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DEVELOPMENT OF A LABORATORY SCALE SAND FILTER WITH MICROCYSTIN-LR DEGRADING BACILLUS CEREUS

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Abstract- A bacterium (KJ 954304 *B. cereus* 12GK) previously demonstrated to degrade the Microcystin- LR, was investigated for bioremediation applications in laboratory scale sand filter. Citric acid treated raw cotton was used as the biofilm of the sand filter. Both control and experimental setups were built up for the study. The experimental sand filter removed 23% of MC-LR from the influent water within three hours of commissioning. The removal rate gradually increased through out 24 hours and showed 90% of removal within four days. The control filter also showed 1% of removal within three hours and 12% after four days. Thus, 12% of MC-LR undergo physical removal via adsorption to sand and rock particles while the remaining 78% of MC-LR was removed entirely through bioremediation.

Key words- Microcystin-LR (MC-LR), *Bacillus cereus*, Sand filter, Biofilm, Citric acid treated raw cotton.

I. INTRODUCTION

Cyanobacteria are a group of prokaryotes found all over the world [1] and grow in well illuminated habitats and prefer neutral or alkaline conditions. They are considered as major primary producers which contribute to fertility of soil. However, under favorable environmental conditions certain cyanobacteria can grow faster and result in blooms. These blooms may last for several weeks. The most dominant bloom forming cyanobacterial genera responsible for these mass occurrences are: *Anabaena, Aphanazomenon, Cylindrospermopsis, Gloeotrichia, Oscillatoria, Rivularia, Lyngbya, Microcystis,* and *Nostoc* [2]. In Sri Lanka, a combination of favorable growth conditions together with global warming and intensive farming using a wide range of materials (e.g. fertilizers and inorganic matter) have resulted in an increase of cyanobacterial blooms throughout drinking water water bodies over the last 20 years [3][4].

These bloom forming cyanobacterial genera can produce a wide range of potent toxins, including a family of heptapeptide hepatotoxins, referred as Microcystins (MCs). MCs are the most frequently detected cyanobacterial toxins, which cause hepatotoxicity and tumor promotion in wild animals, livestock, and humans [6]. There are over 70 variants of MC and among them Microcystin-LR (MC-LR) is considered to be the dominant analogue present in Sri Lankan water bodies [3].

MC-LR has a chemically stable structure and is reluctant to be removed by traditional water treatment methods [7]. Chemical coagulation treatments shown to be inefficient in removal of phytoplankton and especially cyanobacteria from raw water [8]. However, technically advanced water treatment technologies like ozonation and photocatalytic degradation have shown to be effective in removal of MCs [9][10][11]. However, these methods are expensive and inaccessible to the developing part of the world. Therefore there is a need in a country like Sri Lanka to develop a low cost method to remove MC-LR from water prior to distribution.

For small regional communities, biologically active slow sand filtration offers a cost-effective water treatment process which have previously demonstrated some removal of MCs including MC-LR [12][13]. Biological degradation of cyanobacterial toxins in surface waters has been investigated previously [14][15] with bacterial isolates capable of mineralizing the peptide compounds in MC-LR [16][17][18]. Ho et al. [19] investigated applications of potential MC-LR degrader. *Sphingomonas* sp. (MJ-PV), isolated by Jones et al. [15] for remediation of water samples contaminated with MCs. Ho et al. [19] demonstrated his applications by inoculating MJ-PV the sand bed of the sand filter column. The results of Ho et al. [19] showed 100 % removal of MC-LR following 6 days of commission of the sand filter column. However, there are no studies done to date on incorporating potential MC-LR degrading bacteria in to a polymer substrate. Thus, the present study records the development of a laboratory scale sand filter by incorporating MC-LR degrading *B.cereus* (KJ 954304 *B. cereus*12GK), which was previously isolated by authors in to a citric acid treated polymer substrate.

II. MATERIALS AND METHODS

2.1. Chemicals

ACS Reagent citric acid, HPLC-grade Methanol, Milli-Q water, Acetonitrile, Trifluoroacetic acidacid (TFA) for HPLC systems were purchased from Sigma Aldrich. Microcystin-LR (MC-LR), standards were received from Robert Gordon University, UK Tryptone, Yeast extract, Sodium chloride Bacteriological agar, phosphate buffer saline needed for bacteriological studies were purchased from Hardy diagnostics. Molecular grade chemicals, needed for molecular biological studies were purchased from Promega, USA and Thermofischer scientific, USA.

2.2 Development of Polymer substrate

Citric acid treatment was carried out according to the method described by Mc Sweeny et al. [20]. ACS Reagent citric acid (anhydrous) was prepared as an aqueous solution. Aliquots of 10 ml of the aqueous solutions of citric acid (200gl^{-1}) were dispensed into 50ml beakers, each containing a raw cotton cloth of 2g. Samples were mixed with a glass stirring rod and the mixtures allowed equilibrating at room temperature for one-half hour. Water was removed from the samples by placing them in an oven at 60°C for a period of 4 hours. After the reaction period, 25 ml of de-ionized water was added to the sample, mixed with a glass stirring rod, and equilibrated for one-half hour. The excess water containing unreacted citric acid was removed by a vacuum flask and a funnel equipped with paper filter.

