Malays. Appl. Biol. (2019) 48(4): 75–82

REMOVAL OF CYANOTOXIN MICROCYSTIN-LR BY *Lactobacillus plantarum*

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Accepted 9 October 2019, Published online 30 November 2019

ABSTRACT

The natural water resources contaminate high levels of nutrients, especially nitrogen and phosphorus, which can stimulate algae to grow rapidly. *Microcystis* spp., *Cylindrospermopsis* spp. and *Anabaena* spp. are dominant species in water. However, those algae can produce harmful toxins, called microcystin LR (MC-LR) affecting human and animal health. Therefore, this research aimed to eliminate MC-LR by using lactic acid bacteria. *Lactobacillus plantalum* TISTR 854 (LP) was used in this experiment. The effects of glucose concentrations (0, 1 and 3% w/v), pH (4.0, 7.0, and 10.0), and temperatures (25, 30 and 35°C) on LP removal activity were studied. LP was cultured in Lactobacillus MRS medium, centrifuged, and re-suspended in phosphate buffered saline at different pH. Different glucose contents and MC-LR were then added into the cell solutions, before incubate at the mentioned temperatures for 24 hr. The concentrations of MC-LR were then analyzed by Protein Phosphatase Inhibition Assay. The results were expressed as %MC-LR removing. The result showed that LP could reduce MC-LR. The optimal condition for MC-LR was found 1% w/v glucose, pH 7.0, and incubation temperature at 35°C, showed 77±0.6% of MC-LR removal. The study indicated that LP can be used to remove microcystin from water.

Key words: Cyanotoxin, microcystin-LR, *Lactobacillus plantarum* removal

INTRODUCTION

Eutrophication is caused mainly by excessive growth of cyanobacteria, especially *Microcystis* spp., since a source of water acquires a high concentration of nutrients, including phosphorus and nitrogen (Ball *et al*., 2001; Guzzon *et al*., 2001). This cyanobacteria leads to production of cyanotoxins in freshwater, directly harmful to aquatic animals and micro-organism and affecting human life (Barrett *et al*., 1996; Park *et al*., 2006). Points of view, the cyano-toxins are a group of compounds and cyanobacterial toxins, both are from the chemical and the toxico-logical. In terms of their toxicological target, cyanobacterial toxins are hepatotoxins, neurotoxins, cytotoxins, dermatotoxins and irritant toxins (Wiegand & Pflugmacher, 2005). Microcystins are a type of hepatotoxins, probably the most prevalent cyanotoxins in the water resources, and they are presented a high cyanobacterial biomass (Falconer & Humpage, 1996). Chemical structure is cyclic heptapeptide structure classified as hepatotoxins, chemically stable compounds and tumor promoters (Welker & Von Dohren, 2006). In humans, micro-cystins can cause liver inflammation and accelerate development of liver cancer (Gibson *et al*., 1990). It was reported that microcystins are tumor promoters and possible carcinogens in human (Falconer & Humpage, 1996). They are harmful to humans and aquatic animal living in the area that the algae grow rapidly (Lam *et al*., 1995; Jin *et al*., 2009). Most of the available toxicological data on microcystins have been based on microcystin-LR (MC-LR) (Zhao *et al*., 2008). MC-LR has been regarded the most as various toxic variants of microcystins, contains leucine and arginine at the protein amino acid positions (Ito *et al*., 1997; Mulderij *et al*., 2005). In China, the low levels of MC-LR in drinking water has been considered a contributing factor to the high prevalence of primary liver cancer in certain areas (Yu, 1989; Zhao *et al*., 2009). Therefore, the World Health Organization (WHO) has set the drinking water guideline value less than 1.0 μg/L of MC-LR (WHO, 1996). How-

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ever, chemically stable compounds of MC-LR has half-life of 10 weeks in environment. The rate of degradation will be increased under direct sunlight, at high temperatures (>40°C), and low pH (<1) or high pH (>9) (Tsuji *et al*., 1994). MC-LR concentrations can be reduced by boiling or heating in a microwave oven (Harada *et al*., 1996). However, it is high cost in removal process. Thus, lactic acid bacteria was used for degradation of MC-LR process. Our work studied the condition to MC-LR removal by *Lactobacillus plantarum* in experiment.

