

RAPID COMMUNICATION

A simultaneous microwave-assisted extraction and adsorbent treatment process under acidic conditions for recovery and separation of paclitaxel from plant cell cultures

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Abstract—We have developed a simultaneous microwave-assisted extraction and adsorbent treatment process under acidic conditions to increase the recovery and separation efficiency of the anticancer agent paclitaxel from plant cell culture. The simultaneous process under the conditions of extracting solution (90% aqueous methanol), pH 2.2, extraction time, 6 min, ratio of extracting solution to biomass, 1 : 1 (v/w), extraction temperature, 40 °C, adsorbent type, sylopute, and ratio of adsorbent to biomass, 0.08 : 1 (w/w), facilitated 1.97-fold higher recovery of paclitaxel in a shorter extraction time than the conventional solvent extraction process. In addition, biomass-derived tar compounds were successfully removed by the simultaneous process alone (average removal >97%). Using the simultaneous process, the paclitaxel extraction efficiency was improved, biomass-derived tar compounds were removed, and the purification process was simplified at the same time.

Keywords: Paclitaxel, Microwave-assisted Extraction, Adsorbent Treatment, Simultaneous Process, Acidic Condition

INTRODUCTION

Paclitaxel is a diterpenoid anticancer agent originally isolated from the bark of the Pacific yew tree [1]. It was approved by the US Food and Drug Administration for use as a treatment for ovarian and breast cancer, Kaposi's sarcoma, and non-small cell lung cancer [2]. Its application has also been expanded to the treatment of acute rheumatoid arthritis and Alzheimer's disease. Since clinical trials regarding the combined prescription of paclitaxel with various other treatments are underway, the demand for paclitaxel is expected to increase [3,4]. The main methods of paclitaxel production include direct extraction from the yew tree, semi-synthesis involving the chemical combination of side chains after obtaining a precursor from the leaves of the yew tree, and plant cell culture from the main bioreactor after inducing callus from the yew tree and performing a seed culture [5-7]. Among these methods, plant cell culture enables the stable mass production of paclitaxel of consistent quality in a bioreactor without being affected by such external factors as climate and environment [6].

To obtain paclitaxel from a plant cell culture, several separation and purification steps are required. In general, the separation and purification process includes extraction of paclitaxel from biomass (plant cells containing paclitaxel) as a raw material using an organic solvent and then pre-purification and final purification for manufacturing [3,4]. When recovering paclitaxel from plant cells (biomass) using an organic solvent, biomass-derived tar compounds are also extracted. These compounds have a negative influence on the separation/purification of paclitaxel. In particular, a large amount of organic solvent is consumed, the lifetime of the column pack-

ing material (resin) is reduced, and throughput is decreased in the HPLC purification process [2]. Therefore, biomass-derived tar compounds must be removed. Conventional solvent extraction (CSE) has drawbacks, such as a long extraction time, a large amount of organic solvent being required and low extraction efficiency. To make up for these drawbacks, many studies of microwave-assisted extraction (MAE) have recently been conducted [8]. In MAE, a microwave directly heats a solvent or a solvent-mixed material. In addition, direct interaction of a microwave with water molecules contained in biomass leads to tissue rupture and release of active ingredients into an organic solvent for higher extraction efficiency. Therefore, MAE, in comparison to CSE, has many advantages such as a short extraction time, a smaller amount of solvent being required, a high extraction rate and reduction of production costs [9]. The microwave-assisted extraction method is used for extraction of saikosaponin from *Bupleurum falcatum* root [10], extraction of camptothecin, an anticancer substance, from *Nothapodytes foetid* [11], extraction of glycosides from *Populus* bark [12], extraction of ginsenoside, one of the saponins, from ginseng root [13], extraction of glycyrrhizinate from dried licorice root [14], and extraction of polyphenol and caffeine from green tea leaves [15]. Our previous study [16] confirmed the possibility that the extraction of paclitaxel from plant cell cultures using MAE could overcome the aforementioned problems with CSE.

