Effect of Various Polar Solvents to The Improvement of Supercritical CO₂ Extraction of Bacterial Quinones from Activated Sludge

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Abstract
Extraction and identification of bacterial quinones is becoming increasingly important. The main purpose of this research is to investigate the possibility of extracting quinones from activated sludge using supercritical fluid extraction and also to investigate the effect of various polar solvents in order to improve the yield. A ± 0.1 g freeze-dried activated sludge sample was extracted at 35°C, 20 Mpa using supercritical carbon dioxide (scCO₂). This technique was able to extract quinones, yet with very low yield. In order to enhance the yield, performance of scCO₂ was improved by adding any modifiers to the fluid. Various polar solvents such as methanol, acetone, ethanol, and chloroform were investigated. By adding the modifiers, four ubiquinones and 12 menaquinones species were identified in 0.1 g dried activated sludge based on retention time and spectrum analysis. Among the tested various polar solvents, methanol showed to be the best modifier, based on the highest total quinone content extracted and the lowest dissimilarity index.

Keywords: supercritical fluid extraction, polar solvent, activated sludge, bacterial quinones.

Introduction
Microbial community structure is one of the important factors controlling the pollutant-degrading capacity of ecosystem. The capacity of an ecosystem to degrade organic compounds and its response to the changes in environmental conditions depend not only on the total population of microorganisms present, but also on the microbial community structure of that system (Hu et al., 1999). Microbial population dynamics can be analyzed using quinone profile.

Nowadays, the technique of using quinone profiles has gained increased recognition as a simple and useful tool to analyze microbial population dynamics in mixed cultures (Hu et al., 2001; Hedrick and White, 1986). In the conventional method, analysis of quinones is performed by organic solvent extraction (direct extraction) with chloroform–methanol mixture (Collins and Jones, 1981). Samples containing quinones are separated into a complex mixture of lipid and impurities. The impurities are removed by column chromatography, while microbial quinones are separated by thin layer chromatography (TLC). The compositions of purified fractions of ubiquinones and menaquinones are then analyzed by HPLC. Because satisfactory results could not be obtained using TLC, a solid-phase extraction method using Sep-Pak Plus Silica was employed for the purification and separation of quinones (Hu et al., 1999). However, the conventional method has still some disadvantages: time-consuming, tedious and the use of large quantities of various organic solvents, which have detrimental effects on the environment.
Thus, the development of a new method by employing environmentally benign solvent like scCO₂ is crucial and offers solutions to the above-mentioned disadvantages.

Quinones are lipid-soluble substances found in almost all species of organisms (Hiraishi, 1988). Bacterial quinones are located in cytoplasm covered by the rigid cell wall of bacteria. In order to extract quinones from the matrix, the cell wall must be ruptured previously. The bacterial cell may be subjected to extreme conditions which cause disruption. There are two treatments which strongly believed to be the cause of the cell wall disruption, freeze dryer in sample treatment and supercritical fluid CO₂ in main process. Researchers have investigated that freezing process has an effect on cell wall disruption due to the formation of expanding ice crystals. Souzu, H. (1980) has studied about the damage of Escherichia coli cell membrane caused by freezing treatment. Another process which considered to have a main effect in disrupting the cell wall is supercritical CO₂. At high pressure that generally used, supercritical CO₂ can penetrate and rupture the cell wall of bacterial cell. This disruption effect allowed us to sterilize and to extract intracellular products in certain process. Angela Dillow et al. (1999) from University of Minnesota have applied supercritical CO₂ to sterilization process while Polak et al. (1989) were able to extract lipids from algae (Skeletonema costatum) and succeeded to recover about 50% of the total lipids from the organism.

Supercritical fluid extraction (SFE) for analytical purposes has attracted considerable attention from researchers, especially in sample preparation techniques. SFE has shown the ability to compete with conventional organic solvent extraction method (Van der Velde et al., 1992) and usually takes a shorter time than conventional extraction because mass-transfer rates are higher in supercritical fluids than in liquid solvents. For environmental and analytical purposes, scCO₂ is a very suitable extraction fluid, because of its low critical temperature and pressure (Tc = 31.1°C, Pc = 7.4 MPa). Moreover, CO₂ is relatively non-toxic, inert, inexpensive and readily available. These favorable properties of CO₂ have caught our interest to investigate the feasibility of analyzing quinones in activated sludge by applying scCO₂ extraction. This investigation focused mainly on the effect of various solvents as modifier on total quinone contents and quinone profile.

Materials and methods

Activated sludge samples

Activated sludge used in this study was obtained from the aeration tank of domestic wastewater plant at Toyohashi University of Technology, Japan. Prior to quinone extraction experiments, activated sludge samples were dried in a vacuum-freeze dryer for 24 hours and then sieved to collect particles smaller than 500 μm for extraction. A 0.1 g freeze-dried sample was used in each experiment.

Supercritical fluid extraction

All experiments were performed using a SFE system (Jasco, Japan). The system is mainly equipped with two high-pressure pumps (SCF-201, Jasco, Japan), a back pressure regulator (880-81, Jasco, Japan), a 1 ml extraction vessel (Jasco, Japan) and an oven (GC A 353, GL Sciences Inc.) which controls the temperature of the extraction vessel. CO₂ with methanol as modifier was used as supercritical fluid. The operation condition of this experiment as follows: extraction vessel temperature 35°C, pressure 20 MPa, CO₂ flow-rate 2.7 ml/min, methanol flow-rate 0.3 ml/min. This operation was conducted for 15 minutes, during this operation extracted microbial quinones were trapped and collected on the Sep-Pak Plus Silica cartridges (Waters Co., Tokyo) joined in series. The collected quinones were eluted from the cartridges with pure acetone. Finally, prior to HPLC analysis, menaquinones and ubiquinones contained in the acetone were purified and separated using two other Sep-Pak Plus Silica cartridges.

