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Rapid Determination of Microbial Quinones using Supercritical CO_2 Extraction

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Abstract. The supercritical CO_2 extraction of microbial quinones from activated sludge samples obtained from various activated sludge was investigated and compared to the conventional method using organic solvent extraction. The extraction was carried out in a supercritical fluid extraction (SFE) system in the temperature range of 25 to75 ^oC and the pressure up to 30 MPa. Different extraction conditions, such as the temperature, pressure, extraction time and modifier were employed to maximize the SFE efficiency. Significant amount of microbial quinones (ubiquinones and menaquinones) could be extracted rapidly with supercritical CO_2. Results on the value of diversity and dissimilarity suggested that the SFE with supercritical CO_2 extraction was a reliable technique for quinones extraction.

Keywords: quinone profile, microbial community structure, supercritical CO_2, dissimilarity, diversity.

INTRODUCTION

The technique of quinone profiles for the estimation of microbial biomass or for the examination of community structure has been effectively used to study microbial communities in various environments including biological wastewater treatment, natural aquatic systems, agricultural soil and composts (Fujie et al. 1998, Hiraishi 1999, Katayama and Fujie 2000, Tang et al. 2003, Song and Kayama 2005). It has been reported that total amount of quinones reflects the microbial biomass (Saitou et al.1999), and profile of quinones reflects taxonomic diversity of the community since many microorganisms have one major quinone species (Katayama et al. 2001). The technique of quinone profiles has an advantage in terms of quantification, simplicity, and reproducibility (Kunihiro et al. 2002). Comparing with other analysis methods such as PLFA and 16S-rDNA, the quinone profile analysis is also considered to be a useful tool for the analysis of microbial population dynamics in mixed cultures, since it gives the information on the microbial biomass, the diversity and the structure of microbial community (Song and Katayama, 2005). In the conventional method, analysis of quinones from environmental samples is performed by organic solvent extraction (direct extraction) with chloroform–methanol mixture (Collins and Jones 1981), and then continued by using solid phase extraction (Sep-Pak® Plus Silica) for the purification and separation of quinones (Hu et al. 1999), finally analyzed by high performance liquid chromatography (HPLC). However, this conventional method suffers many drawbacks. The need of using a large amount of organic solvents would be the major drawback besides it is labour intensive and time consuming. Thus

the search for an alternative solvent such as supercritical carbon dioxide ($scCO_2$) is crucial.

The use of liquid and supercritical CO_2 (sc CO_2) has been gaining increasing interest of researchers because it is considered as an environmentally benign alternative to organic solvent for a wide range of applications. CO_2 is environmentally acceptable, low toxicity, has convenient critical properties ($Tc=31.1^oC$, $Pc=7.38$ MPa), nonflammable and ease for recycling. Those favorable properties of CO_2 offer the opportunities for selective extraction and fractionation.

The objective of this study is to develop a novel method for rapid determination of bacterial quinones from various activated sludge samples (i.e. domestic, municipal, machinery and fishery sewage) using $scCO_2$. The effect of some parameters such as temperature, pressure, extraction time and modifier on the extracted quinone amount were investigated.

EXPERIMENTAL

Supercritical Fluid Extraction

All experiments were performed using an SFE system (Jasco) as shown in Figure 1. The system is mainly equipped with two high-pressure pumps (SCF-201; Jasco), a back pressure regulator (880-81; Jasco), a 1-ml extraction vessel (Jasco) and an oven (GCA 353; GL Sciences Inc.) which controls the temperature of the extraction vessel. In a typical extraction, 0.1 g of the dried activated sludge was placed into the extraction vessel. The system is then sealed. At the temperature and pressure reached the desired value, the CO_2 and modifier were continuously supplied by the high pressure pump to the extraction vessel. This moment was defined as the beginning of extraction. During the extraction, the extracted microbial quinones were collected in collection flask and then evaporated using rotary vacuum evaporator after completed extractions. These dried extracted quinones, containing menaquinones and ubiquinones, were then loaded into two Sep-Pak® Plus Silica cartridges using 15 ml hexane solution at a fixed flow rate. Prior to HPLC analysis, the menaquinones and ubiquinones were separated by the procedure reported by Gao et al. (2003).

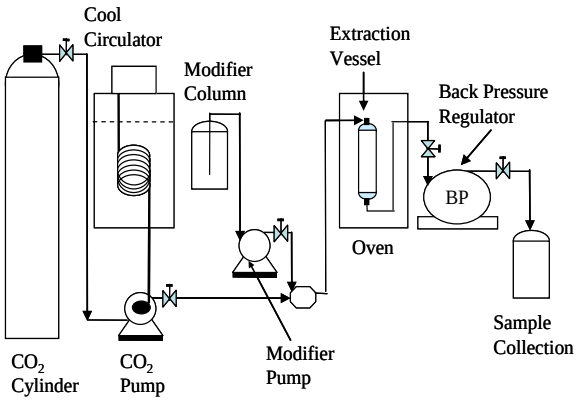

Figure 1. Schematic diagram of supercritical fluid extraction apparatus.

