

Degradation of Microcystin LR, Oxytetracycline and Ampicillin by Four Native Bacteria Species

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Abstract

Pollution reaches its most serious proportion in past few decades and adversely effect on animals and human health. Reduction of pollutant in the environment take place with microbial metabolism and remediation studies by microbes have proved their feasibility on clean up the contaminated environment. Thus, the present study reports the biodegradation of Microcystins (MC-LR) and antibiotics [Oxytetracycline (OTC) and Ampicillin (AMP)] by *Bacillus cereus*, *Enterobacter ulcerans*, *Enterobacter* sp. and *Micrococcus* sp. strains which were previously reported as potential crude oil degraders. A 0.5 ml of overnight starved bacterial suspensions was introduced into medium containing antibiotic (OTC, AMP) at 60 µg/ml and Microcystin-LR at 10µg/ml respectively. Triplicate samples were incubated at 28^oC while shaking at 100 rpm. A 0.5 ml of aliquots was removed at 2 days interval for a period of 14 days and analysis was done by High Performance Liquid Chromatography (HPLC). The highest degradation of MC- LR was shown by *Micrococcus* sp. (97%) where as other stains; *E. ulcerans* (96 %), *Enterobacter* sp. (95 %) and *B. cereus* (88%) also showed comparative high degradation after 14 days of incubation. *B. cereus*, *Enterobacter* sp. and *Micrococcus* sp. were identified as AMP resistance bacteria and degraded AMP at 81%, 22% and 39% respectively. It was found that *B. cereus* was resistance to OTC and showed 56% reduction at 14 days of incubation. The results of the present study revealed that the bioremediation potential of harnessing microbes can cleanup of pollutant in the environment and use as ecofriendly tool for removal of environmental pollutants.

Keywords: *Bacillus cereus*, *Enterobacter* sp., *Enterobacter ulcerans*, *Micrococcus* sp., Antibiotics, Microcystin

1. Introduction

In the last few years a large number of ecosystems have changed due to anthropogenic activities. Petroleum hydrocarbons, microcystin, pharmaceuticals are different type of toxicants in the environment and have become one of the most serious environmental problems around the world (Fereidoun et al., 2007). These contaminates can accumulate via food chain and persist in the environment for a long time. Those pollutants reaches its most serious proportion in the densely settled urban-industrial centers of more developing countries (Kromm, 1973; Havens et al., 2003).

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Occurrence of pharmaceuticals, especially antibiotics in the aquatic environment is a well-established issue and has become a matter of both scientific and public concern (Allen et al., 2010). The introduction of these compounds into the environment through anthropogenic sources can constitute a potential risk for aquatic and terrestrial organisms. Although present at vestigial levels, antibiotics may cause resistance in bacterial populations, making them ineffective in the treatment of several diseases (Baquero et al., 2008; Liyanage and Manage, 2016 c). Several studies have reported the occurrence of antibiotic residues in aquatic ecosystems: surface waters, drinking water, water treatment plant effluents and hospital wastewaters (Berglund et al., 2014; Liyanage and Manage 2015 c, 2016 b).

Further, over the past several centuries, nutrient enrichment in water, particularly nitrogen and phosphorus, associated with urban, agricultural and industrial development, has promoted increase of cyanobacteria which cause eutrophication. This condition favors the mass occurrence of cyanobacterial blooms where cyanobacterial cells grow exuberantly, and reach more than 10,000 cells per milliliter (Manage et al., 2009). Cyanobacteria are the distinct group of bacteria which are photosynthetic and produce several metabolites. These metabolites include several endotoxins known to be cyanotoxins which are commonly found during blooms of cyanobacteria (Manage et al., 2010).