Organic solvent extraction

Conventional analysis of quinones using organic solvent extraction method was also carried out to investigate for the reliability of the SFE method. The procedure reported by Hu et al.(1999) was used to extract ubiquinones and menaquinones from activated sludge. A similar...
0.1 g dried activated sludge sample in SFE method was also used in conventional analysis. 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.

Chromatographic analysis

The extracted microbial quinones were analyzed using HPLC (Shimadzu, Japan) equipped with ODS column (Zorbax-ODS, 4.6 mm I.D. x 250 mm, Agilent Technologies, USA) and two detectors, namely UV-Vis detector (Model SPD-10A, Shimadzu) and photodiode array detector (SPD-M10A, Shimadzu). The temperature of the column oven was maintained at 35°C. A mixture of methanol and isopropyl ether (9:2, v/v) was used as the mobile phase at a flow rate of 1.0 ml/min. The types of quinones were identified according to the retention time and the UV spectrum of each peak observed in UV detector. Ubiquinone 10 (UQ-10) were used as the quantitative standards. The wavelengths used to quantify quinones were 275 nm and 270 nm for ubiquinones and menaquinones, respectively.

Results and Discussion

Non-modified supercritical fluid extraction of bacterial quinones

The extraction of quinones from activated sludge was initially carried out by using pure supercritical carbon dioxide (scCO₂) without any modifier. This technique was able to extract quinones, yet with very low yield and small number of quinone types. The chromatograms of extracted quinones by using nonmodified supercritical CO₂ extraction were shown in Figure 1.

Without adding the modifiers, four ubiquinones (UQ-7, UQ-8, UQ-9 and UQ-10) and nine menaquinones species (MK-6, MK-7, MK-8, MK-8(H₂), MK-8(H₄), MK-9, MK-9(H₂), MK-9(H₄), and MK-10(H₄)) were identified in 0.1 g dried activated sludge. Several minor quinones, such as MK-10, MK-10(H₂) and MK-10(H₄), could not be extracted. This was likely due to the limited ability of pure CO₂ to dissolve polar compounds. It was necessary to add polar solvents to overcome the solubility limitation of pure CO₂ and to enhance the extraction yield of quinone from the activated sludge. Even though only low yield and small number of quinone species were able to be extracted, however this experimental results confirmed that there is a possibility to extract bacterial quinones from activated sludge by using supercritical carbondioxide extraction.

FIGURE 1: HPLC chromatograms of ubiquinones (above) and menaquinones (below) obtained by sc CO₂ extraction without modifier at pressure 25 MPa and temperature 35°C
Modified supercritical fluid extraction of bacterial quinones

Various polar solvents such as methanol, acetone, ethanol and chloroform were investigated in this study. These experiment also used condition operation which similar to the previous experiment. As a result, all of the minor quinones mentioned above were extractable by adding the modifiers to the scCO₂. Four species of ubiquinones and twelve species of menaquinones were observed in the activated sludge, with the dominant quinones being UQ-8, MK-9(H₂), MK-10(H₄), MK-6 and Q-10. The chromatograms of extracted quinones by using methanol modified supercritical CO₂ extraction were shown in Figure 2. By using methanol-modified scCO₂, the total quinone content increased by 750%, while quinone content obtained using other modifiers increased by 250–500%.

The role of modifier in scCO₂ has been investigated elsewhere (Jeong and Chesney, 1999). Addition of polar modifier enhances the ability of scCO₂ to extract quinones due to increased solubility and desorption of quinones from the activated sludge. Figure 3. shows that the use of different organic solvents as modifiers can yield very different total quinone content. The highest total quinone content was obtained by using methanol as modifier compared to acetone, ethanol and chloroform. This is most likely due to the fact that methanol has the highest polarity index among the tested solvents. According to Burdick and Jackson’s polarity index (Lee and Markides, 1990), the solvent’s polarity property in descending order is as follows: methanol, acetone, ethanol and chloroform. Table 1 are listed the most widely used modifier and their polarity index.

Diversity index

The value of diversity index equal to 11.99 for the method employing methanol-modified scCO₂ extraction was almost similar to that obtained by conventional method of 11.95, meaning that the two methods have similar microbial diversity value. Comparing with the conventional method, the values of dissimilarity of quinone profiles by SFE method using pure CO₂, and adding methanol, ethanol, acetone and chloroform as modifier are 0.27, 0.09, 0.10, 0.17 and 0.22, respectively.

The lowest value of dissimilarity, 0.09, was obtained between conventional method and methanol-modified scCO₂ extraction. This value means that the two quinone profiles could be considered similar to each other. 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.
Conclusions

The feasibility of applying supercritical CO$_2$ extraction for bacterial quinone extraction from activated sludge was studied. The experimental showed that the addition of modifier is required to increase the extracted total quinone content. The experimental result also showed that among the various modifiers tested in this study, methanol appeared to be the best modifier based on the highest yield and the value of diversity index.

TABLE 1: Frequently used modifiers in SFE and their polarity indexes.

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Polarity Index</th>
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<tbody>
<tr>
<td>Methanol</td>
<td>5.1</td>
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<tr>
<td>Acetone</td>
<td>5.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4.3</td>
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<tr>
<td>Chloroform</td>
<td>4.1</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>4.0</td>
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<tr>
<td>2-Propanol</td>
<td>3.9</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>3.5</td>
</tr>
<tr>
<td>Water</td>
<td>10.2</td>
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References


