PLASMID MEDIATED BIOREMEDIATION POTENTIAL OF SOME BACTERIAL ISOLATES FROM AQUATIC ENVIRONMENT

Syed Shahid Imran Bukhari and Syed Shahid Ali
Biochemistry and Toxicology Laboratory, Department of Zoology, University of the Punjab,
Quaid-i- Azam Campus, Lahore- 54590, Pakistan.
*Corresponding author Email: shahidimran_bokhari@yahoo.com

ABSTRACT: *Three endosulfan degrading bacterial strains ESDB-1, ESDB-2, ESDB-3 identified as Azotobacter sp. were isolated from water samples collected from Qasur and adjacent areas. These strains were found growing best at their isolated environmental conditions i.e., temperature and pH. Growth curves of the isolates clearly showed preference of endosulfan over glucose as sole source of carbon. All of the isolates were able to grow at 10%. These isolates were found resistant to various heavy metals (Cd, Cr, Cu, Hg and Pb) and antibiotics (ampicillin, chloramphenicol, neomycin, tetracycline and streptomycin). Ethidium bromide curing studies on the plasmid revealed the presence of all resistance genes on the plasmid. Electrophoretic studies showed the one 7 kb plasmid is responsible for developing resistance. The plasmids were used to transform the E. coli C600 competent cells separately. The bacterial strain isolated in the present study seems to have a strong potential for bioremediation of not only endosulfan but also other cyclodiene compounds.*

Keywords: Endosulfan degrading bacteria, Characterization, water, Bacterial strains.

INTRODUCTION

Organophosphates, organochlorinates and pyrethroids are the main groups of insecticides which are in practice since long. Besides their specificity to targeted organisms, these chemicals or their metabolites are generally toxic to many non-targeted organisms including humans [1]. Generally these chemicals are reported to have mutagenic, genotoxic, carcinogenic, fetotoxic and teratogenic effects [2]. Endosulfan is one of the organochlorinated insecticide used in the present study belongs to cyclodiene group, is registered toxicant for major kingdoms [3]. A wide range of microorganisms is reported to have the capability of utilizing chlorinated insecticides as sole source of carbon and/or nitrogen by a process known as biodegradation [4]. The most potential candidate among them used due to their ubiquitous presence and rapid growth are bacteria [5]. Bacteria, due to their continuous exposure to environmental stresses, have developed genetically determined systems against many toxicants [6]. The main objective of the present study was to isolate and characterize insecticide (endosulfan) degrading bacteria from local aquatic environment and to study their mechanism of resistance.

MATERIALS AND METHODS

Sampling sites and sample collection.

Effluent rich water samples were collected in autoclaved screw capped bottles from Rohi Nullah, near Lahore, Pakistan, readily brought to laboratory and preceded on nutrient agar plates. The selected colonies were streaked on M9 in search of endosulfan degrading bacteria.

Preparation of insecticide mixed media

Different volumes of stock insecticides (35 EC) were mixed thoroughly with 100ml media. Fourteen different concentrations in the range of 0.00625% to 10% were prepared and for growth of isolates were used for screening bacterial isolates (Table I).

Isolation of pure cultures of endosulfan degrading bacterial strains

Each isolate in triplicate was inoculated separately in100 ml media (LB-broth) and M9 with glucose. The Solid glucose in M9 agar plates was then replaced by endosulfan at a final concentration of 0.00625% for screening of insecticide degrading bacteria. The colonies appeared were then streaked on gradually increasing concentration of endosulfan from 0.0125% to 10%. Purified cultures of the isolates were kept at -20℃ and refreshed after every 3 months.

Cultivation of bacteria at their growth conditions optima

Selected isolates ESDB-1, ESDB-2, ESDB-3 of bacteria were optimized for temperature (25, 30, 37 and 42ºC) and pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 & 9.0) in the LB-broth. Each isolate with log phase culture in nine (100mL) conical flasks (three for LB-broth, three for M9 with glucose and three for M9 with 0.00625% endosulfan and were incubated in shaking water bath at 37℃ and 60rpm [7]. The absorbance was taken at 600nm after every 2 hours for 24 hours period (Fig 3).