Lactobacillus plantarum is a member of lactic acid bacteria, which is gram-positive, non-sporing, non-respiring cocci or rods and can produce lactic acid during the fermentation process (Emiliano *et al*., 2014). It is commonly found in animal's gastrointestinal tract, fecal sample, respiratory, fermented foods and west water (Hayek & Ibrahim, 2013). It is usually used in production of fermented foods, such as yogurt, peperoni pickles, sauerkraut, Korean kimchi, brined olives, cheeses and fermented sausages (Ray & Panda, 2007). *Lactobacillus plantarum* have been listed on Qualified Presumption of Safety (QPS). QPS is notified by the European Food Safety Authority as recommended microorganism that can be intentionally added to foods or feeds without raising of safety concerns (Anonymous, 2005; Panda *et al*., 2008). Not only to apply in foods and feeds, it has also been reported that lactic acid bacteria could be used to improve water quality in natural resources. *Bifidobacterium lactis*, *L. rhamnosus* and *L. plantarum* were reported in decontamination and MC-LR degradation process (Halttunen, 2008). Thus, *L. plantarum* was tested for ability in removing MC-LR in contaminated water. The applications of water are safe to use in humans and environmentally friendly.

The objectives of this research were to evaluate ability of *L. plantarum* TISTR 854 and optimal conditions in MC-LR removal. The effects of concentration, pH and incubation temperature MC-LR removal. Ability of *L. plantarum* were focused.

MATERIALS AND METHODS

Bacterial strains

Lactobacillus plantarum TISTR 854 was assisted from Thailand Institute of Scientific and Technological Research (TISTR). The bacterial cells were cultured in Lactobacillus MRS (MRS) broth (Himedia, India) at 30°C for harvested after 15 hr incubation by centrifugation (~10^{10} CFU/mL). The cells were centrifuged at 12,000 rpm, 10 min, 4°C, and washed three times with phosphate buffer solution (130 mM sodium chloride, 10 mM sodium phosphate pH 7.0). The freshly bacterial cells were prepared for each experiment.

Microcystin-LR solution

Stock solution of Microcystin-LR (MC-LR) was freshly prepared by dissolving purifying MC-LR (DHI, HØrsholm, Denmark) in phosphate buffered solution (PBS) to obtain the final concentration 50 µg/L. The solution was then passed through 0.2 µm syringe filters in order to sterilization.

Microcystin-LR removal

Completely randomized design was used in this study. The pellet of *L. plantarum* (~10^{10} CFU/mL) was re-suspended in PBS at different pH including 4.0, 7.0 and 10.0, Glucose (SRL, Maharashtra, India) at 0, 1% w/v and 3% w/v was then added. Subsequently, the cell suspension was mixed with MC-LR solution to obtain the final concentration of MC-LR at 2 µg/L. The solutions were incubated at 25, 30, and 35°C for 24 hr. After that, the solutions were centrifuged at 12,000 rpm for 10 min at 4°C. Finally, 50 µL of the supernatant was collected and analyzed for MC-LR contents by Protein Phosphatase Inhibition Assay. The mixture of MC-LR without *L. plantarum* was used as control.

Determination of Mycosistin-LR by protein phosphate inhibition assay

MC-LR concentration was verified by Microcystest kit (Zeulab, Zaragoza, Spain). 50 µL of MC-LR standard and samples of solution were loaded into 96 well microplate. Then, 70 µL of the phosphatase solution and 90 µL of chromogenic substrate were added to each wells and mixed gently. Next, the cover films were closed on bottom of each wells of microplate, incubated the plate for 30 min at 37°C and added 70 µL of stop solution to each wells and mixed gently. Finally, MC-LR standard and samples were read the absorbance at 405 nm by microplate reader (EZ read 2000, Biochrom, Cambridge, England).

