Valuable ingredients and feed toxicity evaluation of *Microcystis aeruginosa* acidolysis product in mice

Shiqun Han¹, Qing Zhou¹, Yudi Xu¹, Floris Vanogtrop², Qijin Guo¹, Guofeng Liu¹ and Shaohua Yan¹

¹Institution of Agricultural Resource and Environmental Sciences, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu 210014, China; ²Faculty of Agriculture and Environment, University of Sydney, Sydney 2006, Australia
Corresponding author: Shiqun Han. Email: ShiqunHann@163.com

Abstract

This research studied the extraction from *Microcystis aeruginosa* using hydrochloric acid method as a potentially valuable protein resource from eutrophic lakes. Amino acid composition, residual algal toxins, and heavy metals of the acidolysis product were studied. After 18 h of hydrochloric acid treatment, the product of *M. aeruginosa* contained 17 amino acids, 51.34% of total amino acid requirements, and 30.25% of the livestock and poultry essential amino acid (Eaa). The residual microcystin-LR (MC-LR) was 0.94 $\mu\text{g kg}^{-1}$, which was less than WHO drinking water limit of microcystins. The removal ratio of microcystins was higher than 99.99% during the process of hydrolysis. The concentration of heavy metals of the product was in compliance with feed standards. Furthermore, using Horn's method, Mouse Micronucleus Test and Sperm Shape Abnormality Test were conducted to study the forage safety of the product. Half lethal dose (LD_{50}) of acidolysis product in mice was $>9.09 \text{ g kg}^{-1}$ body weight, actually belonging to non-toxic grade. Every dose treatment did not significantly increase activities of alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and γ -glutamyltransferase (γ -GT). The results of both micronucleus test and sperm shape abnormality test were negative, which suggested the product with no mutagenicity and sperm malformation effects. This study indicated that the acidolysis product of *M. aeruginosa* was safe to be used as a feed ingredient.

Keywords: *Microcystis aeruginosa*, acidolysis, ingredients, feed safety

Experimental Biology and Medicine 2015; 240: 1333–1339. DOI: 10.1177/1535370214563894

Introduction

Microcystis (*Microcystis aeruginosa*) is a bloom forming cyanobacteria, and seriously deteriorates the quality of eutrophic water bodies. It has become the main constraints of regional social and economic development, and has prevented the maintenance of aquatic ecosystems in large lakes in China, such as Taihu Lake and Dianchi Lake.^{1,2} For the ecosystem restoration of Taihu Lake, *M. aeruginosa* removal from the water is more than 20,000 tons per year. *M. aeruginosa* contains cyclic polypeptides microcystins (for example, microcystin with leucine and arginine [MC-LR], arginine [MC-RR] or tyrosine [MC-YR]), which may cause a secondary pollution when decay if not properly disposed. Producing of biogas and producing fertilizers are two kinds of methods to treat the pollutants.^{3,4} However, these treatments are at high cost and thus uneconomical.

On the other hand, protein-rich *M. aeruginosa*, which is a new type of plant protein resource, might be feasible to be translated into amino acids, and would have wide applications in the feed industry. Previous studies suggested that

feed grade amino acids hydrolyzed from animal and plant protein were economical and technically feasible.^{5,6} *M. aeruginosa* cannot be directly used as feed for poultry and livestock, because of the high level of microcystins which are selectively hepatotoxic to birds and mammals. Currently, there are a number of reports on the amino acid composition of *M. aeruginosa* hydrolysis.⁷ However, they did not report the fate of microcystin residues, which are harmful to animals if the products are in feedstuffs. There are a lot of studies on the toxicology effect of MC-LR and its variants on liver,⁸ median lethal dose (LD_{50}),⁹ mutagenicity,¹⁰ and teratogenicity,¹¹ which used mice or pigs as a model to represent human and other animals.¹²

In this study, the types and compositions of *M. aeruginosa* acid hydrolysate amino acids were studied. At the same time, microcystin residues and heavy metals were determined through standard methods in poultry and livestock industries. The acute toxicity and safety were evaluated. This study provided an experimental basis for utilizing cyanobacterial protein as feed ingredients.