Metal and antibiotic resistance of the isolates

LB agar plates with different concentrations (0.1. 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11, 12, 13, 14, 15, 20 and 40 mM) of five different metals (Cr^{6+} as $K_2Cr_20_4$, Cd^{2+} as $CdCI_2$, Cu^{2+} as $CuS0_4$, Hg^{2+} as $HgCI_2$ and Pb^{1+} 'as lead acetate were used to evaluate the heavy metals resistance of the isolates. Similarly five different antibiotics (ampicillin, chloramphenicol, neomycin, streptomycin and tetracycline) at 5, 10, 20, 30, 40, 80, 100, 120 150, 200 and 250µg concentrations of medium were used to check the resistance of the isolates against antibiotics.

Curing and isolation of plasmids

Plasmids of the isolates were cured with DNA intercalating dye ethidium bromide (Etbr) at concentrations from 100µg/ml to 600µg/ml [8]. Cured cultures of the isolates were checked for their resistance against the above mentioned metals and antibiotics. Loss of or decrease in the resistances of the cultures was taken as an indication of curing [8]. Plasmid was then isolated as described by Holmes (1984) and stored at 0ºC. In these strains, the cultures were taken from the tubes with 550µg/ ml (ethidium bromide). In case these strains, some colonies of EB treated cultures failed to grow on M9 containing glucose which may be due to interference of EB in DNA regions of the enzymes responsible for glucose metabolism.

Electrophoretic studies on plasmids

Agarose gel (1%) was prepared and was pre-run for 30 minutes. From each sample 5µg plasmid DNA (pDNA) was taken on a para film and mixed with loading buffer. The

samples and the standard (Hind-lll cut) were loaded in the wells and power supply was switched on at 20V overnight. The gel was stained, visualized and photographed on a UVP^X gel documentation system. A log graph was plotted between the distance travelled by DNA fragments of marker (along X-axis) and their respective molecular weights (along Y-axis). The approximate size of the plasmids was then calculated [9].

Preparation of competent cells of *E. coli* C600

LB-broth (50ml) was inoculated with 5ml log phase culture of *E. coli* C600 (restriction modification system deficient strain) according to Sambrook *et al*. (1989). This suspension of competent cells was stored at -20ºC.

Transformation of competent cells with pDNA of the isolates

Competent cells (900µl) of competent cells were incubated with 10 µl pDNA of each isolate in a separate eppendorf tube and were incubated at 0ºC for 30 minutes. The M9 plates with 0.5% endosulfan was then inoculated with 100µL of this culture. On a separate plate, competent cells before transformation and *E. coli* C600 cells were also spread as control and incubated at 37ºC overnight [10].

Plasmid incompatibility test

Plasmid Incompatibility Test was performed according to [9]. Resistance genes of antibiotics and metal ions were cross transformed with pDNA of each other. Several transformation crosses were conducted to assess the compatibility of the plasmids of these isolates with each other.

RESULTS

Three endosulfan degrading bacterial strains ESDB-1, ESDB-2, ESDB-3 were isolated from water samples and were characterized. On the basis of various morphological and biochemical tests, ESDB-1, ESDB-2 and ESDB-3 are identified as the members of *Azotobacter spp* (Table I).

Optimum growth temperature of isolates

Three isolates grew best at 30ºC except for ESDB-1 for which 37°C was optimum temperature (Fig. 1).

Optima pH of the isolates

The two isolates, ESDB-2 and ESDB-3 showed maximum growth at pH 7 while ESDB-1 gave best growth at pH 8 (Fig. 2).

Growth curves of isolates

Media preference order for above three isolates were found to be LB-broth > M9 containing 0.00625% endosulfan > M9 containing glucose (Fig. 3).

Metal and antibiotic resistance of the isolates

The order of the toxicity of the five metal ions tested against above three isolates was found to be: Hg > Cu > Cd > Pb > Cr (Table 5). The isolated strains however, showed a uniform resistance against tested antibiotics.

Growth of the isolates on different concentrations of endosulfan

All the three isolates were found capable of growing on M9 plates containing endosulfan up to 10% concentration within 7 days of incubation (Table 2). However, after overnight incubation these isolates showed growth maximum up to 0.05% concentration of endosulfan. All of the isolates showed capability of utilizing endosulfan crystals as a clear area around the growth in all concentrations of endosulfan checked after 7 days of incubation.

Curing of plasmids

A very high percentage yield of curing (74-90) was obtained for all of the isolates (Table 4).

Plasmid encoded resistance against metals and antibiotics

Cured strains of all isolates lost their ability to grow even on 0.05% endosulfan and to resist any of the metals except for Pb and antibiotics except for that of tetracycline.

