Bioresource Technology 181 (2015) 72–77

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of urea on growth and microcystins production of *Microcystis aeruginosa*

Xuanhao Wu, Yangwei Yan, Pinfei Wang, Lanqi Ni, Jiayi Gao, Ruihua Dai *

Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, China

HIGHLIGHTS

- MC-LR production of *M. aeruginosa* was restricted by high urea concentration.
- Growth and MC-LR production was promoted by low urea concentration.
- The first urea-N was biosynthesized into the Ala or Leu residue.
- Mdha was the last residue to assimilate urea-^{15}N.

ARTICLE INFO

Article history:
Received 4 November 2014
Received in revised form 7 January 2015
Accepted 9 January 2015
Available online 17 January 2015

Keywords:
Urea
Microcystis aeruginosa
Microcystin-LR
^{15}N

ABSTRACT

The effects of urea on the growth and toxin content of *Microcystis aeruginosa* isolated from Dianchi Lake in China were investigated. Experiments were carried out in lab using ^{15}N isotopic technique to characterize urea-N biosynthesis to microcystins. High urea concentration (3.6 mmol-N L^{-1}) would restrict the growth of *M. aeruginosa* and the production of microcystin-LR, while low urea concentration (0.4–1.4 mmol-N L^{-1}) would promote the growth of *M. aeruginosa* and the production of microcystin-LR. The ^{15}N labeling experiment further demonstrated that there existed selectivity when *M. aeruginosa* assimilated urea to form its structure. The majority of *M. aeruginosa* assimilated 1 urea molecule at first which was biosynthesized into the Ala or Leu residue. On day 18, The m/z = 1004 parent ion assimilated 9 ^{15}N except that the Mdha residue did not assimilate any urea-^{15}N. The results give deeper insight to the biosynthesis of urea into microcystins.

1. Introduction

Microcystis aeruginosa (*M. aeruginosa*) is one of the most common freshwater cyanobacteria in nature that may produce a suite of hepatotoxins known as microcystins (MCs). The MCs have caused several poisonings of domestic animals and the wildlife around the world, hence it poses health hazard on human through the consumption of contaminated water for drinking and recreation (Guo, 2007). The generalized structure of microcystins is described as cyclo (D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where MeAsp is D-erythro-β-methylaspartic acid, Mdha is N-methydehydroalanine, and *X* and *Z* are two variable L-amino acids, with their one-letter abbreviations used as the suffix of names (Miles et al., 2013). Adda, (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E-dienoic acid) is a characteristic residue for the C_{20} amino acid plays an important role in microcystin activities (Dawson, 1998). Microcystin-LR (MC-LR), which is one of the variants of microcystins substituted at *X* by leucine (Leu) and at *Z* by arginine (Arg) is one of the most toxic microcystins with an LD_{50} of 36–122 g kg^{-1} in mice (Dawson, 1998).

The dissolved inorganic nitrogen (DIN, ammonium, nitrate) and dissolved organic nitrogen (DON, urea, free amino acids, amides, and vitamins) are common in water. *M. aeruginosa* utilizes ammonium, nitrate nitrogen and other dissolved inorganic nitrogen in preference (Takamura et al., 1987; Yang et al., 2012). Nonetheless, when algae blooms occur and ammonium and other forms of DIN decline to the minimum, DON compounds become the dominant nitrogen forms in water (Flynn and Butler, 1986). DON compounds could act as the main source of nitrogenous nutrition in summer because of the excessive consumption by massive algae (Donald et al., 2011).

Urea is used widely in agricultural fertilizer application, animal feed and manufacturing. Global urea production is approximately 70 million metric tons per year and is predicted to exceed 200 million metric tons per year in 2020 (Glibert et al., 2006). Increasing

* Corresponding author. Tel.: +86 21 55664354; fax: +86 21 65643597.
E-mail address: rhdai@fudan.edu.cn (R. Dai).

http://dx.doi.org/10.1016/j.biortech.2015.01.035
0960-8524/

urea use contributes to high available nitrogen concentration in natural waters. Significant amount of urea (generally 0.1–10 μmol-N L^{-1}, depending on the different areas and sampling times), originating from both natural and anthropogenic sources, is gathered in water (Glibert et al., 2006; Kudela et al., 2008). Urea is an important substrate for enhancing phytoplankton growth (Strom and Bright, 2009; Solomon et al., 2010; Yuan et al., 2012). Moreover, it has been reported that urea produces the highest growth rate of *Alexandrium tamarense* (Xu et al., 2012). However, comprehensive studies on the effect of urea on the *M. aeruginosa* have not been examined. Understanding the effects of urea on the growth and microcystin production in eutrophic environments is important for understanding the different roles of urea in the ecophysiology of *M. aeruginosa* and algal bloom formation.

At present, most studies focused on the effects of inorganic and organic nitrogen on microcystin algae growth and toxin production (Davis et al., 2010; Donald et al., 2011; Xu et al., 2012; Yuan et al., 2012). However, the inner mechanism and the relationship between urea and the structure of microcystin are unclear. A complete understanding of algal cell metabolic products and the molecular properties of urea is required in order to unravel the role of urea in the growth and microcystin biosynthesis of *M. aeruginosa*. The development of ^{15}N enrichment techniques, which is used to trace these processes, can give a deeper insight to the biosynthesis pathway of nitrogen into microcystins (Sano et al., 2011). Liquid chromatography tandem mass spectrometry (LC/MS/MS) is one of the main technical tools for environmental analysis which has a very low detection limit, and a powerful qualitative ability. In addition, it has a high sensitivity and a strong specificity, which can be used to measure the advantage of multiple indicators at a time (Mayumi et al., 2006). With the promotion of chromatography-mass spectrometry technology, isotope tracer technique has been widely used in the research of trace metabolites in organisms. Fewer studies have combined the ^{15}N labeling technique with LC/MS/MS to deeply investigate the relationship between urea and microcystin biosynthesis by *M. aeruginosa*.