The standard curve was obtained by plotting standard absorbance at 405 nm in the y-axis and MC-LR concentration in a logarithmic x-axis. The MC-LR concentration in the samples were calculated by using the following equation:

$$y = a\ln x + b$$

Where,
x = MC-LR concentration equivalent in the samples.
y = absorbance at 405 nm.

Statistical analysis

Mean values of triplicate experiments were reported along with their standard deviations. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Analysis of Variance and Duncan's Multiple Range test were used at a 0.05 significance level.

RESULTS AND DISCUSSION

Effects of glucose concentrations on Microcystin-LR removal by *L. plantarum*

Figure 1 shows that after 24 hr of incubation at 35°C, MC-LR was continuously decreased from 2.06±0.03 µg/L to 0.58±0.01, 0.47±0.01, and 0.67±0.11 µg/L in 0, 1, and 3% of glucose, respectively. No changes were detected in the control, the MC-LR solution without *L. plantarum* TISTR 854. This indicated that *L. plantarum* had ability in reducing MC-LR. Moreover, it was found that addition of 1% of glucose enhanced *L. plantarum* TISTR 854 removal activity. However there was no significant difference in the treatment of 3% glucose when compared with the control. This meant that high concentration of glucose could negatively affect the removal activity of *L. plantarum* TISTR 854.

Glucose is an energy source of microorganisms (Samuel *et al*., 1980). *Lactobacillus plantarum* use glucose not only for cell growth but also for cell maintenance and survival. It took up glucose and change to lactic acid (Gobbetti *et al*., 1994; Sudhanshu *et al*., 2018). However, high glucose concentration accelerated microcystin consumption of the cells, weakening the cells and causing the cell death eventually. It was reported that the presence of 2 and 3% glucose, bacterial cells took up toxin faster and died faster too. While microcystin may be either directly toxic to *L. plantarum* or its elimination could exhaust the cells. No eliminated toxin was released by non-viable cells (Surono *et al*., 2008). Therefore, to enhance ability of *L. plantarum* to remove microcystin, optimal glucose concentration was required (De Vries *et al*., 2006).

Many bacterial communities can degrade MC-LR along with other organic compounds. Some probiotic bacteria such as *L. rhamnosus* GG, *L. rhamnosus* LC-705, *L. Plantarum* IS-10506 and IS20506, *Bidobacterium longum* 46, and *B. lactis* 420 and Bb12, have also been reported to be involved in enzymatic degradation of MC-LR (Nybom *et al*., 2007; Surono *et al*., 2008). The presence of specic gene clusters namely mlrA, mlrB, mlrC and mlrD in certain group of bacteria encoding enzymes were reported (Bourne *et al*., 1996; Ho *et al*., 2010; Jiang *et al*., 2011). The cyclic hepato-toxins structure of MC-LR was removed by an enzyme encoded by the rst gene mlrA and the linear molecule of MC-LR was hydrolysed successively by peptidases encoded by the mlrB and mlrC genes. Finally the hydrolysed product of MC-LR is transported into the cell for bacterial uptake by means of a putative transporter protein encoded by the gene mlrD (Bourne *et al*., 1996). Various methods have been found to analyse the respondent genes for MC-LR degradation (Saito *et al*., 2003).

Effects of temperature on Microcystin-LR removal by *L. plantarum*

To enhance MC-LR removal ability of *L. plantarum* TISTR 854, 1% of glucose was added into the MC-LR solutions. The result showed that