Plasmid isolation and transformation

Agarose gel electrophoresis revealed the presence of one 7 kb plasmid in each strain.

Plasmid Incompatibility Test

Test and back crosses made between plasmid of one strain and the competent cells of the other. The results showed that only pESDB 2 and pESDB 3 can express together in the same cell so they are compatible.

DISCUSSION

Three isolates *i.e*., ESDB-1, ESDB-2 and ESDB-3 are solely identified as the members of the genus Azotobacter which is a well-known candidate of insecticide biodegradation [11]. All the three categories of insecticides are detoxified/degraded/utilized in different organisms by DDT-dechlorinase, the microsomal mixed-function oxidase system, GSH-S-transferases, epoxide hydrases and a variety of esterases, amidases and monooxygenases [12]. Microorganisms including bacteria generally derive energy from the chlorinated insecticides by removing halogen group from closed carbon ring [13]. All three bacterial strains isolated from aquatic environment during this study are cocci. Moreover, isolates namely ESDB-1, ESDB-2 and ESDB-3, are Gram negative. All strains were isolated from the samples with alkaline pH. One may suppose from this observation that Gram negative cocci bacteria isolated from the samples with alkaline pH may act as biological indicators of chlorinated insecticide pollution in the environment. This hypothesis has the support of another report of [14]. All three isolates showed maximum growth at 37°C in LB-broth with the exception of ESDB-1which grew best growth at 30°C. These observations support the idea of "*in situ bioremediation*" originally presented by [15]. It would involve the use of microorganisms at the site from where they are' to be isolated without providing them managed situation to do their task. Growth curves of the isolates indicate their preference for endosulfan over glucose as the only sole carbon source which again supported the accuracy of the biochemical tests which revealed that the isolates do not utilize glucose as a sole source of carbon. The short lag phases (ranging from 2-4 hrs) and log phases of the isolates (ranged from 8-12 hrs) suggest these isolates as the rapidly growing bacterial strains and would help in constructing the industrial strains.

These three strains of *Azotobacter* were found to be resistant to the increasingly high levels of all of the tested metal ions and antibiotics. Metal resistance in bacteria may be via adsorption of metal to cell surfaces [16], complexion by exo polysaccharides [17], binding with bacterial cell envelops [18], intracellular accumulation, biosynthesis of metal binding proteins [19], precipitation and transformation to volatile compounds [20]. Likewise there are several mechanisms in bacteria to resist antibiotics [21]. Multiple resistances of the isolates against metal ions like Hg, Cd, Cu, Pb and Cr and antibiotics like ampicillin, tetracycline streptomycin, neomycin

and chloramphenicol reveals the presence of one or more of several resistance systems and recommends them to be used in metal removal from the environment. These data also suggest them as the best candidates to act as model organisms for *in situ* bioremediation.

Plasmids of the isolates were cured using ethidium bromide as curing agent [22]. The intercalation of ethidium bromide in DNA double helix prevents plasmid DNA to replicate at the time of replication of chromosome. As a result, after a few generations, the cells get cured from plasmids. Ethidium bromide, in this study, was proved to be a good curing agent yielding a curing percentage ranging from 74% to 90% thus denying some of the reports that it is not a good curing agent [23]. There is a body of literature to support the plasmid encoded resistance of four metal ions (Hg, Cd, Cu and Cr) and four antibiotics (ampicillin, chloramphenicol, streptomycin and neomycin) according to several previous reports [24]. Chromosome-determined resistance of all isolates against lead and of ESDB-1 against tetracycline, in addition has already been reported elsewhere [25, 26].

It is a well-known fact that most of the antibiotic resistance genes like those of chloramphenicol, tetracycline, ampicillin and streptomycin and some of the metal resistance genes like those of cadmium and mercury are located on transposons either of simple or of complex type [27]. The isolates of this study also seemed to have such genes on transposons. This conclusion comes from the fact that Pb^{+2} is a chromosome-determined trait in all isolates and tetracycline resistance is encoded by the chromosome in ESDB-1. It might be possible that the resistance against these two strains were also plasmid encoded. Under permissive conditions of selection pressure and environmental stress, the transposons carrying the resistance genes for these two antimicrobial agents jumped from plasmid to the chromosome. If this prediction is true, then these traits showed the genetic plasticity of the isolates against environmental stresses.