Materials and methods

Materials

M. aeruginosa was obtained from Taihu Lake, China. The contents of dry matter, crude protein (based on the dry weight), ash, MC-RR, and MC-LR (based on the dry weight) were 5.15%, 59.46%, 7.69%, 50810 $\mu\text{g kg}^{-1}$, and 32300 $\mu\text{g kg}^{-1}$, respectively.

The experimental mice: 150 germ-free healthy mice with ICR strain were purchased from the Animal Hospital of Nanjing Medical University, China. The weights ranged from 25 to 35 g, and male:female = 1:1.

Methods

Acid hydrolysis of *M. aeruginosa* and microcystin degradation. Acidolysis was carried out in a 50 L closed fermenter by hydrochloric acid. Hydrolysis temperature was 110°C, the ratio (v/w) of hydrochloric acid (12 M) and dried *M. aeruginosa* weight was 10:1. There were nine treatments (hydrolysis time was 6, 8, 10, 12, 14, 16, 18, 20, and 22 h, respectively) with three replications.

Chemical and biochemical analysis.

1. Amino acid analysis: The amino acids were quantitatively determined by HPLC (Agilent Series 1100, Agilent, USA). The separation column was Venusil-AA (4.6 mm $\times$ 250 mm, 5 μm). The diluted sample (100 μL) and norleucine (10 μL , 10 mM) as internal standard were absorbed in 1.5 mL plastic centrifuge tube, and then dried in an oven at 60°C for 8 h. The samples were dissolved with 110 μL 0.1 M HCl, and 50 μL triethylamine acetonitrile (1 M) and 50 μL phenyl isothiocyanate acetonitrile (0.1 M) were added, and then the samples were shaken and kept for 1 h at room temperature. Then, 200 μL n-hexane was added, and maintained for 10 min at room temperature after being shaken. When the samples were settled, the lower solution was filtered through a 0.22 μm membrane and 20 μL of this mixture was injected into HPLC. The following gradient elution was employed: 15 min linear gradient from 100% mobile phase A (sodium acetate 0.1 M pH 6.5/acetonitrile 185/14, v:v) to 5% mobile phase B (80% acetonitrile, v:v), hold 15 min, 5% B for 10 min, linear gradient from 22% B to 36% B, hold 9 min, 2 min gradient to 100% B, hold 5 min, return to 100% A. The equilibration time was 20 min, the flow rate was of 0.8 mL/min, the wavelength was 254 nm, and the column temperature was 40°C.
2. Microcystin analysis: Microcystin in water was determined following the method of Enzyme-Linked Immunosorbent Assay (ELISA).¹³
3. Heavy metal analysis: Cd and Pb were determined by atomic absorption (SavantAA Sigma, GBC Scientific Equipment, Australia), and As and Hg were determined by atomic fluorescence spectrometry (CN/61M-XGY-6060, Midwest Huabo co., Ltd, China).¹⁴

4. Lactate dehydrogenase (LDH), γ -glutamyltransferase (γ -GT), and alkaline phosphatase (ALP) analysis: mice of acute toxicity assay groups were used for collecting blood from retro-orbital plexus at 30 min and at mean time to death. Samples for the determination of ALP activity were collected from liver, and were washed free of adhering extraneous material. Serum was used to determine serum activity of LDH and γ -GT by commercial diagnostic kits. The ALP kit was purchased from Jiu Qiang Ltd., Beijing and the LDH and γ -GT kits were from Applygen Technologies Inc., Beijing. ALP, LDH, and γ -GT were estimated by kinetic method using p-nitrophenol, NADH, and γ -glutamyl-p-nitroanilide as substrates, respectively, at 340 nm (LDH) and 405 nm (γ -GT, ALP) by Shanghai 752 Spectrophotometer.¹⁰

Acute toxicity assay in mice. According to Horn's method, four groups with different doses were set: 2.15, 4.64, 10, and 21.5 g kg^{-1} body weight. Negative control was given 0.9% saline at 0.2 mL kg^{-1} body weight, and positive control was given cyclophosphamide 40 mg kg^{-1} body weight. The negative and positive controls of other experiments were the same.