Organic Solvent Extraction

Conventional analysis of quinones using organic solvent extraction method was also carried out. The procedure reported by Hu et al. (1999) was used to extract ubiquinones and menaquinones from activated sludge. A sample of 0.1 g of a dried activated sludge (similar to that used in the case of $scCO_2$ extraction method) was used in the case of the organic solvent extraction. Quinones were initially extracted from the sludge using chloroform-methanol mixture (2:1, v/v) and subsequently extracted into hexane. The hexane extract, containing the ubiquinones and menaquinones, was separated and purified using two Sep-Pak Plus Silica cartridges joined in series before analyzed by HPLC.

The extracted bacterial quinones were analyzed using HPLC (Shimadzu, Japan) equipped with ODS column (Zorbax-ODS, 4.6 mm I.D. × 250 mm, Agilent Technologies, USA) and two detectors, UV-Vis detector (Model SPD-10A, Shimadzu)

and photodiode array detector (SPD-M10A, Shimadzu). Ubiquinone 10 (UQ-10) was used as the quantitative standards for ubiquinones and menaquinones. The wavelengths used to quantify quinones were 275 nm and 270 nm for ubiquinones and menaquinones, respectively.

RESULTS AND DISCUSSION

Comparison between Sc CO_2 Extraction and Conventional Method

Figure 2 shows quinone profiles of the activated sludge samples extracted by using $scCO_2$ and conventional solvent extraction. The detected quinones consisted of ubiquinones with 7 to 11 isoprenoid units and menaquinones with 6 to 11 isoprenoid units.

Both quinone profiles from different extraction methods were almost similar. However, interesting trend was shown from these profiles where most ubiquinones species extracted by using $scCO_2$ extraction are fewer than those by using conventional method. On the other hand, most menaquinoes species extracted by using conventional method are fewer than those by using $scCO_2$ extraction. Since the polarities of ubiquinones are higher than those of menaquinones, so ubiquinones are more easily extracted by using $scCO_2$ than menaquinones.

Table 1. Advantages and disadvantages of using conventional extraction and $scCO_2$ extraction.

		Conventional Extraction	**Sc-CO_2 Extraction**
1	Extraction time / one sample	3.5 hr	15 min
2	High pressure instrument	No need	Need
3	Automation	Difficult	Easy
4	Chemicals / one sample:		
	Chloroform	70 ml	0
	Methanol	35 ml	4.5 ml
	Hexane	95 ml	15 ml
	Acetone	3 ml	0
	CO_2	0	40.5 ml

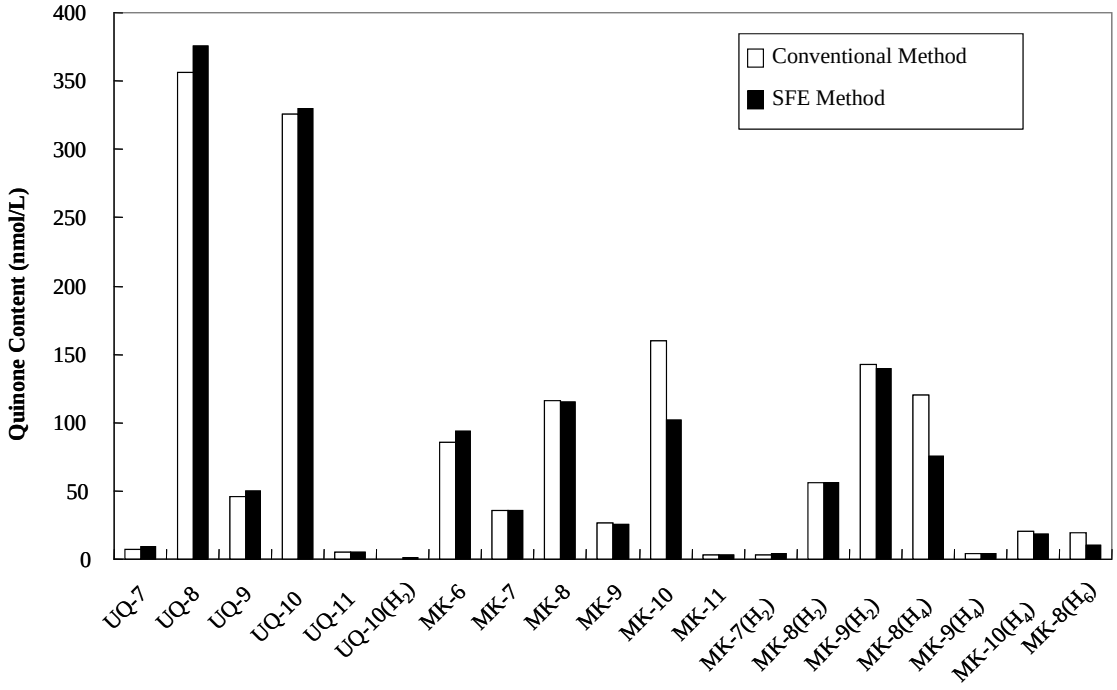

Figure 2. Quinone profiles of the activated sludge samples extracted by using $scCO_2$ and conventional solvent extraction.