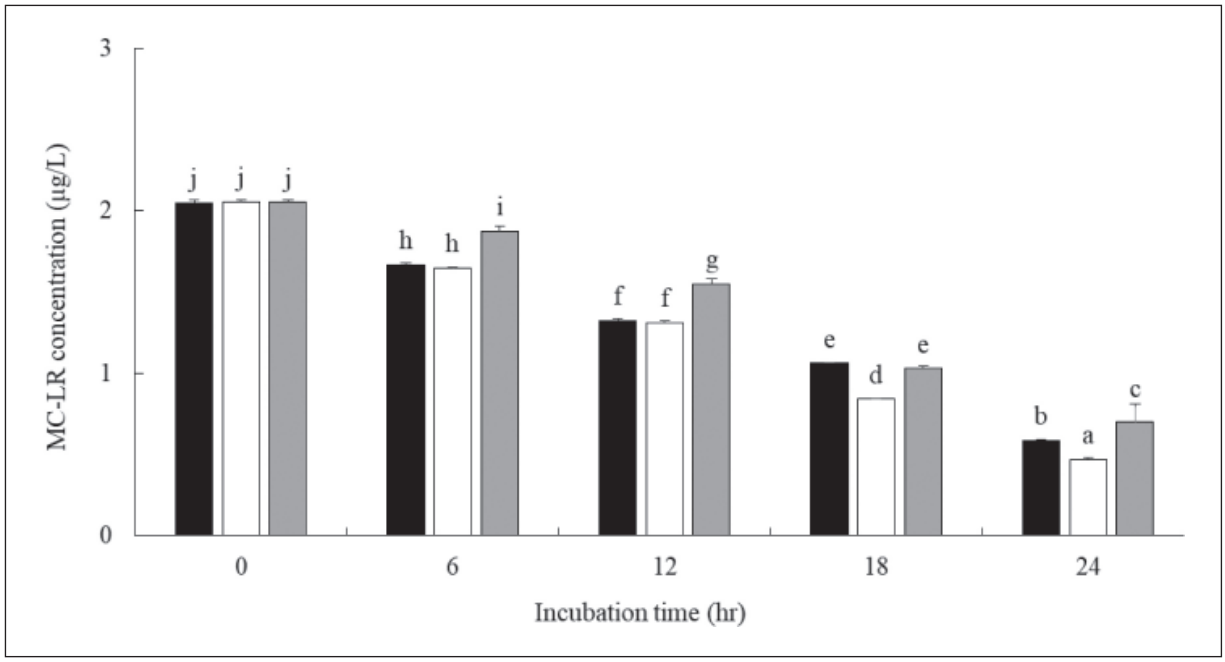

Fig. 1. Microcystin-LR concentrations removed *L. plantarum* in the mixture without glucose (■+) and with 1% (□+) and 3% of glucose (▯+) after 24 hr. Error bars show the SD of the mean of three experiments. The differences among the three experiments were statistically significant at the level of 0.05.

temperatures of incubation played an important role in MC-LR reduction. It was found that after incubating for 24 hr, MC-LR contents were decreased from 2.01±0.5 µg/L to 0.72±0.01, 0.51± 0.01, and 0.47±0.03 µg/L, when the mixtures were incubated at 25°C, 30°C, and 35°C, respectively (Figure 2). MC-LR with *L. plantarum* TISTR 854 incubated at 35°C had significantly lower concentration than the control. However, it was not significantly different when compared with the 25°C and 30°C.

Lactobacillus plantarum is a member of LAB, which had optimum growth temperature of LAB ranked between 20°C and 35°C (De Angelis *et al*., 2004). However, *L. plantarum* had better growth at 35°C than 25°C and 20°C. Surono *et al.* (2008) reported *L. plantarum* IS-20506 and IS-10506 from dadih behaved similarly in response to glucose supplementation, with faster and higher removal ability of MC-LR at 37°C. Besides, *L. plantarum* FH185 was found the optimum growth temperature at 40°C (Park & Lim, 2015).

The effect of pH

In addition to temperature, pH influenced MC-LR removal activity of *L. plantarum* TISTR 854. The results showed that at pH 7.0 MC-LR had less concentration than that at pH 4.0 (0.67±0.08 µg/L) and pH 10.0 (1.05±0.03 µg/L). MC-LR at pH 7.0 and 4.0 were significantly different when compared with the control (2.06±0.13 µg/L) after 24 hr incubation, while no significant difference was observed in MC-LR at pH 10.0 (Figure 3). The percentages of MC-LR removal by *L. plantarum* were 75%, 78%, and 48% for pH 4.0, 7.0 and 10.0, respectively.