The present study investigated the effect of urea on the growth and microcystin-LR production of *M. aeruginosa* using ^{15}N isotopic tracer. The goal of this study is to reveal the mechanism of the influence of urea on the microcystins production and to provide a scientific basis for exploring the production of microcystins and controlling the pollution caused by *Microcystis*.

2. Methods

2.1. M. aeruginosa and cultures

An axenic strain of *M. aeruginosa* isolated from Dianchi Lake in China was obtained from Institute of Hydrobiology, Chinese Academy of Science. It was demonstrated that the *M. aeruginosa* only produced MC-LR (Dai et al., 2008). The *M. aeruginosa* was maintained in 1 L flask with about 500 mL BG-11 medium in a climatic chamber (BIC-300, Boxun, Shanghai, China) at 25 °C, a photon flux of 8–12 μw cm^{-2} with a 12 h photoperiod. The flasks were shaken each day and rearranged randomly in order to reduce any effects caused by minor differences in the light intensity on the algae growth (Vezie et al., 2002).

2.2. Different urea concentrations experiment

Cultures in exponential phase were concentrated by centrifugation at 5000 rpm for 20 min and washed 3 times with sterile distilled water and then inoculated in BG-11 medium, but without a nitrogen source for a week to exhaust the nitrogen in the cells (Vezie et al., 2002). The *M. aeruginosa* were then added to a series of 2.5 L flat-bottomed bottles which contained 2 L BG-11 medium and was supplemented with 0.4 mmol-N L^{-1}, 0.7 mmol-N L^{-1}, 1.4 mmol-N L^{-1} and 3.6 mmol-N L^{-1} of urea instead of nitrate. Control bottles were incubated with the same algal inoculums in the medium without nitrogen (Blank group).

Subsamples were taken at predetermined intervals after inoculation during the incubation period to count the cell number, extract MC-LR and measure nitrogen in cultures. The data presented here were the average values of three parallel samples with the standard deviation.

2.3. ^{15}N labeling experiment

After conducting the experiment, in order to analyze the effect of urea on the microcystin synthesis, two more flasks were set at the concentration of 1.4 mmol-N L^{-1}, which contained urea ^{15}N.

As for 1.4 mmol-N L^{-1} urea-^{15}N flasks, sub-samples were extracted to analyze the molecular structure of microcystins on day 0, 6, 12 and 18 using the LC/MS/MS (TSQ Quantum). Separations were carried out using Agilent 1100 HPLC equipped with Thermo C_{18} chromatographic column (2.1 mm × 150 mm). The mobile phase consisted of 30% acetonitrile and 70% water (0.5% glacial acetic acid). The flow rate was 250 μL/min and sample injection volumes were 10 μL. The Mass spectra were obtained using a Scientific TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Fisher, U.S.A.) with ESI mode. The ESI source was operated as follows: typical source voltage, 4 kV; source gas pressure, 15 (arbitrary units); auxiliary gas pressure, 5 (arbitrary units); transfer capillary temperature, 320 °C; argon collision gas pressure, 1.5 mTorr. For MS/MS operation the collision energy was 53 eV. MS tuning and optimization were achieved by infusing microcystin-LR and monitoring the $[M+H]^+$ ion at m/z = 995. The mass spectrogram was analyzed to identify the exact position of ^{15}N added to the microcystin molecular. The first mass spectrometry (MS^1) showed the difference between the molecular weight of the MC-LR labeled by ^{15}N and unlabeled (M_W = 994, m/z = 995). Furthermore, the secondary mass spectrometry (MS^2) indicated the position of ^{15}N located in the MC-LR molecule. The difference in m/z values observed from the LC/MS/MS spectra using ^{15}N labeling technique is applicable to the structural characterization of MC-LR and to determine the sequence of MC-LR utilization of urea nitrogen, suggesting new methods for studying MC-LR.

2.4. Analysis methods

2.4.1. Urea analysis

Urea concentrations were determined by NH_4^+ analysis after sample incubation with urease in a 50 °C water bath to convert urea N into NH_4^+ quantitatively (McCarthy, 1970). The concentrations of both urea and ammonium were analyzed in supernatant solutions. Ammonium was analyzed according to the standard method (APHA, 1995). For every flask, 50 mL algae culture was extracted into four 150 mL Erlenmeyer flasks washed with dilute HCl and rinsed thoroughly with deionized distilled water. Two of them were added to 5 mL dilute urease solution and put into 50 °C water bath for 20 min. These two samples were subsequently cooled at the room temperature and analyzed for ammonia. Then, the other two flasks were added to 5 mL dilute urease solution without a water bath and analyzed. A standard and two blanks were run for each batch. Absorbances were read at 640 nm with a spectrophotometer using 10 cm cells. The F factor is determined from the expression of $F = S/(A_S - A_b)$, where S is the concentration of the urea standard (in mmol-N L^{-1}), A_s is the absorbance of the standard, and A_b is the absorbance of the blank. The concentration of urea would be $F \times (E_S - E_B)$ (in mmol-N L^{-1}), where E_s is the