2.3 Attachment of bacteria into polymer substrate

Overnight grown bacterial culture of KJ 954304 *B. cereus*12GK was starved in 0.01M of PBS and suspensions were equalized at A_{590nm}=0.35. Then 0.5 ml of bacterial suspensions were introduced into 100ml Erlenmeyer flasks containing 2g of polymer substrate and 10ml of filter sterile lake water inoculated with MC-LR at a final concentration of 5µgml^{-1}. Control samples were maintained without bacterial inoculation. The flasks were closed with cotton wool bungs and placed in shaker at 28 ^{0}C for 14 days at 100 rpm. Sample aliquots (0.5ml) were removed every two days interval including the first day of the experiment. Turbidity of the removed sample aliquots were recorded spectrophotometrically at 590nm. Following spectrophotometric analysis the sample aliquots were used for PDA-HPLC according to the procedure explained in 2.5 in order to see the MC-LR removal by the attached bacteria.

2.4 Development of laboratory scale sand filter

Control and experimental filter setups were developed for the study. Both setups consisted of a water input column and a sand filter column, each with a diameter of 50 mm and a height of 210 cm (Fig 1).

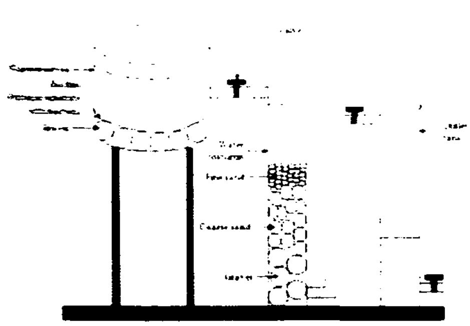

Fig 1. Structure of the Sand filter

The filter bed of the sand filter column was supported by gravel layers. The column was made of PVC tubes. The minimum supernatant level was controlled by an outflow weir. The flow rate was maintained at 0.4 lmin^{-1} using valves set up to the sand filter column. A minimum supernatant level of 30 cm was maintained above the sand bed. Filter design, material and operating conditions are listed in Table 1. The retention time in biological layer was 30 minutes.

Table 1. Filter design, material and operating conditions of the sand filter column

Components	Size (mm)	Depth (cm)
Fine Sand	<2	50
Coarse sand	2-4	5
Gravel	4-8	5

Sand and gravel used for the experiment were washed in distilled water and autoclaved at 121^{0}C for 15 minutes at 1.5 atm. Then they were dried in an oven at 100 ^{0}C for 40 minutes prior to introduction to the sand filter column. The biological layer composed of Citric acid treated cotton and MC-LR degrading bacteria. Overnight grown and starved bacterial culture was inoculated to Citric acid treated substrate. MC-LR was spiked at a final concentration of 10µgml^{-1} for the water reservoirs of both control and experimental setups. 1ml aliquots were collected from the inlet and outlet tanks of both control and experimental filters at 0, 3, 6, 12, 24, 48 and 72 hours. Collected samples were frozen (-20 °C) immediately and freeze dried used for PDA-HPLC.

2.5 Analysis of MC-LR removal by sand filter

Samples for PDA-HPLC analysis were prepared from freeze dried samples, reconstituted in 200 µl of 50% (v/v) aqueous methanol and centrifuged at 15000 g for 10 min, room temperature (RT) and the supernatant (100 µl) was removed for PDA-HPLC analysis. MC-LR removal percentages of both filters were calculated using the following equation.

MC-LR removal percentage = [(a-b)/a] x 100

Where,

a- Initial MC-LR concentration,

b - MC-LR concentration on sampling day

III. RESULTS AND DISCUSSION

3.1 Attachment of bacteria in to polymer substrate

Citric acid treated cotton substrate was very efficient in attachment of bacteria (Fig 2). The turbidity levels of both control and experimental setups varied parallel, proving attachment of bacteria into the cotton substrate.

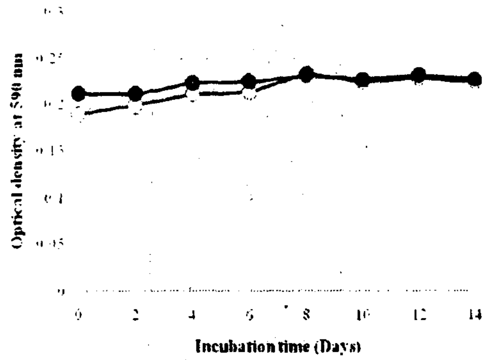

Fig 2. Optical densities of samples when Citric acid treated cotton was used as the substrate Closed circles (Experiment), Open circles (Control).