Cyanobacteria may produce toxins such as hepatotoxic peptides, neurotoxic alkaloids and dermatotoxic phenolic compounds, in addition to lipopolysaccharides (Sivonen et al., 1990). Microcystin is a group of cyclic heptapeptide hepatotoxins, which are produced by some strains of cyanobacteria, *Microcystis* spp., *Anabaena* spp., *Oscillatoria* spp. (Romanowska-Duda et al., 2002). These substances are natural endotoxins and release into water in high concentration when cell lysis takes place. (Manage et al., 2000; Romanowska-Duda et al., 2002). Cell lysis occurs under stress, mostly because of natural mortality or algacide treatments. This cyclic heptapeptide causes serious damage to liver architecture, cellular organelles and reorganization of microfilaments. They inhibit the activity of protein phosphate 1 and 2A and act as tumor promoters (Zhao et al., 2006).

The mechanical and chemical methods that are used to remove antibiotics and Microcystin from contaminated sites have limited effectiveness and are not accessible to all parts of the world due to high operating and material costs (Liyanage and Manage 2016 b, Idroos and Manage, 2014, Manage et al., 2009). Bioremediation is one of the most promising technologies for the treatment of antibiotics and Microcystin as it is cost effective and identified as the best alternative eco-friendly method (Liyanage and Manage, 2015 b., Manage et al., 2009). In bioremediation specific bacteria use to remove either specific type of contaminant or different type of contaminants. A number of studies have reported biological degradation of microcystin in samples collected from lakes and sediments (Manage et al., 2009; Manage et al., 2010), but only a few bacterial strains with the ability to degrade microcystins have been isolated and characterized. For instance, many of the MC degraders are reported as *Sphingomonas* or *Sphingomonas*. Until Manage et al. (2009), identified MC degrading Gram-positive *Actinobacteria*, all of the MC degraders had belonged to Gram-negative *Proteobacteria*.

In degradation of antibiotics; Ding et al (2016) reported the tolerance of *Acinetobacter*, *Stenotrophomonas maltophilia* and *Aeromonas veronii* to OTC in freshwater environments of Ireland and England. Maki et al., (2006) has isolated flavobacterium strains responsible for the degradation of a group of antibiotics including OTC and AMP. Liyanage and Manage (2016a; 2015a.) reported the antibiotic resistance activity by bacterial strain *Enterococcus* sp., *Acinetobacter* sp.

Thus, present study was focused on the Microcystin and Antibiotics degradation feasibility of *Bacillus cereus*, *Enterobacter* sp., *Micrococcus* sp. and *Enterobacter ulcerans* stains, since very limited information is available regarding multiple pollutant degradation ability of bacteria in Sri Lanka. These four stains were previously reported to be degraders of crude oil (Liyanage and Manage, 2016b).

2. Material and method

2.1 Chemicals and reagents

OTC and AMP standards, HPLC and Bacteriological grade chemicals were purchased from Sigma Aldrich, USA.

2.2 Preparation of bacterial cultures

Each bacterial strain was transferred into 5ml of liquid Luria Bertani (LB) medium and incubated 28°C. Then followed centrifugation to remove carbon source from the LB media and then the bacteria suspension was subjected to the starvation procedure, in phosphate buffer solution (PBS). Thereafter equalized the turbidities of the bacteria suspension at $A_{590}=0.35$ (Manage et al., 2009; Liyanage and Manage 2016).

2.3 Microcystin degradation

A 0.5 ml of starved, equalized bacterial suspension was inoculated into filtered sterilized lakewater containing microcystin at a final concentration of 10µg/ml. The treated flasks were incubated at 28°C while shaking at 100 rpm. 0.5ml of sub-samples were collected at two days intervals for a period of 14 days. Then the subsamples were centrifuged (12000 rpm) and the supernatant of each sample was subjected to immediate freezing and stored at -20°C (Manage et al., 2010). Then the frozen samples were freeze-dried and reconstituted in 1ml of 100% aqueous HPLC grade methanol and subjected to the HPLC analysis (Idroos and Manage, 2014). The control was treated in the same manner as the experiment without bacterial inoculation. All the experiments were done for each tested bacterial strain.

