The Comparison of Purification Microcystins Extractant *

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Abstract: The study investigated the extraction and purification of microcystin' extractant from natural cyanobacterial bloom. The extracting method was assessed with variant solvents, and 80% methanol had the highest efficiency while ethanol solvent could extract microcystins safely. The mass of phycobiliproteins, which having negative effects on ODS(C_{18}) cartridges, was removed by adjusting the pH of solvents to their isoelectric points. The results provide a solid basis for obtaining pure microcystins efficiently.

Key words: natural bloom; cyanobacterial powder; microcystins extract; purify
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1 Introduction

Toxin-producing cyanobacteria often occur in eutrophicated fresh and brackish water bodies[1]. The types of toxin commonly found in freshwater are microcystins (MCs), hepatotxic cyclic heptapeptides produced by different genera of cyanobacteria, like Microcystis, Nostoc. Anabaena and Oscillatoria. The general structure of MCs is cyclo-(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha) (where D-Ala is *D*-alanine, *D*-Glu is γ-linked *D*-glutamic acid, *D*-MeAsp is β-linked *D*-erythro-β-methylaspartic acid, Mdha is N-methyldehydroalanine, and Adda is the unique C_{20} β-amino acid, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-Adda dienoic acid). The structural variants of MCs are named according to the one-letter abbreviation of amino acid. For example, the commonest microcystin contains leucine and arginine in these positions and is therefore named microcystins-LR. Possible modifications in loss or addition of methyl groups and variations in the Adda group lead to more than 60 structural variations of MCs, and the number will increase in the future[2].

MCs are potential toxins due to their inhibition of the regulatory enzymes protein phosphatases 1 (PP1) and 2A (PP2A). Furthermore, MCs are regarded as tumor promoters, and are involved in many cases of animal poisoning and human health problems[3]. The use of MCs in enzymes study in related processes makes them important biochemical tools and further enhances the demand for purified MCs. There are several reports about extraction and analysis of MCs[4-5]. Typical process is to extract cyanobacterial cells with solvents, and the extract is then concentrated and purified by a range of sample separation techniques. However, the methods vary greatly and there is no consensus on the most efficient purification approach to date. This paper presents the comparison of various extracts on MCs extractant from cyanobacterial bloom, and for the first time reports the removal of phycobillisome from the extractant by pH adjustment. The pretreatment remarkably reduces the pollution to the expensive C_{18} cartridges and makes the following separation feasible.

2 Materials and methods

2.1 Cell material

Cyanobacterial field samples were harvested from April to October in 1998 from Dianchi Lake, Yunnan Province, China, using machine collection and air dry. The cyanobacteria powder was stored in dark bags under room temperature. The sample contained 85% Microcystis spp with the granularity of about 0.25mm, and 40% ~ 50% of crude protein. The moisture was approximately 8% ~ 10%. The powder was blue-green or slightly Kelly, and had fishy smell of the algal.

2.2 Extraction of microcystins

One gramme of cyanobacterial powder in tubes was extracted with 50 mL of the following solvents respectively: distilled water, 5% acetic acid, aqueous methanol (10% grades increasing), and aqueous ethanol

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(10% grades increasing). The samples were sonicated for 5 min and subsequently shaken for 10 min during each extracting step. After centrifugation, the supernatants were pooled and stored in darkness.

2.3 Purification of extractant

The extractant was purified with three methods. The first method is salting out, in which sufficient ammonium sulfate (20 g for each extractant) was added in the solution to salt out the coextracted phycobiliprotein and other pigments. The second method is flocculation, in which $(FeCl_3)_n$(0.5 g for 50 mL extractant) was put in the solution to flocculate the coextracted materials. The last method is sedimentation at isoelectric point, in which pH of the solution was adjusted by H_2SO_4 to about 4.0, the solution was then centrifuged for 5 min at 5000×10^{-6} after 10 min. The supernatants was then decanted and the pH was djusted to 8.0 by $NH_3 \cdot H_2O$.

2.4 Preparative HPLC

The purified extractant was loaded to preparative HPLC, which the process was seen elsewhere.

3 Results and discussion

The methods of MCs extraction and purification from algal cells have been extensively developed[6]. Extracting solvents used in the purification of MCs vary greatly and yet thereis no agreement as to which is the most appropriate. This maybe because the range of microcystin variants among samples and the non-uniform behaviors of different toxins. There is a widerange of solvents been utilized, such as butanol-methanol-water[7], 5% acetic acid[8] and aqueous methanol[5], often without efficiency report. Those frequently used and/or found to be satisfactory solvents were evaluated for the collected bloom cyanobacteria. The results were shown in Fig.1.

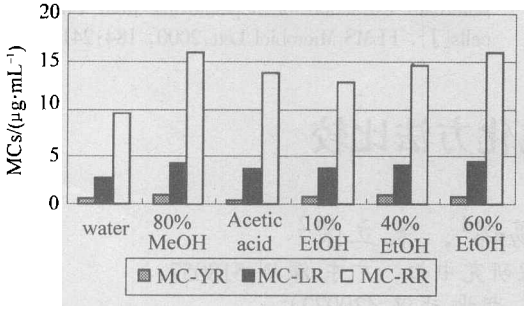

Fig.1 MCs concentration in various solvents

The results indicated that efficient extraction of microcystins from field samples could be achieved using 40% ~ 90% methanol, and 80% aqueous methanol was the most efficient. However, the results also showed that the aqueous methanol could be replaced by aqueous ethanol for safety with similar efficiency. The simplest water extraction was not recommended because the extractant smelled due to phycocyanins degradation.