Based on the above results, the extraction of quinones with $scCO_2$ is promising and more simple compare to that of conventional extraction method. The comparison of the two methods is shown in Table 1. As can be seen that the $scCO_2$ extraction method could reduce the extraction time and the consumption of organic solvents significantly. The main disadvantage of the $scCO_2$ extraction is the fact that this technique requires high pressure instruments.

$ScCO_2$ Extraction of Bacterial Quinones from Different Activated Sludge

Figure 3 shows quinone profiles from different activated sludge samples extracted by using $ScCO_2$ extraction. Five species of ubiquinones and 16 menaquinones were extracted from activated sludge waste water plant at toyohashi university of technology (TUT), 6 ubiquinones and 16 menaquinones from plant A, 6 ubiquinones and 13 menaquinones from plant B, 6 ubiquinones and 14 menaquinones from plant C. Figure 3 also showed that all of the tested sludges contained UQ-8 as the most predominant ubiquinone type, followed by UQ-10, UQ-7, and UQ-7, although these sludge are obtained from different sources. On the contrary, the menaquinone composition of tested activated sludge was complex as compared with the ubiquinone profiles recorded. Menaquinones with six to twelve isoprene units (including hydrogenated types) occurred as homologues. MK-10(H_4) is most predominant menaquinone in activated sludge waste water plant at TUT. The remaining three plant sludges; plant A, B and C contained MK-6, MK-9(H_2), and MK-8(H_2) as the most predominant menaquinones, respectively. All the tested sludges had MK-6 as the major component. Although HPLC analyses revealed that there were marked differences in menaquinone systems among the sludges tested, one should note that MK-6 constituted a significant proportion of the total menaquinones in all cases and the next lower and higher homologues are MK-8, MK-7 and MK-9(H_2), occurred frequently in considerable amounts.

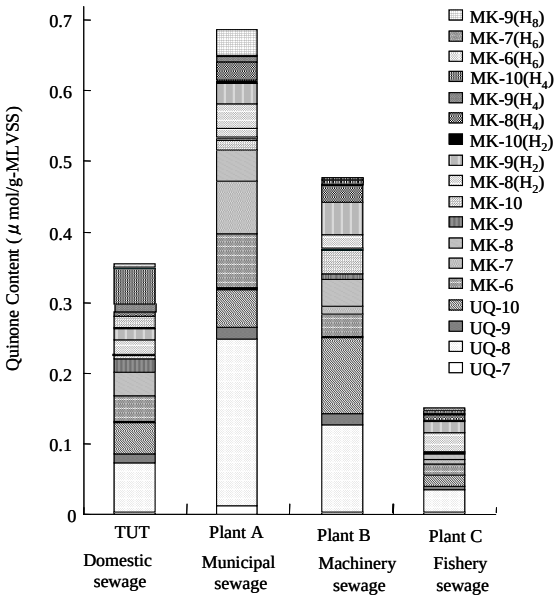

Figure 3. Quinone profiles from different activated sludge samples

Dissimilarity and diversity

The difference in the results obtained in each sample by using the two methods was estimated by comparing the quinone profile data. To enhance the objectivity of the information and to make quantitative estimates, two statistical indices, diversity index (DQ) and dissimilarity index (D) were used. The value of diversity index equal to 11.99 for the method employing $scCO_2$ extraction was almost similar to that obtained by organic solvent extraction method of 11.95, meaning that the two methods have similar diversity value. Furthermore, the low value of dissimilarity, 0.09, was obtained between organic solvent extraction and $scCO_2$ extraction. Hu et al. (2001) suggested that two quinone profiles could be considered different when the dissimilarity index is higher than or equal to 0.1. Therefore, this value means that the two quinone profiles could be considered similar to each other. It proves that $scCO_2$

extraction is a usefull method in quinone analysis for activated sludge.

CONCLUSION

The total amount of extracted quinones with $scCO_2$ extraction has been compared with that of obtained by organic solvent extraction. The diversity indices of the two methods were relatively similar (11.99 and 11.95 for extraction with $scCO_2$ and solvent extraction, respectively) and the dissimilarity index between the two methods was low (0.09). Based on those values/indices, it was found that $scCO_2$ extraction is a reliable technique for ubiquinones and menaquinones extraction from activated sludge in quinone analysis. Compared to the conventional method, the $scCO_2$ extraction technique extremely can reduce the procedures, the consumption of organic solvents and extraction time.

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