2.4 Analysis of MC-LR by HPLC

MC-LR analysis was carried out by using the HPLC system consisting of Agilent 1200 series following the modified method of Manage et al (2009). The injected volume was 25µl at 30°C. The C₁₈ column was used and the eluents solvent were deionized water – 0.05% trifluoroacetic acid (TFA) and acetonitrile – 0.05% trifluoroacetic acid (TFA). Concentration of MC-LR was determined by calibration of the peak areas in 200-330 nm. The HPLC method has a detection limit of 0.5µg/ml and MC-LR recovery was obtained greater than 90% (Manage et al., 2010).

2.5 Antibiotic degradation experiment for bacteria isolates

A 0.5µl of equalized bacterial suspension was inoculated into filter-sterile freshwater, containing antibiotic at a final concentration of 60µg/ml respectively. All flasks were incubated at 28°C with continuously shaking at 100 rpm. A 0.5ml of sub samples were collected at two days intervals for a period of 14 days (Liyanage and Manage 2016 b). Then the subsamples were centrifuged (12000 rpm) and supernatant of each sample was subjected immediate freezing and stored at -20°C. Then the frozen samples were freeze-dried and reconstituted in 1 ml of 100% aqueous HPLC grade methanol and subjected to the HPLC analysis. Control samples were prepared in triplicates following same protocol without bacteria inoculation (Liyanage and Manage. 2016 a).

2.6 Minimum Inhibition Concentration

The Minimum Inhibition Concentration (MIC) was determined by using an agar dilution method according to CLSI guidelines (CLSI.2015).

2.7 Analysis of antibiotics by HPLC

The target antibiotics were quantified by using Agilent 1200 series HPLC equipped with diode array and fluorescence detector (Fernandez-Torres et al., 2010; Liyanage and Manage, 2016 b). The

injected volume was 20µl and chromatography was performed at 30°C. The mobile phase considered of a mixture of 0.1% Glacial acetic acid in water (Component A): 0.1% Glacial acetic acid in acetonitrile (Component B), 99:1 (v/v) was pumped in beginning at a flow rate of 0.7 ml/min for OTC and TET. Then followed linear elution gradient from 99% to 70% A in 25 min for the above antibiotics (Liyanage and Manage, 2016 b). Concentration of each antibiotic was determined by calibration of the peak areas (at 280 nm for OTC, 230 nm for AMP) with that of an external standard. OTC and AMP recoveries were greater than 90%.

2.8 Determination of degradation rate and half-life time

The degradation rate (h) of antibiotic/MC-LR by bacteria was calculated according to the equation given below. Half life time was calculated as the time taken to degrade 50% of antibiotic/MC-LR from the initial concentration.

$$h = \ln(C/C_0)/t \quad (1)$$

Where:

C_0 = the concentration of antibiotic/MC-LR at the beginning

C = the concentration of antibiotic/MC-LR at the end of the time interval respectively (Manage et al., 2000).