There has been an interest in using citric acid and other multifunction carboxylic acid compounds to improve mechanical properties of paper and textile products, without using formaldehyde [21]. The objective of the present study was to create two or more ester crosslinkages per acid molecule between the cellulose units of raw cotton. The crosslinkages improve wet strength for raw cotton and enhance permanent press properties in textiles.

Furthermore, Bacterial cell walls are composed of an inner membrane and a thick peptidoglycan which functions to maintain cell shape. The polysaccharide portion makes up 50% percent of the cell wall and consists of a neutral polysaccharide composed of N-acetylglucosamine, N-acetylmannosamine (ManNac), N-acetylgalactosamine and glucose in a molar ratio of 4: 1: 1: 1. The acidic portion of the cell wall is characteristic in having a repeating tetrasaccharide unit . 5% of the cell wall is made up of techoic acids, consisting of N-acetylglucosamine, galactose, glycerol, and phosphorus in a molar ratio of 1: 1.4: 1: 1. Thus Citric acid treated cotton material could easily form cross linkages with the bacterial cell walls and adsorb the cells into the surface. Fig 3 shows the MC-LR removal by *B. cereus* attached to the citric acid treated cotton substrate.

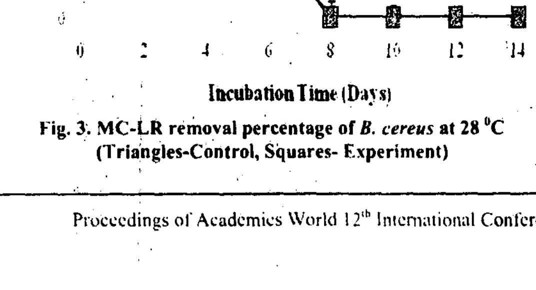

Fig. 3. MC-LR removal percentage of *B. cereus* at 28 °C (Triangles-Control, Squares- Experiment)

B. cereus showed a complete removal of MC-LR within 8 days of incubation in the experimental flask whereas the control samples did not show any removal of MCs. Therefore, it is evident that *B. cereus* attached to the citric acid treated cotton are able to utilize MC-LR as their sole carbon source.

3.2 Removal of MC-LR using the developed sand filter

Sand filters have been used for the production of clean drinking water for nearly 200 years. Additional advantages of sand filter in comparison to other treatment techniques are its simplicity and the low cost of construction and maintenance. Batch and column experiments with soil and sand by Lahti et al. [7] and Miller et al. [22] have shown low adsorption for MCs on sandy material and biodegradation as the most important elimination process for of the system. This, as well as the efficacy of slow sand filters to remove particulate substances, indicated slow sand filter to be an effective option for removing MCs from surface waters used as a drinking water source.

Experiments conducted by Gru¨tzmache et al. [12], with MCs on full-scale slow sand filters showed a high elimination potential for dissolved as well as cell-bound MCs. The overall elimination rates ranged from 43% to 99%, with values below 85% occurring in late autumn at low temperatures.

In the present study, MC inoculated water was passed through both the sand filters and MC removal was monitored by removal of sample aliquots at 0,3,6,12,24,48 and 72 hours of incubation (Fig 4.). The results showed that the experimental sand filter removed 23% of MCs from the influent water within three hours of commissioning. The removal rate gradually increased through out 24 hours and showed 90% of removal within four days.

On the other hand the control filter also showed 1% of removal within three hours and 12% after four days. Thus, it is clear 12% of MCs undergo physical removal through via adsorption to sand and rock particles as the control filter did not contain MC-LR degrading bacteria. Hence, 90% removal of experimental filter shows that 12% has been removed through physical adsorption into sand particles while the remaining 78% was removed entirely through bioremediation.

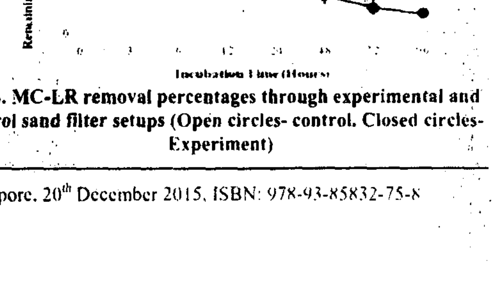

Fig 4. MC-LR removal percentages through experimental and control sand filter setups (Open circles- control. Closed circles- Experiment)

Previous study of Ho et al. [19] has shown that 100 % removal of MC-LR through the columns was possible only following 6 days of implementation. However, the sand filter developed in the present study showed that 90% of MC-LR was removed within 4 days of implementation . This may due to the attachment of MC-LR degrading bacteria as a biofilm in the present study, where as Ho et al. [19] merely inoculated MC-LR degrading bacterium, MJ-PV directly in to the water column.

Thus, the laboratory scale sand filter developed during the current study has provided a practical view on implementing small scale filters especially for domestic purpose to minimize the consumption of MC-LR contaminated water.

CONCLUSION

The present study has practically demonstrated the introduction of these microbes into sand filters. Therefore, MC-LR degrading forms of the present study can be employed as a low cost effective strategy in provision of safe drinking water by responsible authorities.

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