A trial of 14 days was conducted to derive the LD₅₀ values of *M. aeruginosa* acidolysis product by the comparison of mice mortality in the test with the Horn's LD₅₀ values. According to the acute toxic dose, the product toxicity level was determined.

Micronucleus assay of mice bone marrow polychromatic erythrocytes. Three groups with different doses (10,000, 5000, 2500 mg kg^{-1} body weight), negative control, and positive control were set. The test samples were dissolved with sterile water into 500, 250, and 125 mg mL^{-1} , respectively. A 30-h test and gavage method were employed for each mouse with 20 mL kg^{-1} body weight.¹⁴

Bone marrow preparations were made using sternum from each mouse. Slides were stained and fixed according to conventional producer, and then examined with microscope. The polychromatic erythrocytes (PCE): normochromatic erythrocytes (NCE) ratio was scored in 1000 cells. The incidences of micronuclei (MN), observed in 2000 PCE/mouse were calculated to find the clastogenic property of the acidolysis products.¹⁵ All animal care and procedures were done in accordance with the Animal Care Guidelines from Nanjing Medical University's Animal Ethics Committee.

Sperm shape abnormality test in mice. Three groups with different dose were set with 10000, 5000, 2500 mg kg^{-1} body weight, respectively. Negative and positive controls were used. Gavage doses and method of the test sample were the same as "Micronucleus assay of mice bone marrow polychromatic erythrocytes", once a day and continuous for 5 days. Mice were sacrificed by over dose of anaesthesia at 35 days after the last exposure. The epididymis was removed and minced in 1 mL PBS and the suspension was then filtered

through 80 μm nylon mesh. One drop of 1% eosin-Y was added to the filtrate, and was kept for 30 min. One thousand sperms per mouse were screened and classified into normal and different abnormal types based on a similar procedure as described by Narayana et al.¹⁶

Calculation and statistics. The analysis of variance was performed using SPSS software. $P < 0.05$ was considered as significant level and $P < 0.01$ as extremely significant.

Results

Important ingredients of the acidolysis products

Different acidolysis time affect the amino acid compositions. There were 17 kinds of amino acids identified from the acidolysis products of *M. aeruginosa*, and the contents were different according to the time treatments (Table 1). The highest contents of total amino acid and essential amino acid (Eaa) of livestock and poultry which were derived after 14 h of treatment were 52.63% and 30.25%, while they were 51.34% and 29.51% after 18 h of treatment. The contents of Eaa accounted for 57.48% of the total amino acid after 18 h of treatment. The total contents of four kinds of amino acids (Arg., Thr., Lys., and Met.) which are common feed additives, first increased with the hydrolysis time and then decreased slightly, except for Met, whose content was very low and independent from the hydrolysis time (Figure 1). The Arg. reached its maximum content at 16 h ($P < 0.01$, $n = 9$), while the others reached their maximum contents at 14 h ($P < 0.01$, $n = 9$). The results suggested that the duration of hydrolysis had a great influence on the contents of the amino acids compositions ($P < 0.01$, $n = 9$).

Microcystin residues of the acidolysis products at different time. Before hydrolysis (0 h, Table 2), the concentrations of microcystins in *M. aeruginosa* were very high (MC-RR and MC-LR were $50810 \mu\text{g kg}^{-1}$ and $32300 \mu\text{g kg}^{-1}$). Acidolysis reaction could greatly degrade microcystin concentrations, and the longer the hydrolysis time, the lower the microcystin concentration (Table 2). The concentrations of MC-RR and MC-LR in hydrolysate were $0.90 \mu\text{g kg}^{-1}$ and $0.94 \mu\text{g kg}^{-1}$ at 18 h. The removal ratio of MC-RR and MC-LR was up to 99.99% after 18 h of hydrolysis, so that the hydrolysis time of 18 h was acceptable for the requirements of feed demand.