3. Results

3.1 Microcystin-LR degradation kinetics

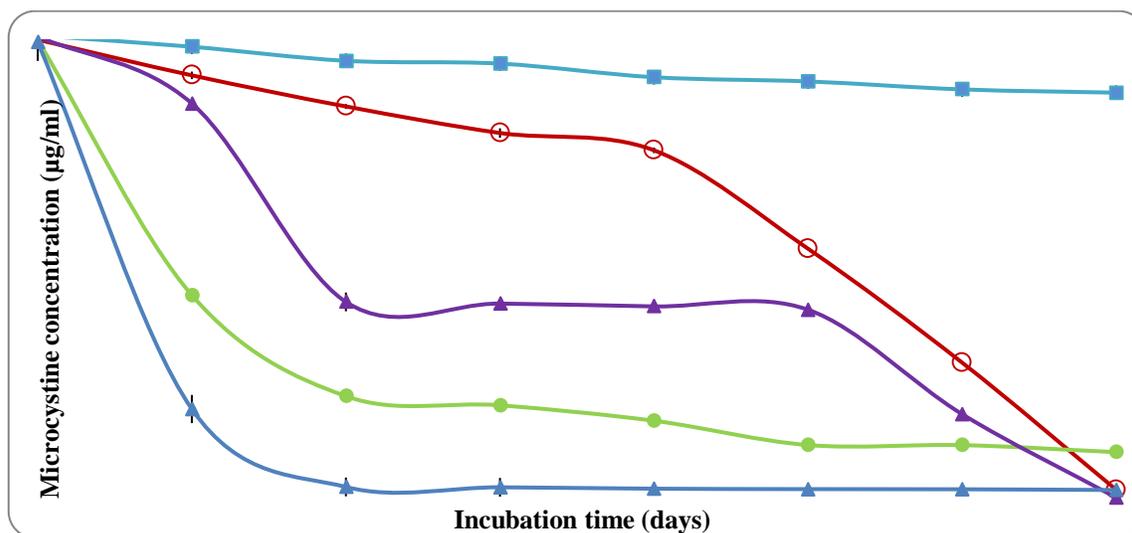


Figure 1: Microcystin-LR (MC-LR) degradation by four bacterial strains after 14 days of incubation at 28 °C ± 1°C. When error bars are not shown standard error was less than the width of the symbol. (Closed square; control, open circle; *Enterobacter* sp., closed circle; *B. cereus*, open triangle; *Micrococcus* sp., closed triangle; *E. ulcerans*).

Figure 1 showed the degradation of MC-LR by different bacterial strains. At 4 days of the incubation *Micrococcus* sp. showed significant degradation, after that remaining constant until day 10 and thereafter decreased up to 97%. In contrast, *Enterobacter* sp. maintained gradual decrease of MC-LR by day 8 of incubation and then initiated rapid degradation and ended up with achieving 98% degradation at 14 days of incubation. *B. cereus* only showed 82% of degradation even at 14 days of incubation. Analysis

of the sterile controls showed no significant loss of MC-LR during the incubation and only 9% of decrease was detected may due to natural degradation and photo degradation effect.

Table 1:Rate of degradation in MC-LR (given as mean value of triplicates).

Incubation time (Days)	Degradation rate (d ⁻¹)			
	<i>Enterobacter</i> sp.	<i>B. cereus</i>	<i>Micrococcus</i> sp.	<i>E. ulcerans</i>
0	0±0.000	0±0.000	0±0.000	0±0.000
2	0.04±0.02	0.39±0.04	0.07±0.02	0.77±0.01
4	0.04±0.01	0.36±0.06	0.20±0.04	0.76±0.05
6	0.04±0.03	0.25±0.04	0.14±0.02	0.51±0.02
8	0.03±0.05	0.21±0.02	0.10±0.05	0.39±0.03
10	0.06±0.06	0.19±0.01	0.08±0.01	0.31±0.02
12	0.10±0.06	0.17±0.01	0.13±0.04	0.26±0.04
14	0.23±0.04	0.16±0.02	0.26±0.02	0.22±0.02

E. ulcerans showed the highest degradation rates at second day of incubation (0.77±0.01d⁻¹) where as other strains showed low degradation rates during first two days. The degradation rates of *Enterobacter* sp. were more or less constant (~0.04±0.02 d⁻¹) up to day 10 and thereafter increased significantly up to 0.23±0.04d⁻¹ at 14 days of incubation. *B. cereus* showed high degradation rate (0.39±0.04d⁻¹) at the beginning and pronounced gradual decrease was observed onwards. *Micrococcus* sp. showed higher degradation rate at end of the 14 days of incubation (0.26±0.02d⁻¹).

Table 2:Half life time of the MC-LR degradation by four bacteria strains.

Name of the bacteria	Half life time (days)
<i>Enterobacter</i> sp.	10.5
<i>B. cereus</i>	1.8
<i>Micrococcus</i> sp.	3.7
<i>E. ulcerans</i>	1.3

E. ulcerans showed the lowest half life time of 1.3 days of incubation whereas *B. cereus* and *Micrococcus* sp. showed 1.8 and 3.7 days respectively. A half life time of 10.5 days were detected for *Enterobacter* sp.

Antibiotic degradation kinetics

Resistance of the bacteria to tested antibiotics

Table 3:Antibiotic resistant against Oxytetracycline and Ampicillin by the tested bacterial strain.

