a controversial topic. Controlling vectors through commercially available insecticides is currently the method of choice, but these insecticides are mainly chemically synthesized and are non-specific. Hence, it is necessary to search for novel natural molecules which are species-specific, produce no side effects, are commercially viable for mass production and are biodegradable.

From the characterization results obtained in this study, we could successfully elucidate the probable structure of biosurfactant as cyclic lipopeptide having 10 amino acids. The results suggest that microbial surfactants have vast potentiality in remediating environmental contaminants and have an advantage over commercially available mosquito larvicidal as well. The presence of Cd in soil and continuous exposure of microflora to Cd severely affects the development and normal cellular processes. Remediating it through the use of secondary metabolites such as biosurfactant which efficiently chelate the metal ions can be helpful for plants and other flora to regain their normal cellular processes. Thus, it can be concluded that the biosurfactants obtained from the microbial sources have economically viable and environmentally friendly compounds. They can be efficiently used against vector-borne diseases and are suitable to be applicable for eliminating larvae from drainage and other water repository systems. From the results obtained for detoxification ability of CL against Cd, it can be inferred that CL from *B. tequilensis* is efficient in detoxifying the heavy metal such as Cd and can be applied to large-scale processes.

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Compliance with ethical standards

Conflict of interest No conflict of interest.

References

- Das K, Mukherjee AK (2006) Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains. *Acta Trop* 97:168–173. <https://doi.org/10.1016/j.actatropica.2005.10.002>
- de Faria AF, Teodoro-Martinez DS, de Oliveira Barbosa GN, Vaz BG, Silva ÍS, Garcia JS, Tótila MR, Eberlin MN, Grossman M, Alves OL, Durrant LR (2011) Production and structural characterization of surfactin (C14/Leu7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry. *Process Biochem* 46(10):1951–1957
- Fiskesjo G (1985) The Allium test as a standard in environmental monitoring. *Hereditas* 102:99–112. <https://doi.org/10.1111/j.1601-5223.1985.tb00471.x>
- Geetha I, Manonmani AM (2010) Surfactin: a novel mosquitocidal biosurfactant produced by *Bacillus subtilis* ssp. *subtilis* (VCRC B471) and influence of abiotic factors on its pupicidal efficacy. *Lett Appl Microbiol* 51:406–412. <https://doi.org/10.1111/j.1472-765X.2010.02912.x>
- Ghribi D, Chaabouni SE (2011) Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnol Res Int* 2011:653654–653656
- Ghribi D, Elleuch M, Abdelkefi L, Ellouze-Chaabouni S (2012) Evaluation of larvicidal potency of *Bacillus subtilis* SPB1 biosurfactant against *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae and influence of abiotic factors on its insecticidal activity. *J Stored Prod Res* 48:68–72. <https://doi.org/10.1016/j.jspr.2011.10.002>
- Juwarkar AA, Nair A, Dubey KV, Singh SK, Devotta S (2007) Biosurfactant technology for remediation of cadmium and lead contaminated soils. *Chemosphere* 68(10):1996–2002
- Kaustuvmani P, Rupshikha P, Mohan CK, Suresh D (2017) Characterization of biosurfactant produced during degradation of hydrocarbons using crude oil as sole source of carbon. *Front Microbiol* 8:279
- Kuiper I, Lagendijk LE, Pickford R, Derrick PJ, Lamers EMG, Lugtenberg JJB, Bloemberg VG (2004) Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. *Mol Microbiol* 51:97–113
- Liu D, Jiang WGX (2003) Effects of cadmium on root growth, cell division and nucleoli in root tip cells of garlic. *Biol Plant* 47:79. <https://doi.org/10.1023/A:1027384932338>
- Manahan SE (2003) Toxicological chemistry and biochemistry, 3rd edn. CRC Press, Limited Liability Company (LLC), Boca Raton
- Maoxue L (1989) Silver staining of plant chromosomes—techniques, principle and application. *J Wuhan Bot Res* 7:87–96
- Mukherjee AK (2007) Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis* strains in laundry detergent formulations. *Lett Appl Microbiol* 45(3):330–335
- Nabar BM, Lokegaonkar S (2015) Larvicidal activity of microbial metabolites extracted from extremophiles against vector mosquitoes. *Int J Mosq Res* 2:161–165
- Nielsen TH, Sorensen D, Tobiasen C, Andersen JB, Christophersen C, Givskov M, Sorensen J (2002) Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. *Appl Environ Microbiol* 68:7 3416–3423
- Panneerselvam C, Murugan K, Kovendan K et al (2013) Mosquito larvicidal and pupicidal activity of *Euphorbia hirta* Linn. (Family: Euphorbiaceae) and *Bacillus sphaericus* against *Anopheles stephensi* Liston. (Diptera: Culicidae). *Asian Pac J Trop Med* 6:102–109 doi: [https://doi.org/10.1016/S1995-7645\(13\)60003-6](https://doi.org/10.1016/S1995-7645(13)60003-6)
- Prabhukumar G, Matsumoto M, Mulchandani A, Chen W (2004) Cadmium removal from contaminated soil by tunable biopolymers. *Environ Sci Technol* 38(11):3148–3152
- Pradhan AK, Pradhan N, Mall G et al (2013) Application of lipopeptide biosurfactant isolated from a halophile: *Bacillus tequilensis* CH for inhibition of biofilm. *Appl Biochem Biotechnol* 171:1362–1375. <https://doi.org/10.1007/s12010-013-0428-3>

- Pradhan AK, Pradhan N, Mohapatra P et al (2014a) Cytotoxic effect of microbial biosurfactants against human embryonic kidney cancerous cell: HEK-293 and their possible role in apoptosis. *Appl Biochem Biotechnol* 174:1850–1858. <https://doi.org/10.1007/s12010-014-1168-8>
- Pradhan AK, Pradhan N, Sukla LB et al (2014b) Inhibition of pathogenic bacterial biofilm by biosurfactant produced by *Lysinibacillus fusiformis* S9. *Bioprocess Biosyst Eng* 37:139–149. <https://doi.org/10.1007/s00449-013-0976-5>
- Priya T, Usharani G (2009) Comparative study for biosurfactant production by using *Bacillus subtilis* and *Pseudomonas aeruginosa*. *Bot Res Int* 2:284–287
- Priyadarshini E, Pradhan N, Pradhan AK, Pradhan P (2016) Label free and high specific detection of mercury ions based on silver nano-liposome. *Spectrochim Acta Part A Mol Biomol Spectrosc* 163:127–133. <https://doi.org/10.1016/j.saa.2016.03.040>
- Saravanan V, Vijayakumar S (2012) Isolation and screening of biosurfactant producing microorganisms from oil contaminated soil. *J Acad Ind Res* 1:264–268
- Tran H, Kruijt M, Raaijmakers JM (2008) Diversity and activity of biosurfactant-producing *Pseudomonas* in the rhizosphere of black pepper in Vietnam. *J Appl Microbiol* 104:839–851
- Wegler K, McLaughlin MJ, Graham RD (2004) Effect of Chloride in soil solution on the plant availability of biosolid-borne cadmium. *J Environ Qual* 33:496–504
- Wen J, Stacey SP, McLaughlin MJ, Kirby JK (2009) Biodegradation of rhamnolipid, EDTA and citric acid in cadmium and zinc contaminated soils. *Soil Biol Biochem* 41:2214–2221. <https://doi.org/10.1016/j.soilbio.2009.08.006>
- Wuana RA, Okieimen FE (2011) Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecol* 2011: 20