Factors including temperature and ultrasonic effects were tested for the extraction of MCs. The results are illustrated in Figure 2 and Figure 3.

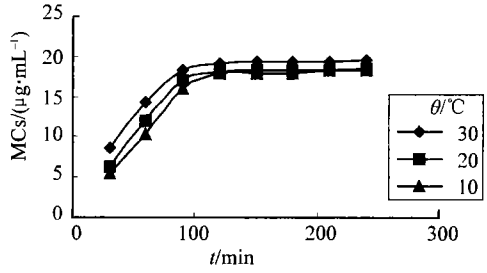

Fig.2 The effect of temperature

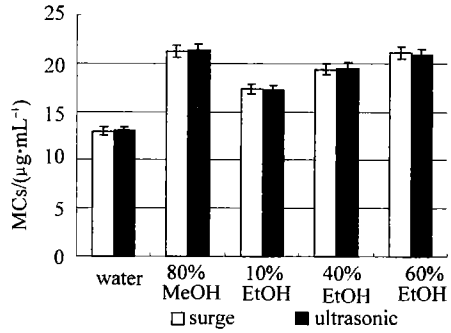

Fig.3 The effect of ultrasonication

The microcystins could be extracted directly and kept stable within a temperature range of 10 ℃ to 30 ℃ (Fig. 2). And there was little notable difference among extractions from 10 ℃ to 30 ℃. Metcalf (2000) used boiling water bath and microwave oven, and found both resulted in a quicker (> 10 min) and efficient extraction of microcystins with no recorded degradation[10]. However, very small cell mass was used in his study since it was developed primarily as a routine quantification method. Therefore, further tests are necessary before the method can be recommended for large scale purification. In addition, the degenerated protein due to boiling would produce disgusting smell.

Although sonication was used in a significant number of papers about microcystins extract[5], the results of our investigation suggested that it had little effect on microcystins' yield (Fig.3).

As the phototrophic prokaryotes, there are kinds of pigments can be coextracted with MCs, including water-soluble phycocyanin and solvent soluble pigments such as chlorophyll a, carotenoids β-carotene, zeaxanthin, ketocarotenoid echinenone, and carotenoid-glycoside myxoxanthophyll[9]. The appearance and scanning spectrum of extracts confirmed that massive pigments were coextracted with MCs (Fig. 4).

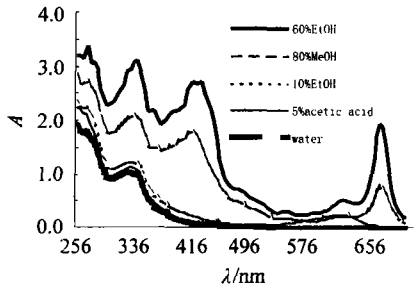

Fig.4 Scanning spectrum of various extracts

The large amount of coextracted compounds would interfere with subsequent column purification and separation steps, thus redeemed simple purification by reversed-phased flash chromatography impossible. In many cases, these pigments were loaded on RP-column, and caused seriouspollution on expensive C_{18} cartridges. Many workers have used size exclusion, usually Sephadex LH-20 from Pharmacia, as an initial separation technique that allows the removal of pigments and large interfering molecules. Recently, Saito reported sample preparation with ammonium sulfate and DEAE anion exchange chromatography to remove interfering compounds. Those methods are either expensive or time-consuming.

In order to wipe off those pigments, several methods were investigated. Both salting out with ammonium sulfate and flocculation with $(FeCl_3)_n$ had little effect on precipitation of the pigments. However, pH adjustment gave good results. Massive pigments were precipitated after the pH was adjusted to 4. Because the isoelectric point of phycocyanin is ~4.3, the sedimentation may be caused by isoelectric precipitation. The purification method shows its virtues of simplicity and no negative effect on the following separating processes, thus making preparation of microcystins in large scale feasible.

4 Conclusions

It was shown that in all extracts 80% methanol was the most effective for extracting MCs. Aqueous ethanol is a good alternation with similar efficiency and higher safety.

pH adjustment is a simple and effective method to remove pigments from extracts and makes following separating processes feasible.

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微囊藻毒素的提取纯化方法比较

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摘 要: 研究了从天然蓝藻水华藻粉中提微囊藻毒素, 以及对提取物纯化的方法。通过对比不同提取剂的提取效果, 发现浓度为80%的甲醇溶液提取效率最高, 但采用乙醇作为替代提取剂也有较好的效率, 且方法更为安全。对于提取物的纯化, 可通过调节溶剂的pH至等电点以除去对反相填料具有负作用的藻胆蛋白。研究结果为更高效地纯化微囊藻毒素提供了依据。

关键词: 天然水华; 藻粉; 藻毒素提取; 纯化
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