Bacteria	Oxytetracycline (OTC)	Ampicillin (AMP)
<i>Enterobactersp.</i>	-	+
<i>B. cereus</i>	+	+
<i>Micrococcus</i> sp.	-	+
<i>E. ulcerance</i>	-	-

(+) resistance(-) not resistance.

The bacteria resistance for the 60µg/ml concentration of OTC and AMP was recorded in table 3. Only *B. cereus* was resistance to OTC whereas *Enterobacter* sp., *B. cereus*, *Micrococcus* sp. were recorded as resistance strain of bacteria for AMP.

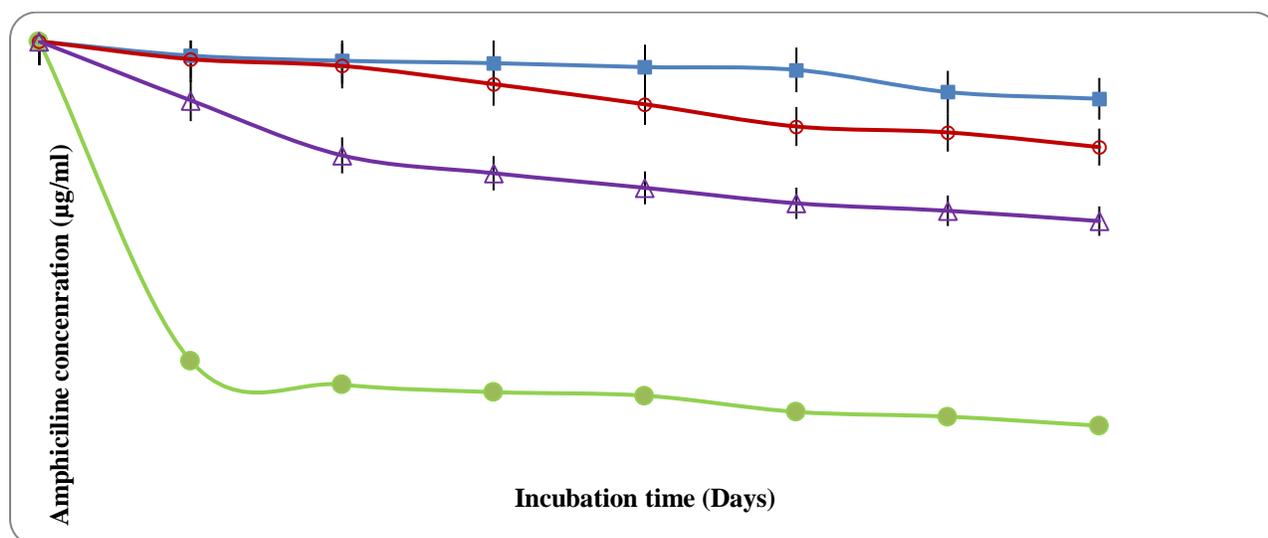


Figure 2: Degradation of AMP by three native bacterial strains, which are resistance to AMP. When error bars are not shown standard deviation was less than the width of the symbol. (Closed square; control, open circle; *Enterobacter* sp., closed circle; *B. cereus*, open triangle; *Micrococcus* sp.).

Figure 2 shows the degradation of AMP (60 µg/ml) by bacterial strains during the incubation period. The AMP resistance bacteria; *B. cereus*, *Enterobacter* sp. and *Micrococcus* sp. were showed 81%, 22%, 39% degradation of AMP respectively at 14 days of incubation. Except *B. cereus*, other two bacterial strains were not showed a significant degradation thus it was not possible to calculate half life time during the incubation. Half life time of AMP for *B. cereus* was 1.5 days and it was identified as a potential AMP degrader among the bacteria strains employed in the present study.

Table 4: Degradation rate of Ampicillin in 60µg/ml (given as mean value of triplicate).