Heavy metal contents in acidolysis products. The contents of heavy metals (As, Pb, Hg, and Cr) in the acid

Figure 1 Effect of hydrolysis time on four kinds of amino acid contents (110°C, HCl:BGA = 10:1)

Table 1 Effect of different hydrolysis time on amino acid content

Acidolysis time (h)	6	8	10	12	14	16	18	20	22
Lys (Eaa) (%)	2.45	2.68	2.74	2.85	3.02	2.99	2.96	2.84	2.74
Met (Eaa) (%)	0.86	0.87	0.87	0.95	0.99	0.98	0.92	0.87	0.88
Val (Eaa) (%)	2.01	2.38	2.60	2.79	3.12	2.96	3.12	3.16	3.14
Phe (Eaa) (%)	1.63	1.76	1.75	1.9	2.02	1.96	1.99	1.90	1.87
Leu (Eaa) (%)	3.78	4.14	4.38	4.58	5.00	4.84	4.85	4.78	4.55
Ile (Eaa) (%)	1.42	1.71	1.91	2.12	2.50	2.49	2.51	2.61	2.63
Thr (Eaa) (%)	2.49	2.77	2.92	2.95	3.12	3.09	3.00	2.89	2.75
Arg (Eaa) (%)	2.59	2.87	3.03	3.14	3.27	3.33	3.32	3.25	3.09
Gly (Eaa) (%)	2.51	2.61	2.69	2.74	2.96	2.85	2.78	2.74	2.61
Tyr (Eaa) (%)	2.06	2.20	2.29	2.33	2.53	2.43	2.42	2.37	2.21
His (Eaa) (%)	0.59	0.64	0.65	0.70	0.73	0.71	0.72	0.69	0.67
Cys (Eaa) (%)	0.61	0.62	0.78	0.84	0.99	0.91	0.92	0.90	0.89
Asp (%)	4.66	4.86	4.91	5.07	4.98	5.00	5.01	4.59	4.52
Glu (%)	5.79	6.34	6.64	6.77	6.82	6.77	6.76	6.44	6.38
Ser (%)	2.86	3.03	3.10	3.13	3.24	3.21	3.09	2.94	2.86
Ala (%)	4.40	4.64	4.71	4.83	5.07	5.01	4.87	4.73	4.53
Pro (%)	1.90	2.01	2.09	2.11	2.27	2.19	2.10	2.06	1.95
Total essential amino acid (Eaa) (%)	23.0	25.25	26.61	27.89	30.25	29.54	29.51	29.0	28.03
Total amino acid (%)	42.61	46.13	48.06	49.80	52.63	51.72	51.34	49.76	48.27

Table 2 Effect of different hydrolysis time on the concentration of microcystins

Hydrolysis time (h)	0	10	14	16	18	20	22
MC-RR ($\mu\text{g kg}^{-1}$)	50810 $\pm$ 0.55	3.45 $\pm$ 0.021	1.33 $\pm$ 0.011	0.98 $\pm$ 0.0031	0.90 $\pm$ 0.0030	0.90 $\pm$ 0.0033	0.88 $\pm$ 0.0022
MC-LR ($\mu\text{g kg}^{-1}$)	32300 $\pm$ 0.32	2.89 $\pm$ 0.018	1.99 $\pm$ 0.0012	1.0 $\pm$ 0.0040	0.94 $\pm$ 0.0028	0.88 $\pm$ 0.0025	0.83 $\pm$ 0.0025

Table 3 Comparison of heavy metal contents between acidolysis products and feed standard of the People's Republic of China

Heavy metals (mg kg^{-1})	Acidolysis products	PRC National standards (2008)
As	0.24	≤ 2.0
Pb	3.97	≤ 5.0
Hg	0.002	≤ 0.1
Cr	1.23	≤ 10.0

hydrolysis products of *M. aeruginosa* were far below the limit of PRC national feed standard (Table 3). Thus, acid hydrolysis products of *M. aeruginosa* were safe to be used as feed ingredients.

Acute toxicity assay in mice fed with the acidolysis products

There was no gender difference of the mortality in mice (Table 4). The mice mortalities of negative control, doses 1, 2, 3, and 4, were 0, 20%, 20%, 20%, and 100%, respectively.

