An Efficient Separation of Pyrroloquinoline Quinone Using Chemical Complexation Extraction

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Abstract—Pyrroloquinoline quinone (PQQ), an important natural product, is biosynthesized by *Gluconobacter oxydans*. **In order to prepare the product in large scale, a rapid and selective chemical complexation extraction (CCE) is proposed to separate and purify PQQ from fermentation broth. In this work, the effect of extraction solvent system, stripping agent, and inorganic ion on the extraction were investigated, respectively. As a result, trioctylamine-octanol system showed the most extraction constants (log K_{ex} = 5.07). Ammonia water was the most stripping agent (log K_{ex} = 4.85). Several inorganic salts were determined to have negative effect on the extraction, which suggest that inorganic salts should be removed from broth before extraction. Finally, the optimal extraction condition is Trioctylamine-octanol system (1:1, v/v), 28 °C, and pH=6.5, which leads to more than 90% of extraction rate. In conclusion, trioctylamine-octanol system and ammonia water showed excellent extraction efficiency, which can be used to prepare PQQ.**

Keywords: Nature Products; Pyrroloquinoline quinone; Separation; Chemical Complexation Extraction

I. INTRODUCTION

Pyrroloquinoline quinone (PQQ, Fig. 1) is an important natural product with many physiological functions, such as antioxidation, promoting growth, and protecting cells [1-2]. Though it shows potential application, plants and animals do not produce PQQ. The source of PQQ in these organisms was shown to be from microorganisms [3]. Several bacteria are able to synthesize PQQ including *Acinetobacter calcoaceticus*, *Pseudomonas fluorescens CHA0*, *Klebsiella pneumonia*, *Methylobacterium extorquens AM1*, *Gluconobacter oxydans*, and *Methylovorus sp. strain MP688* [4-8]. Their synthesis gene cluster has also been revealed in these bacteria [4-8].

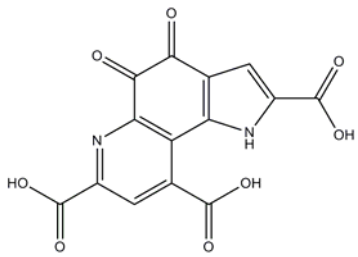

Fig. 1 The structure of PQQ

Although PQQ can be biosynthesized in bacteria, the concentration of PQQ in the fermentation broth is very low [9-11]. Furthermore, due to the polar property of PQQ, it cannot easily dissolve in organic solvent, the traditional solvent extraction or solvent sublation is unsuitable to separate PQQ from fermentation liquid. Previously, Zhao reported that ion exchange or reverse column was used to separate and purify PQQ. However, the yield was very low [12]. Therefore, in order to prepare PQQ in large scale, an efficient process of separation for PQQ is very important.

We noted that there are three carboxyl groups in the structure of PQQ, which can be used as functional group for chemical complexation. In this paper, a chemical complexation extraction process has been developed for separation of PQQ. The total extraction rate of the target compound is more than 90%, and the purity of product is 89%. These results suggested that this method can be used to prepare PQQ efficiently.

II. EXPERIMENTAL

Reagents and chemicals

Acetonitrile (CH_3CN) of HPLC grade was purchased from Sigma–Aldrich (Dorset, UK). Water (H_2O) (18.2MΩ) was obtained from a Purelab Ultra system from Elga (Bucks, UK). The standard of PQQ was purchased from J&K. Trioctylamine (AR) was purchased from Sigma–Aldrich (Dorset, UK). Octanol (AR) was purchased from J&K.

Microorganisms and fermentation conditions

The strain *G. oxydans* ATCC 621H was applied to produce PQQ. The fermentation medium was composed of 80 g/L sorbitol, 20 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, and 5 g/L $MgSO_4{\cdot}7H_2O$. All media were sterilized by autoclaving at 115 °C for 15 min. Pre-culture was inoculated with a single cell colony from an agar plate and incubated in shaker with 250 rpm at 28 °C for 24 h. A 10% (v/v) of cells was transferred into the 100 mL medium in the flask and grown at 28 °C for 24 h.

Procedure

Fermentation broth and extraction agent (1:1, v/v) were put in conical flask, and vibrated for 30 min under 28°C. A series of mixture liquid, such as trioctylamine mixing with ethyl acetate, petroleum ether, and octanol, was used as

extraction agents. To preparation PQQ and reused extraction solvent, the extraction solvent and stripping agent (1:1, v/v) were put in conical flask, and vibrated for 30 min under 28°C.

Analysis method of PQQ

According to the literature reported by Yang [13]. The procedure is described as below:

Ten microliters of the PQQ solution was injected into an Agilent UPLC system equipped with waters Acquity UPLC BEH C18, 2.1×100 mm, 1.7 μm preceded by a Waters Acquity BEH VanGuard C18 guard column packed with 1.7 μm packing material. Elution was performed at a flow rate of 200 μL/min. The mobile phase was CH3CN/H2O (3:7, v/v). The total UPLC run time was 10 min. The gradient was increased linearly from CH_3CN/H_2O (3:7, v/v) to CH_3CN/H_2O (9:1, v/v) for 6 min, and then the initial conditions were restored and allowed to equilibrate for 4 min.