Incubation time (Days)	Degradation rate (d ⁻¹)		
	<i>Bacillus cereus</i>	<i>Enterobacter</i> sp.	<i>Micrococcus</i> sp.
0	0±0.000	0±0.000	0±0.0000
2	0.56±0.014	0.02±0.007	0.07±0.001
4	0.32±0.005	0.01±0.001	0.07±0.007
6	0.22±0.025	0.01±0.012	0.05±0.000
8	0.17±0.002	0.02±0.016	0.05±0.007
10	0.15±0.035	0.02±0.000	0.04±0.014
12	0.13±0.005	0.02±0.014	0.04±0.000
14	0.11±0.032	0.02±0.007	0.03±0.014

B. cereus showed highest degradation rate (0.56±0.014 d⁻¹) after two days of incubation while *Enterobacter* sp. and *Micrococcus* sp. showed 0.02±0.007d⁻¹ and 0.07±0.001 d⁻¹ degradation after two days of incubation respectively.

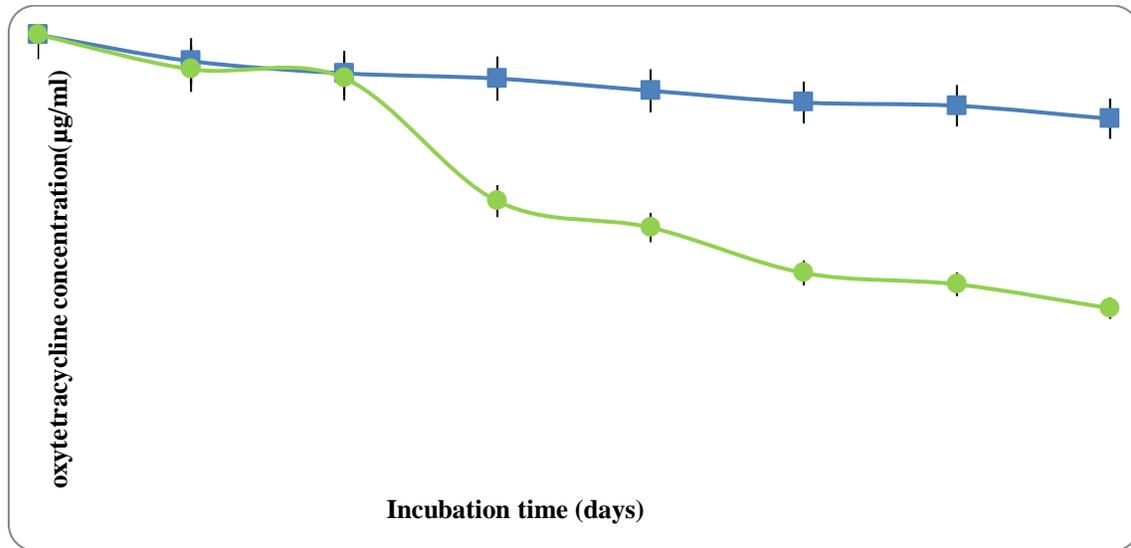


Figure 3: Degradation of OTC by *B. cereus*. The error bars are not shown standard deviation was less than the width of the symbol. (Closed square; control, closed circle; *B. cereus*).

Figure 3 showed the degradation of OTC (60µg/ml) by *B. cereus* which was the only strain recorded to resistant against OTC. After 14 days incubation at 28±1°C the bacterial strains achieved 56% reduction of OTC.

Table 5: Rate of degradation in Oxytetracycline by *B. cereus* (given as mean value of triplicate).

Days	Degradation rate (d ⁻¹)
0	0±0.0000
2	0.04±0.004
4	0.02±0.004
6	0.07±0.002
8	0.06±0.006
10	0.07±0.002
12	0.06±0.003
14	0.06±0.009

Low degradation rate was detected until day 4 of incubation (0.02±0.004d⁻¹) where as it was increased up to 0.07±0.002 d⁻¹ at 6th day of incubation.