LD₅₀ values from the Horn's LD₅₀ appendix showed that when the mortalities of dose 1 (20%), dose 2 (20%), and dose 4 (100%) kept unchanged, with the increasing mortality of dose 3 (from 40% to 80%), there would be a gradual decrease of LD₅₀ (from 9.09 to 6.19 g kg⁻¹). In this test, the mortality of dose 3 was 20% which was less than 40%, and according to Horn's LD₅₀ appendix, LD₅₀ was greater than 9.09 g kg⁻¹. It was estimated that the grade was 5001 to 15,000 mg kg⁻¹ of once oral acute toxicity in mice. This result suggested that the acidolysis products of *M. aeruginosa* were actually non-toxic.

Effects of *M. aeruginosa* acidolysis products on mice serum enzymes of hepatic ALP, LDH, and γ -GT were shown in Figure 2. There was no significant difference in these enzyme activities compared with negative control ($P > 0.05$), but they were significantly lower than positive controls compared to the three dose treatments ($P < 0.01$). There were no significant differences among the three dose treatments ($P > 0.05$), which meant dose treatment did not increase ALP, LDH, and γ -GT activities.

Impact on mouse bone marrow micronucleus

The micronucleus ratios of normal mature erythrocyte were not significantly different between negative control and each dose group ($P > 0.05$). The micronucleus ratios between male and female in the same dose groups were also not significantly different ($P > 0.05$). These results indicated that, within the range of tested concentrations, the acidolysis products of *M. aeruginosa* did not significantly

increase the micronucleus forming ratio of bone marrow PCE. The results of micronucleus assay were negative in this study, indicating that the acidolysis product of toxic *M. aeruginosa* did not have impact on mice bone marrow cell micronucleus formation and PCE/red blood cell (RBC) ratio (Table 5).

The effect on mice sperm shape abnormalities

The number of each type of mice sperm abnormality in three dose groups was approximate to those in negative control, with hookless and amorphous types accounting for the most proportion among all the abnormal types (Table 6). However, when compared with cyclophosphamide group (positive control), the differences were significant. There was no significant difference of total sperm abnormality proportion between the dose groups and negative control ($P > 0.05$), while there was significant difference between positive control and each dose group, and between positive control and negative control ($P < 0.01$). Increasing the dose of cyanobacteria acid hydrolysate, total sperm abnormality proportion was not significantly increased among the three dose groups ($P > 0.05$). The results were negative, which indicated that the acidolysis products of *M. aeruginosa* did not affect mice sperm deformity proportion.

Discussion

Amino acid composition and feeding value of the acidolysis products

Amino acid is one kind of nutrition fortifier in poultry and livestock feed. It can significantly improve feed nutrition and increase its biological potency. During early, middle, and late broiler growth stages, lysine requirements of feed were 1.00%, 1.15%, and 0.87%, respectively, and total methionine and cystine demands were 0.91%, 0.76%, and 0.65%, respectively.¹⁴ The acidolysis products of *M. aeruginosa* (18 h treatment) contained 2.96% lysine, 0.92% methionine, and 1.84% of total methionine and cystine, which were more than the required standards of broiler feed for different growth stages. Thus, our study showed that *M. aeruginosa* acid hydrolysate can be potentially used as feed additives. The study also indicated that *M. aeruginosa* acidolysis products had high nutritional value. The products could be used in poultry feed as amino acids additives. Chamruspollert et al.¹⁷ reported that the three nutritional requirements of arginine, methionine, and lysine for animals were interrelated. The *M. aeruginosa* acidolysis products (18 h treatment) contained high levels of arginine (3.32%) and lysine, and therefore, additional synthetic lysine or high-lysine feed ingredients were not needed in order to balance the two amino acids in the preparation of a good feed. The ratio of arginine and lysine was 1:12, and it

Table 4 The mortality of male and female mice in each dose group

Dose (g kg ⁻¹ body weight)	Number for of experiments		Alive number		Mortality (%)	
	Male	Female	Male	Female	Male	Female
Negative control	5	5	5	5	0	0
2.15 (Dose 1)	5	5	4	4	20%	20%
4.64 (Dose 2)	5	5	4	4	20%	20%
10.00 (Dose 3)	5	5	4	4	20%	20%
21.50 (Dose 4)	5	5	0	0	100%	100%

Figure 2 The effect of *M. aeruginosa* acidolysis products on mice hepatic ALP, serum LDH, and serum γ -GT. Data with same letters were not significantly different ($P > 0.05$), while with different letters were very significantly different based on Duncan's new multiple range method

reached the optimum Arg/Lys ratio, which was in the range from 0.90 to 1.18.¹⁸ Therefore, *M. aeruginosa* acidolysis is a potentially safe and nutritious feed stock.