The presence of metal/drug resistance genes on the plasmids also qualifies these plasmids for their use in recombinant DNA technology. In addition, DNA sequence coding for this resistance may be used as DNA probe for the search of new metal resistant and/or insecticide degrading bacterial strains [28]. Plasmids of the isolates were found to be of 7 Kb so these plasmids can fall into the category of small molecular sized plasmids. All of the plasmids have at least one gene for insecticide degradation, four metal resistance genes and five drug resistance genes (except for pESDB2 which do not code for tetracycline resistance).

It can be concluded from these findings that these isolates grow best at their environmental conditions, prefer endosulfan over glucose as a source of carbon and have plasmid-borne insecticide degradation genes. In addition, their plasmids also confer resistance against several antimicrobial agents and fall into similar or closely related plasmid incompatibility grouping. This whole scenario reflects the strong conduity of these isolates to be used for environmental cleanup. This can be suggested from this study that these insecticide degrading bacteria can be used in collaboration with certain members of genus *Bacillus* especially *B. thuringiensis* which are very well known bio insecticides [29].

Table 1: Morphological and biochemical characterization of endosulfan degrading bacteria.

Test	ESDB-1	ESDB-2	ESDB-3
Colony: colour	Creamy White	Creamy White	Creamy White
size	2 mm	0.75 mm	2 mm
Shape	Rounded	Rounded	Rounded
Grams reaction:	Negative	Negative	Negative
Cell shape	Cocci	Cocci	Cocci
Growth on: Blood agar	β Hemolysis	β Hemolysis	β Hemolysis
MacConkyagar	Lactose fermentation	Lactose fermentation	Lactose fermentation
Activity of: Oxidase	+ve	+ve	+ve
Coagulase	-ve	-ve	-ve
Catalase	+ve	+ve	+ve
Urease	+ve	+ve	+ve
Tyrosine Degradation	+ve	+ve	-ve
Starch hydrolysis	+ve	-ve	+ve
Citrate utilization	+ve	-ve	+ve
Nitrate reduction	-ve	-ve	-ve
Indole production	-ve	-ve	-ve
v.p test	+ve	+ve	+ve
Motility	+ve	+ve	+ve
Acid release from: Glucose	-ve	+ve	-ve
Galactose	+ve	+ve	+ve
Fructose	+ve	+ve	+ve

Sucrose	-ve	-ve	-ve
Lactose	+ve	+ve	+ve
Maltose	+ve	+ve	+ve
Mannose	+ve	+ve	+ve
Raffinose	-ve	-ve	+ve
Rahminose	+ve	+ve	-ve
Xylose	-ve	-ve	-ve
Sorbitol	-ve	-ve	-ve

Table 2: The incubation period (7 days) taken by three isolates for appearance of growth in different concentrations of endosulfan in M9 medium in place of glucose

Endosulfan in thee medium (%	Growth of ESDB-1(hours)	Growth of ESDB-2 (hours)	Growth of ESDB-3 (hours)
0.00625	24	24	24
0.5	72	72	72
1	72	72	72
5	120	120	120
10	168	168	168

Table 3: Data showing the percentage of plasmid curing after treatment with Eth Br (600µg/ml) of th

Strain	Total colonie streaked	Colonies appeared o M9+glusose	Colonies appeared on M9- endosulfan	Mutation %	Curing %
ESDB-1	100	100	24	ND*	76
ESDB-2	100	100	26	ND	74
ESDB-3	100	50	05	50	90

*ND= Not determined

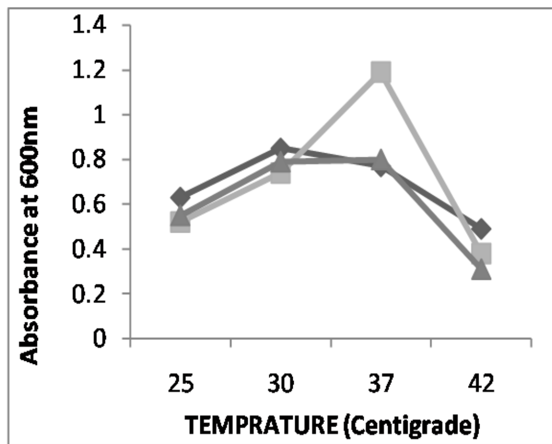

Fig 1: Effect of different temperatures on growth of ESDB-1(□), ESDB-2(■) and ESDB-3(▲).