The qualitative analysis of PQQ was performed using DAD detector on an Agilent Technologies DAD 1290 system (Agilent Technologies Co. Ltd., Palo Alto, CA, USA) and electrospray-ionization tandem mass spectrometer on a Thermo LTQXL® (Thermo Fisher Scientific Inc., Waltham, MA, USA). The ion spray voltage and temperature were optimized for production of the requisite precursor ions in negative- and positive-ion modes. PQQ was analyzed in negative-ion mode. Universal mass spectrometric settings included capillary voltage of 3500 V, cone voltage of 49 V, extractor voltage 9 V, source temperature of 198 °C, desolvation temperature of 300 °C, cone gas flow 110 L/h, desolvation gas flow 750 L/h, collision energy of 15, and dwell times of 150 ms. SRM peak integration sand data analyses were performed using the Analyst® program (Agilent Technologies Co. Ltd., Palo Alto, CA, USA). The collision energy and gas pressure were then optimized for dissociation of deprotonated molecule $[M-H]^-$ into the characteristic product ions.

III. RESULT AND DISCUSSION

A series of concentrations of trioctylamine mixing with ethyl acetate, petroleum ether, and octanol were extraction efficiency. The extraction ratios of a series of extraction systems were displayed in the Table-1.Among them, trioctylamine-octanol system showed a high extraction efficiency with 90% of rate. The effects of volume ratio of Trioctylamine-octanol, tempreture, and pH were evaluated. The optimal extraction condition is Trioctylamine-octanol system (1:1, v/v), 28 °C, and pH=6.5.

Table-1 The extraction ratios of a series of extraction systems

type of extraction agent system	extraction ratio
ethylacetate-trioctylamine	53.4
n-butyl alcohol-trioctylamine	52.1
dichloromethane-trioctylamine	56.3
petroleum ether-trioctylamine	57.8
octanol--trioctylamine	92.1

Table-2 Effect of inorganic salts on the extraction of PQQ

type of inorganic salts	extraction ratio	
	sorbitol	PQQ
no	6.4±0.1	91.3±1
NaCl	0.5±0.1	28.6±1
$MgCl_2$	3.8±0.1	28.5±1
Na_2SO_4	0.4±0.1	53.4±1
$MgSO_4$	0.4±0.1	53.6±1

Table-3 Screening of the stripping agent

type of stripping agent	extraction ratio
NaOH	90.1±1
Na_2CO_3	90.7±1
$NaHCO_3$	89.2±1
Na_2HPO_4	68.5±1
NH_4OH	91.2±1
H_2O	20.1±1

Table-4 Effect of molar ratio (ammonia: succinic acid load in organic phase) on the reextraction

molar ratio of ammonia: PQQ load in organic phase	extraction ratio
4:5	29.3±1
6:5	32.5±1
8:5	43.2±1
10:5	50.1±1
12:5	51.2±1
14:5	51.2±1
16:5	51.3±1

When using chemical complexation extraction for separation of water-soluble compound, the first step, the

extracted complex was transferred from water phase into organic phase. The preparation of the target compound is the end objective, and then the extraction solvent was reused, which is a key factor for chemical complexation extraction. In the process of chemical complexation extraction, organic carboxylic acid, under free state, was transferred into organic phase. When pH increasing, the rate of dissociated carboxylic acid goes up. This is so