Microcystin residues and heavy metals of the acidolysis products

The utmost concern of *M. aeruginosa* acid hydrolysate for feed utilization is its microcystin residues. From the 1980s till date, MC-LR has been the most frequently reported and the most toxic congener. There is no standard limitation on Cyanobacterial toxin for animal feeds. The value of

microcystin guideline¹³ for drinking water quality only covers total MC-LR, which is 1.0 $\mu\text{g/L}$. In this study, the content of MC-LR was less than 1 $\mu\text{g kg}^{-1}$ after hydrolysis for 18 h. Although 14 h treatment of acid hydrolysis had the highest total amino acids, considering a well degradation of microcystins, 18 h of acid hydrolysis was appropriate.

Another concern is about heavy metals. The results in this study indicated that the contents of heavy metals in the acid hydrolysis products of *M. aeruginosa* were below the heavy metal limitations of feed standards. *M. aeruginosa* used in this study were obtained from the Taihu Lake, which is a drinking water source for millions of people

Table 5 The effect of *M. aeruginosa* acidolysis products on bone marrow micronucleus ratio and PCE/RBC (Red Blood Cell) ratio in mice

Gender	Dose (mg kg ⁻¹ body weight)	Mice number	PCE	PCE of micronucleus	Micronucleus ratio $\bar{x} \pm SD$ (‰)	PCE/RBC $\bar{x} \pm SD$
Female	10,000	5	5000	10	2.0 ± 1.0	1.09 ± 0.05
Female	5000	5	5000	6	1.2 ± 0.4	1.07 ± 0.04
Female	2500	5	5000	8	1.6 ± 0.5	1.08 ± 0.03
Female	Negative control	5	5000	7	1.4 ± 0.9	1.09 ± 0.03
Female	Positive control	5	5000	118	23.6 ± 3.0*	0.90 ± 0.04
Male	10,000	5	5000	9	1.8 ± 0.8	1.07 ± 0.07
Male	5000	5	5000	7	1.4 ± 0.9	1.06 ± 0.05
Male	2500	5	5000	9	1.8 ± 0.8	1.09 ± 0.03
Male	Negative control	5	5000	6	1.2 ± 0.4	1.10 ± 0.06
Male	Positive control	5	5000	122	24.4 ± 3.1*	0.89 ± 0.04

*Much significant difference with negative control ($P < 0.01$).

Table 6 The effect of *M. aeruginosa* acidolysis products on malformation type and proportion of mice sperm

Dose (mg kg ⁻¹ body weight)	Hookless N %	Amorphous N %	Fat-head N %	Banana-shaped N %	Double-headed N %	Double-tailed N %	Coiled/folded N %	Total sperm abnormality	Total sperm abnormality rate (%)
10,000	31 24.4	84 66.1	9 7.1	1 0.8	0 0.0	0 0.0	2 1.6	127	2.5 ± 0.4 a B
5000	35 25.4	91 65.9	9 6.5	2 1.4	0 0.0	0 0.0	1 0.7	138	2.8 ± 0.5 a B
2500	33 29.2	70 61.9	6 5.3	2 1.8	0 0.0	0 0.0	2 1.8	113	2.3 ± 0.3 a B
Negative control	31 25.4	80 65.6	8 6.6	2 1.6	0 0.0	0 0.0	1 0.8	122	2.4 ± 0.6 a B
Positive control	161 28.0	292 50.8	69 12.0	23 4.0	9 1.6	12 2.1	9 1.6	575	11.5 ± 1.4 b

Note: In total, 5000 sperms were tested. *N* is the number. Data with different letters were very significantly different ($P < 0.01$).

and is well reserved. It might be attributed that heavy metals in Taihu Lake do not exceed the PRC national standards limitations,¹⁴ hence, the heavy metal contents need to be monitored and be further studied for the algae from other lakes.