Degradations of MC-LR by bacteria, such as *Sphingomonas* sp., *Paucibacter toxinivorans*, and *Pseudomonas aeruginosa* have been reported (Maruyama *et al*., 2006; Passos *et al*., 1996). The LAB including *L. plantarum* in fermented foods is an advantage when isolating for removing MC-LR. The fermentation of glucose can be decreased pH in culture media. The pH has dropped from pH 7.6 to 4.5 and the bacterial cell start to die (Eleuterio & Batista, 2010). *L. plantarum* produces lactic acid, which in turn causes cell stress as *L. plantarum* strains are unable to withstand the low pH for a long time (Surono *et al*., 2008). Vijayakumar *et al*. (2015) was reported *L. plantarum* KCC-24 was tolerant to a different range of salts especially NaCl and bile salts, pH of 4.0–8.0, temperatures of 28-45°C, and with optimum cell growth at a temperature of 37°C and pH 7.0 respectively. Neutral pH values may preserve bacterial viability cell in solution. While, accumulation of the metabolic products may decrease bacterial viability by changing the pH and decrease bacterial activity on MC-LR degradation (Jieming *et al*., 2011). At pH 7.0, *L. plantarum* was removed higher than each level.

The optimal conditions for MC-LR removal process was 1% concentration of glucose, incubation temperature at 35°C and pH 7.0 at MC-LR dose 2 µg/L in phosphate buffer solution. The removal

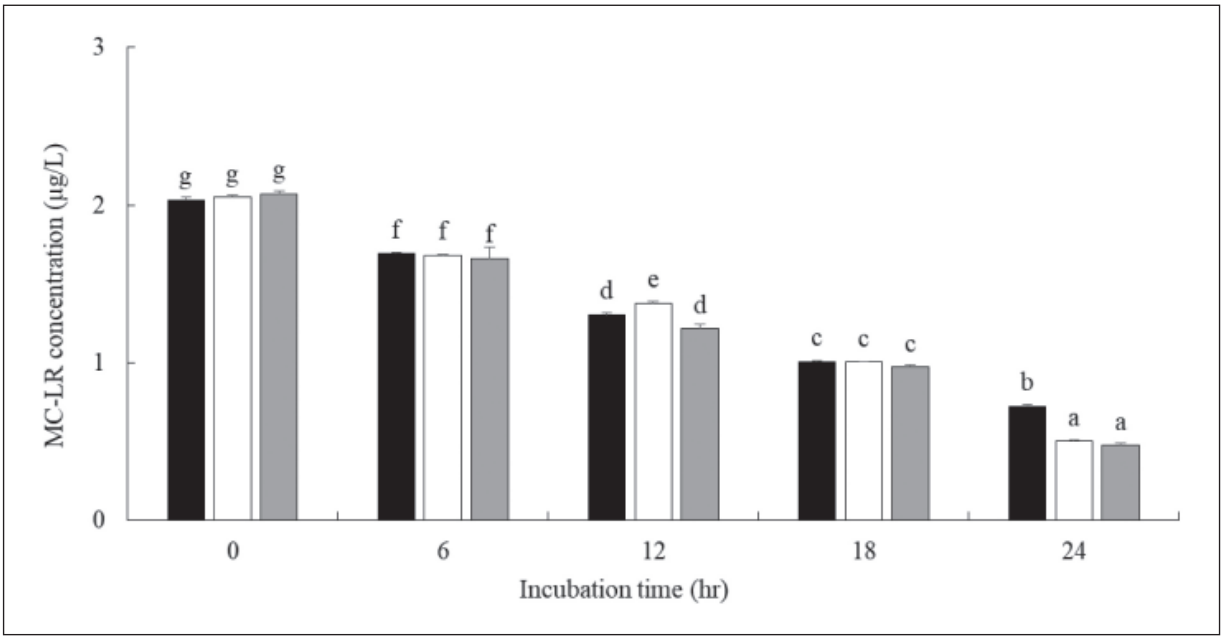

Fig. 2. Microcystin-LR concentrations removed *L. plantarum* in the mixture incubated at 25°C (■+), 30°C (□+) and 35°C (▒+) after 24 hours. Error bars show the SD of the mean of three experiments. The differences among the three experiments were statistically significant at the level of 0.05.