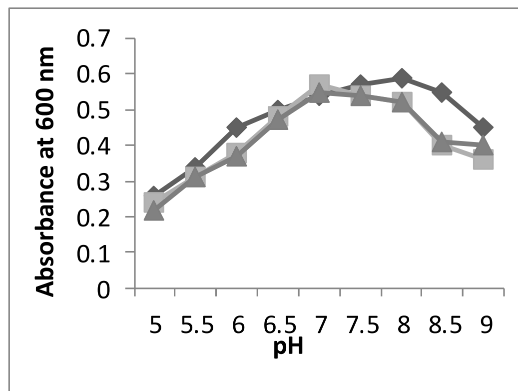

Fig 2: Effect of pH on the growth of ESDB-1(▲), ESDB-2(■) and ESDB-3(□) isolates.

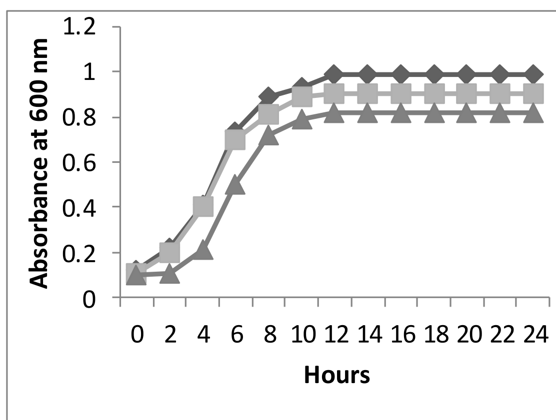

Fig 3: Growth curves of ESDB-1(□) in LB-broth (■), M9 containing 0.00625% endosulfan (▲) and M9 containing 20% glucose (◊).

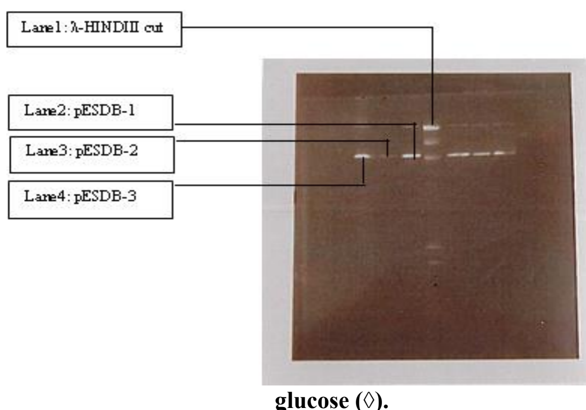

Fig 4: Agarose gel showing plasmids isolated from the bacterial isolates.

REFERENCES

[1] Pandey, G. and Jain, R. K., "Bacterial chemotaxis toward environmental pollutants." Applied and Environmental Microbiology, 68(12): pp. 5789-5795 (2002).

[2] Karami-Mohajeri, S. and Abdollahi, M., "Toxic effects of organophosphate, carbamate, and organochlorine pesticides on cellular metabolism of lipids, proteins, and carbohydrates: A comprehensive review." *Human & experimental toxicology*, 59 (2010).

[3] Gupta, V. K. and Ali, I., "Removal of endosulfan and methoxychlor from water on carbon slurry." *Environmental Science & Technology.* ***42*** (3): 766-770 (2008).

[4] Kanaly, R. A., and Harayama, S., "Biodegradation of high molecular weight polycyclic aromatic hydrocarbons by bacteria." *J. Bacteriology*, **182**(8): 2059-2067 (2000).

[5] Siddique' T., Okeke, B. C., Arshad, M. and Frankenberger, W. T., "Enrichment and isolation of endosulfan degrading microorganisms**.** *J. Env. Quality.* **10:** 2134 **(2003)**.

[6] Ogawa, N., Miyashita, K. and Chakrabarty, A. M., "Microbial genes and enzymes in the degradation of chlorinated compounds." *Chemical Record*, **3**(3):158.(2003).

[7] Benson, H.J., "Microbiological Applications, Complete Version. Laboratory Manual in general microbiology." 8th Edition. W.C. Brown Publishers, Dubuque, Melbourn, Oxford, (1994).

[8] Haq, R. and Shakoori, A.R., "Use of DNA probes for determination of homologies in genomes of different Thiobacillus ferroxidans strains isolated from various localities of the world and their relationship with adaptation and evolution of the strains. Pakistan J. Zool., **29:** 193-198.(1997).