4. Discussion

Present study reports the biodegradation of some peptides (microcystins and some antibiotics) by four native bacteria species namely *B. cereus*, *E. ulcerans*, *Enterobacter* sp. and *Micrococcus* sp. The bacteria species were previously isolated from soil and water from three distinguishes cites where exposed to crude oil contamination with regular shipping activities and reported as degraders of crude oil (Liyanage and Manage, 2016).

There is a growing number of isolated organisms reported as having the ability to degrade microcystin in water and so far the majority of bacteria appears belonging to the class proteobacteria (Havens et al., 2003). However, Manage et al., (2009) reported actinobacteria species as

microcystin degraders. In the present study four bacterial isolates were shown significant degradation of microcystin-LR (MC-LR) at 14 days incubation. At 2 days incubation significant degradation of MC-LR was achieved by *E. ulcerans* (78%) and *B. cereus* (54%). At 4 days of the incubation *Micrococcus* sp. (56%) showed significant degradation of MC-LR (Figure 3). Several studies were reported that *Bacillus* spp. as microcystine degrading bacteria (Zhoa et al., 2006). *Bacillus* sp. utilize microcystine as sole carbon source and concentration of microcystine become zero within 7 days according to Enzyme Linked Immunosorbant Assay (ELISA) and within 8 days according to protein phosphatase inhibition assay (Alamri, 2013). Accordingly the present study *B. cereus* degraded 88% of MC-LR after 14 days incubation and *E. ulcerans* (96%), *Enterobacter* sp. (95 %) and *Micrococcus* sp. (97%) have ability to degrade high amount of MC-LR (Figure 3) compare to the other bacterial strains. Out of the four strains *E. ulcerans* was shown short half-life (1.3 days) for MC-LR (Table 1) compared to other three bacteria species employed in the present study.

Antibiotics are considered as emerging micro contaminants in water due to their potential adverse effects on ecosystems and human health. Major negative effect of high concentration of antibiotics in the environment is developing antibiotic resistance bacterial species and those bacteria become more virulent (Tan et al., 2012). Antibiotic resistance can occur via three general mechanisms: prevention of interaction of the drug with target, efflux of the antibiotic from the cell, and direct destruction or modification of the compound (Samah et al., 2006). The WHO has recommended a guide line value of less than 1 µg/l of antibiotic residues in the aquatic environment and less than 100 µg/l in soil respectively (WHO, 2015). Liyanage and Manage (2015 c.) recorded that both water and sediment samples collected from wastewater discharge points in Sri Lanka contains higher OTC (0.664-0.841 µg/ml) and AMP (0.115-0.139 µg/ml) levels than the recommended values given by the WHO. Therefore it causes an increase of antibiotic-resistant bacteria. Accordingly present study *B. cereus* is resistance to OTC and the tested other bacteria species were resistance to AMP except *E. ulcerans*.

B. cereus, *Enterobacter* sp. and *Micrococcus* sp. showed 81%, 22%, 39% degradation of AMP respectively. *B. cereus* was shown highest degradation and it was found that the *B. cereus* was the only bacteria specie showed 56% reduction of OTC at 14 days incubation.

Hence introduction, establishment and development of such ecofriendly, cost effective bio technologies for the country is timely important to help for the development of the country. The results of the present study revealed that the bioremediation technology with harnessing microbes will provide sound information for the cleanup of pollutant in the environment as ecofriendly tool for green solution. Further studies with combination of biotechnology approaches are needed to development of eco friendly methods for cleanup environmental pollutants.

5. Conclusions

The highest degradation of MC-LR was shown by *Micrococcus* sp. (97%) where as other stains; *E. ulcerans* (96%), *Enterobacter* sp. (95%) and *B. cereus* (88%) also showed comparative high degradation after 14 days of incubation. *B. cereus*, *Enterobacter* sp. and *Micrococcus* sp. were identified as AMP resistance bacteria and degraded AMP at 81%, 22% and 39% respectively. It was found that *B. cereus* was resistance to OTC and showed 56% reduction at 14 days of incubation. The results of the present study revealed that the bioremediation potential of harnessing microbes can cleanup of pollutant in the environment and use as eco friendly tool for removal of environmental pollutants.

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