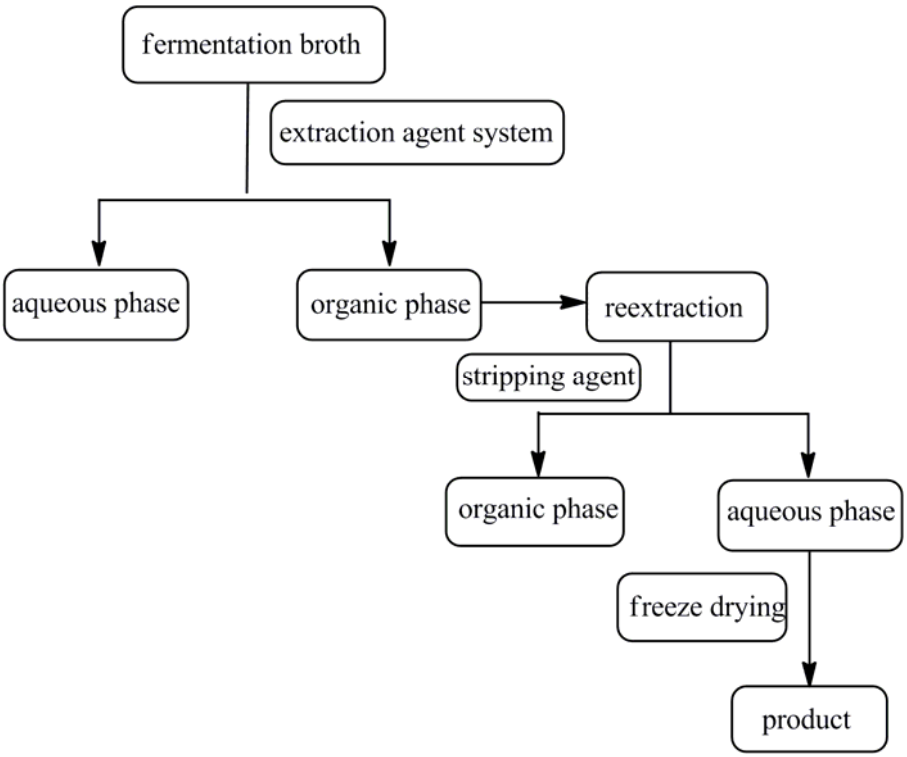

Fig. 2 The technology roadmap of Chemical Complexation Extraction

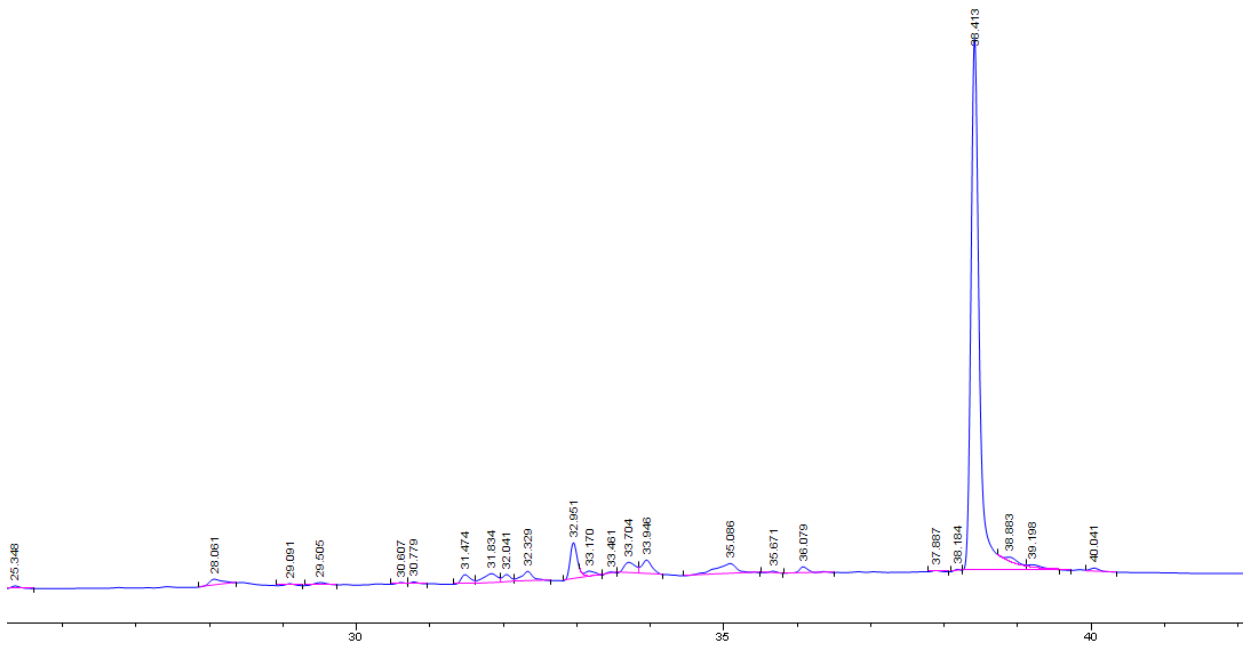

Fig. 3 A profile of the product in HPLC detected by DAD detector at 239 nm

called pH stripping affection. The result of screening of the stripping agent were displayed in Table 3 and 4.Among them, the ammonia water showed an excellent property which leads to a high extraction rate (>90%).

The effects of several inorganic salts on extraction were displayed in the Table-2. The results suggested that the inorganic salts have negative effect on extraction. The anion have the similar effect.

The reason may be the anion could compete with trioctylamine to connect the positive ion. Based on this phenomenon, the inorganic salt should be removed. Therefore, the fermentation broth needs to be pretreated using macroporous resin column, which can remove the inorganic salt.

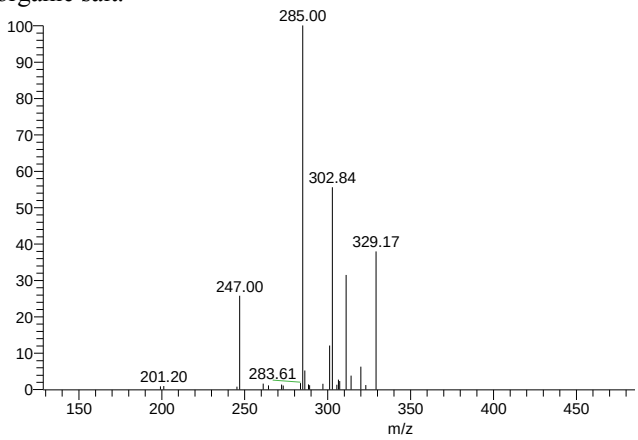

Fig. 4 The characteristic ion fragments for PQQ in the MS

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