[9] Ohman, D.E., "Experiments in gene manipulation." Printice Hall Inc. Englewood Cliffs, New Jersey, 07632.(1988).

[10] Sambrook, J., Fritsch, Eif. & Maniatis, T., "Molecular Cloning: A Laboratory Manual" 2nd Edition. Cold Spring Harbor Laboratory (CSH), NY, USA.(1989).

[11] Ramani, V., "Effect of pesticides on phosphate solubilization by *Bacillus sphaericus* and *Pseudomonas cepacia*. *Pesticide Biochemistry and Physiology*, ***99***(3): 232-236 (2011).

[12] Hans, R.K., Khan, M.A., Farooq, M. and Beg, M.U., "Glutathione-S-transferase activity in an earthworm, Pheretima posthuma exposed to three insecticides." Soil. Biol. Biochem., **25:** 509-511.(1993).

[13] Kanekar, P. P., Bhadbhade, B. J., Deshpande, N. M. and Sarnaik, S. S., "Biodegradation of organophosphorus pesticides." *Proceedings-Indian National Science Academy Part B*, **70**(1): 57-70 (2004).

[14] Kalevitch, M. V. and Kefeli, V. I., "Study of bacterial activity in fabricated soils." *International journal of environment and pollution*, **29**(4): 412-423 (2007).

[15] Madsen, E.L., "Determining in situ biodegradation: Facts and challenges." Environ. Sci. Technol., **25:** 1663-1673.(1991).

[16] Malik, A., "Metal bioremediation through growing cells" Environment international, 171:33(2004).

[17] Rodrigues, C. M., Takita, M. A., Coletta-Filho, H. D., Olivato, J. C., Caserta, R., Machado, M. A. and De Souza, A. A.' "Copper resistance of biofilm cells of the plant pathogen xylellafastidiosa." *Applied microbiology and biotechnology*, **77**(5): 1145-1157 (2008).

[18] Percival, S. L., Bowler, P. G. and Russell, D., "Bacterial resistance to silver in wound care." *Journal of Hospital Infection*, **60**(1): 1-7 (2005).

[19] Klaus-Joerger, T., Joerger, R., Olsson, E. and Granqvist, C. G., "Bacteria as workers in the living factory: metal accumulating bacteria and their potential for materials science." *Trends in Biotechnology,* **19**(1): 15-20 (2001).

[20] Valls, M. and De Lorenzo, V., "Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution." *FEMS microbiology Reviews*, **26**(4), 327-338 (2002).

[21] Costerton, J. W., Ellis, B., Lam, K., Johnson, F. and Khoury, A. E., "Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria." *Antimicrobial agents and chemotherapy*, **38**(12): 2803-2809 (1994).

[22] Pickett, M. A., Everson, J. S., Pead, P. J. and Clarke, I. N., "The plasmids of *Chlamydia trachomatis* and *Chlamydophilap neumoniae* (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents." *Microbiology*, **151**(3): 893-903 (2005).

[23] El-Mansi, M., Anderson, K. J., Inche, C. A., Knowles, L. K. and Platt, D. J., "Isolation and curing of the *Klebsiella pneumoniae* large indigenous plasmid using sodium dodecyl sulphate." *Research in microbiology*, **151**(3): 201-208 (2000).

[24] Ivask, A., Virta, M. and Kahru, A., "Construction and use of specific luminescent recombinant bacterial sensors for the assessment of bioavailable fraction of cadmium, zinc, mercury and chromium in the soil." *Soil Biology and Biochemistry*, **34**(10): 1439-1447 (2002).

[25] Patra, M., Bhowmik, N., Bandopadhyay, B. and Sharma, A., "Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance." *Environmental and Experimental Botany*, **52**(3): 199-223(2004).

[26] Chopra, I. and Roberts, M., "Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance." *Microbiology and molecular biology reviews*, **65**(2): 232-260 (2001).

[27] Roberts, M. C., Update on macrolide–lincosamide–streptogramin, ketolide, and oxazolidinone resistance genes. *FEMS microbiology letters*, **282**(2): 147-159 (2008).

[28] Desantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K. and Andersen, G. L., "Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB." *Applied and environmental microbiology*, **72**(7): 5069-5072 (2006).

[29] Bravo, A., Likitvivatanavong, S., Gill, S. S. and Soberón, M., "Bacillus thuringiensis: a story of a successful bioinsecticide." *Insect biochemistry and molecular biology*, **41**(7): 423-431 (2011).