Feed safety of the acidolysis products

Acute toxicity trial is important to clarify the toxicity of the test materials, understand its target organ, and gain important indicators for feed safety assessment. While there are few reports about microcystins in feed safety studies, LD₅₀ and some biochemical indicators were extensively used to measure the impact of microcystins on mice. Gupta et al.¹⁰ found that, when the mice were administered LD₅₀ dose of MC-LR, MC-RR, or MC-YR (43, 235.4, and 110.6 µg kg⁻¹ body weight, respectively), microcystin variants significantly increased activity of hepatic enzymes γ-GT, glutamic oxaloacetic transaminase (AST), and glutamic pyruvic transaminase (ALT). When Sprague-Dawley rats were infused continuously with 16, 32, and 48 µg kg⁻¹ body weight of MC-LR, the result showed that ALP was dose-dependent and had a significant increase with the dose increasing.⁸ In our study, the result of mice LD₅₀ suggested that the acidolysis products of *M. aeruginosa* were non-toxic. It further demonstrated that the products did not increase mouse biochemical

metabolisms related with serum LDH and γ-GT, and liver ALP activities. Our toxicity test did not change the mouse LDH, γ-GT, and ALP activity levels on different doses of the *M. aeruginosa* acidolysis products, because of its very low residual microcystin level.

Intraperitoneally injecting MC-LR could significantly decrease RBC count, hemoglobin (Hb), and hematocrit (Ht) in treatments of 2 and 8 µg kg⁻¹ body weight, and significantly elevate micronucleus frequency in bone marrow cells in treatments of 0.5, 2, and 8 µg kg⁻¹ body weight in mice.¹⁹ Spontaneous frequency average of bone marrow micronucleus was 0.8‰ to 2.3‰ in ICR mouse species with the weighted average of 1.5‰. In our micronucleus test, the ratio of PCE-RBC of mice in each dose group was basically consistent with the reported results of normal range which was from 0.6 to 1.2,²⁰ indicating no suppression of PCE formation.

Other researchers reported that 6 and 12 µg kg⁻¹ body weight MC-LR could increase the DNA-protein crosslinks formation in testicle cell of mice, and MC-LR could also damage the chromosome in early stage of sperm cell.²¹ Usually, the sperm abnormality proportion in negative control was 0.8% to 3.4%.²⁰ In our study, the sperm malformation proportion was 2.5 ± 0.4 in the maximum dose experimental group, so that our results were in line with the referenced standard.

In summary, amino acid was abundant and of essential balance in the *M. aeruginosa* hydrolysate, and its microcystin residues and heavy metals were in line with relevant standards. Evaluating from the aspect of toxicology, *M. aeruginosa* acid hydrolysis products were non-toxic, non-mutagenic, and non-teratogenic. Therefore, the feed utilization of cyanobacterial as protein resource was feasible. However, its chronic toxicology and nutrition values need to be further studied.

Authors' contributions: SH, YX, and QG designed the experiments; FV and QZ did and interpreted the experiments; GL and SY wrote the manuscript. All authors have read and approved the content of the manuscript.

ACKNOWLEDGEMENTS

This work was supported by grant 2012ZX07103-005 from Ministry of Science and Technology of the People's Republic of China for Lake Chaohu projects: "Pollution control technology and demonstration of bioremediation on heavily polluted confluence Bay in Lake Chaohu" and grant TH2010205 from project: "Jiangsu special funds for the Lake Taihu".