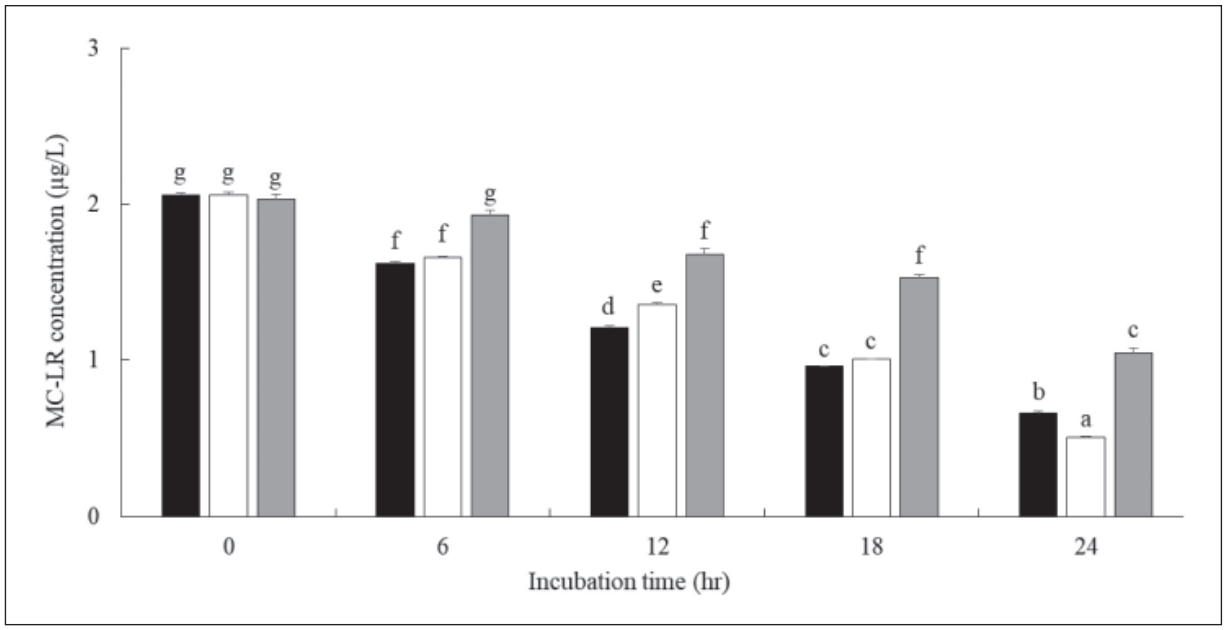

Fig. 3. Microcystin-LR concentrations removed *L. plantarum* in the mixture incubated at pH 4.0 (■+), 7.0 (□+) and 10.0 (▯+) after 24 hr. Error bars show the SD of the mean of three experiments. The differences among the three experiments were statistically significant at the level of 0.05.