REFERENCES

- Qin B, Zhu G, Gao G, Zhang Y, Li W, Paerl HW, Carmichael WW. A drinking water crisis in Lake Taihu, China: linkage to climatic variability and lake management. *Environ Manage* 2010;**45**:105–12
- Shen Y, Liu Y, Wu G, Ao H, Qiu C. Mechanical removal of heavy cyanobacterial bloom in the hyper-eutrophic lake Dianchi. *Acta hydrobiologica Sinica/Chung-kuo ko hsueh yuan shui sheng sheng wu yen chiu so* 2003;**28**:131–6
- Dong S-x, Dong J-y, Song H-c, Zhou J-s, Xie J, Xia C-f, Gao T-r. Study on the biogas fermentation with blue algae from Dianchi lake. *Renew Energy* 2006;**2**:16–8
- Han S-Q, Yan S-H, Wang Z-Y, Song W, Liu H-Q, Zhang J-Q, Huang J-P. Harmless disposal and resources utilizations of Taihu Lake blue algae. *J Nat Resour* 2009;**3**:007
- Esteban MB, Garcia AJ, Ramos P, Marquez MC. Sub-critical water hydrolysis of hog hair for amino acid production. *Bioresour Technol* 2010;**101**:2472–6
- Rogalinski T, Herrmann S, Brunner G. Production of amino acids from bovine serum albumin by continuous sub-critical water hydrolysis. *J Supercrit Fluid* 2005;**36**:49–58
- Qian Y-T, Chang Z-Z, Wang S-M, Wu J-W, Xu X. Hydrolyzation of cyanobacteria into amino acid solution. *Jiangsu J Agri Sci* 2008;**5**:030
- Solter PF, Wollenberg GK, Huang X, Chu FS, Runnegar MT. Prolonged sublethal exposure to the protein phosphatase inhibitor microcystin-LR results in multiple dose-dependent hepatotoxic effects. *Toxicol Sci: Official J Soc Toxicol* 1998;**44**:87–96
- Rao PV, Bhattacharya R. The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver in vivo. *Toxicology* 1996;**114**:29–36
- Gupta N, Pant SC, Vijayaraghavan R, Rao PV. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. *Toxicology* 2003;**188**:285–96
- Abramsson-Zetterberg L, Sundh UB, Mattsson R. Cyanobacterial extracts and microcystin-LR are inactive in the micronucleus assay in vivo and in vitro. *Mutat Res* 2010;**699**:5–10
- Falconer IR, Burch MD, Steffensen DA, Choice M, Coverdale OR. Toxicity of the blue-green alga (cyanobacterium) *Microcystis aeruginosa* in drinking water to growing pigs, as an animal model for human injury and risk assessment. *Environ Toxicol Water Qual* 1994;**9**:131–9
- WHO. *Cyanobacterial toxins: Microcystin-LR in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality*. Geneva: World Health Organization (SDE/WSH), 2003, pp.4–57.
- Standards PN. *National standards glossary of the People's Republic of China*. Beijing: Published by China Standard Press, 2008
- da Silva J, Herrmann SM, Heuser V, Peres W, Possa Marroni N, Gonzalez-Gallego J, Erdtmann B. Evaluation of the genotoxic effect of rutin and quercetin by comet assay and micronucleus test. *Food Chem Toxicol: Int J Br Indust Biol Res Assoc* 2002;**40**:941–7
- Narayana K, D'Souza UJ, Seetharama Rao KP. Ribavirin-induced sperm shape abnormalities in Wistar rat. *Mutat Res* 2002;**513**:193–6
- Chamruspollert M, Pesti GM, Bakalli RI. Dietary interrelationships among arginine, methionine, and lysine in young broiler chicks. *Br J Nutr* 2002;**88**:655–60
- Balnave D, Barke J. Re-evaluation of the classical dietary arginine: lysine interaction for modern poultry diets: a review. *World's Poultry Sci J* 2002;**58**:275–89
- Zhou W, Zhang X, Xie P, Liang H. The suppression of hematopoiesis function in Balb/c mice induced by prolonged exposure of microcystin-LR. *Toxicol Lett* 2013;**219**:194–201
- Zhang Q. *Toxicology base*, 3rd edn. Beijing: Published by People's Health, 2000
- Dong L, Zhang H, Duan L, Cheng X, Cui L. Genotoxicity of testicle cell of mice induced by microcystin-LR. *Life Sci J* 2008;**5**:43–5

(Received July 15, 2014, Accepted October 28, 2014)