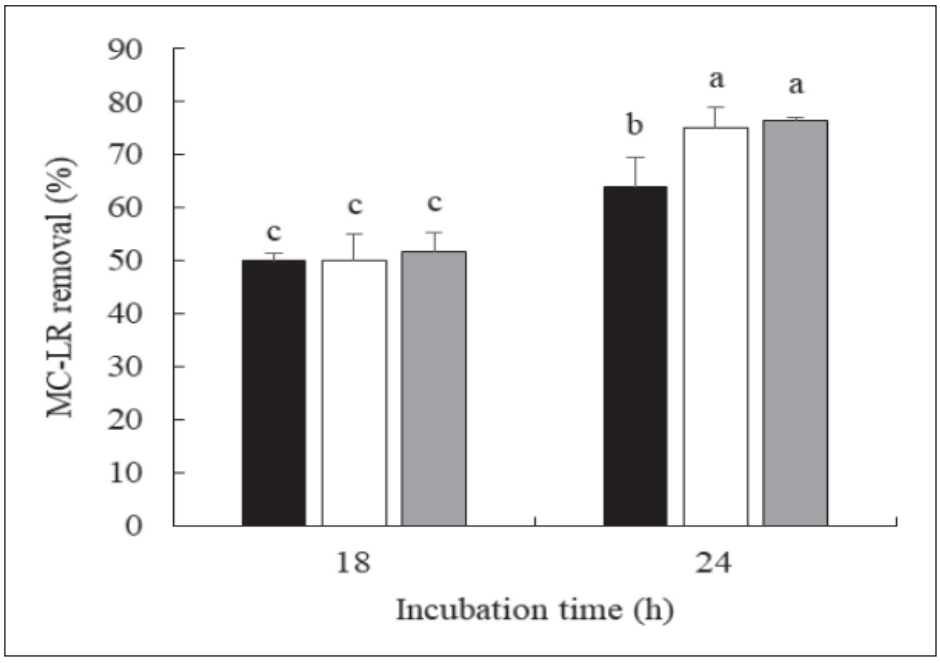

Fig. 4. Microcystin-LR concentrations removed *L. plantarum* in the mixture with 1% concentration of glucose, and pH 7.0 incubation temperature at 25°C (■+) 30°C (□+) and 35°C (▯+) after 18 and 24 hr. Error bars show the SD of the mean of three experiments. The differences among the three experiments were statistically significant at the level of 0.05.

percentages of MC-LR by *L. plantarum* TISTR 854 was 77±0.6% and the rate of breakdown was 67 ng/hour after 24 hr (Figure 4).

Fitzgeorge *et al*. (1994) reported LD_{50} values in the range 2.5-3.0 µg/g for MC-LR was hazardous to mouse. The levels of MC-LR in the natural lakes and ponds ranged from 4-605 µg/g dry weight of biomass (Kotak *et al.*, 1993). The presence of the toxic cyanobacterial blooms is a potential threat to the health and survival of aquatic wildlife such as sh, turtle, frog, water birds and other organisms including humans (Metcalf *et al.*, 2012). Whereas,

the raw water for drinking-water production had MC-LR level ranged from 0.15-4.3 µg/L. After 11.5 hr of treating process, MC-LR levels were reduced to 0.09-0.64 µg/L (Kotak *et al*., 1993). However, in the presence of natural microbial populations in the raw water, MC-LR concentrations can rapidly decrease (Tsuji *et al*., 1994). *Lactobacillus plantarum*, a commensal bacterium present in humans, has been reported to reinforce the intestinal barrier and to reduce intestinal permeability in animal studies (Charalampopoulos *et al.*, 2002; White *et al*., 2006). Also, it can be used in the water treatment process. The removal efficiency was dependent on temperature, pH and cell density, and increased significantly when glucose was added to the medium. A maximum removal of 95% was observed for *L. plantarum* strain IS-20506 (37°C, 10^{11} CFU/mL) with 1–2% glucose supplementation (Nybom *et al*., 2008a; Nybom *et al*., 2008b). Therefore, MC-LR can be reduced by adding *L. plantarum* in natural water resources. It could be useful for treating water in drinking water industry.

CONCLUSION

In this study, *L. plantarum* TISTR 854 was demonstrated to properties of MC-LR removal in laboratory. We investigated the optimum condition of *L. plantarum* for MC-LR removal. The removal percentages of freshly grown *L. plantarum* was 77±0.6% and the rate of breakdown was 67 ng/hour after 24 hr. The condition, 1% glucose, incubation at 35°C and pH 7.0 are optimum conditions for removing MC-LR in this experiment. These results indicate that *L. plantarum* TISTR 854 can be used to remove MC-LR in the water reservoirs of harmful algal blooms.

ACKNOWLEDGMENTS

This research was partly supported by the Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Thailand. Many thanks go to Institute of Research and development, Rajamangala University of Technology Thanyaburi, Thailand is granting the financial support for this research.

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