In 2011, Lee and Kim [17] developed a simultaneous MAE and adsorbent treatment process to extract paclitaxel from biomass and also effectively remove biomass-derived tar compounds to improve separation/purification efficiency (process simplification). According to Kim and Kim [18] in 2014, the pH of an extracting solution was reduced for acid hydrolysis of glycoside contained in biomass in order to increase paclitaxel recovery. In other words, when the pH of the extracting solution (90% aqueous methanol) was decreased to 2.2 (acidic conditions) using HCl in the CSE pro-

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cess, the recovery was 1.7-fold higher than with the CSE process without adjusting the pH (pH: 8.29) because the paclitaxel recovery was increased due to acid hydrolysis of glycoside contained in biomass.

In the present study, we first developed a simultaneous process where MAE with a high extraction efficiency is linked with adsorbent treatment under acidic conditions for the effective recovery and separation of paclitaxel from biomass. In other words, efficient extraction of paclitaxel from biomass by MAE, efficient removal of biomass-derived tar compounds by adsorbent treatment, and higher paclitaxel recovery under acidic conditions due to acid hydrolysis of glycoside (sugar-binding paclitaxel, 7-xylosyl paclitaxel) contained in biomass as well as cost reduction due to simplification of the separation/purification process were obtained. Furthermore, we suggest optimum strategies for the recovery of paclitaxel by investigating the effect of critical process parameters, such as the adsorbent used for the simultaneous process, the ratio between adsorbent and biomass and the ratio between extractant and biomass.

MATERIALS AND METHODS

1. Plant Materials

A suspension of plant cells originating from *Taxus chinensis* was cultured in a bioreactor [19]. Following cultivation, biomass (plant cell and debris) was recovered by using a decanter (CA150 Clarifying Decanter; Westfalia, Germany) and a high-speed centrifuge (BTPX 205GD-35CDEFP; Alfa Laval, Sweden). The biomass was provided by Samyang Genex Company, South Korea.

2. Paclitaxel Analysis

An HPLC system (SCL-10AVP; Shimadzu, Japan) and a Capcell Pak C18 column (250×4.6 mm, Shiseido) were used to analyze the paclitaxel contents. Acetonitrile/water (35:65-65:35, v/v gradient) was used as the mobile phase. Using a UV detector, paclitaxel was analyzed at 227 nm [20]. In addition, the flow rate and sample injection volume were 1.0 mL/min and 20 μ L, respectively. Authentic paclitaxel was purchased from Sigma-Aldrich (purity: 95%), and used as a standard. Each sample was analyzed in triplicate.

3. Quantitative Analysis of Tar Compounds

GC (GC-2014, Shimadzu, Japan), an HP-5 column (0.20 mm ID×25 m, 0.33- μ m film) and a flame ionization detector were used for quantitative analysis of tar compounds. The separation temperature within the column was programmed in increments of 5 °C/min from 50 °C to 250 °C. The carrier gas used was helium and it was analyzed at a flow rate of 1.0 mL/min. Authentic 2-picoline, o-xylene, 2,5-xyleneol, 1-methylnaphthalene, and acenaphthene, which are the major tar compounds in cells of *Taxus chinensis* [21], were purchased from Sigma-Aldrich (purity: 99%) and used as standards.

4. Conventional Solvent Extraction (CSE)

The biomass from plant cell cultures was mixed with methanol and stirred at a room temperature for 30 min. The mixture was filtered under vacuum in a Buchner funnel through filter paper (150 mm, Whatman), where the methanol was preferably added to biomass at a ratio of 1:1 (mL/g, v/w). Extraction was performed four times with new methanol [22]. Each methanol extract was collected, pooled, concentrated and dried at 40 °C under vacuum

(635 mm Hg) for HPLC analysis. The paclitaxel yield in CSE was defined as follows:

$$\text{Yield (\%)} = \left(\frac{\text{quantity of paclitaxel in extract}}{\text{quantity of paclitaxel in biomass}} \right) \times 100$$

5. Simultaneous MAE-adsorbent Treatment Process

To extract paclitaxel from biomass by simultaneous process, a microwave extraction device (2,450 MHz Model 1501, Korea Microwave Instrument Co., Korea) was used and the extraction temperature was consistently maintained by microwave generator. In the microwave extraction device, a thermocouple to measure extraction temperature, an agitator to properly mix biomass and an organic solvent and the cooling system to prevent evaporation were equipped. In our previous study [18], the optimal pH of the extractant, extraction time, extraction temperature, ratio of extractant to biomass, and extractant in MAE-acid hydrolysis were 2.2, 6 min, 40 °C, 1:1 (v/w), and 90% aqueous methanol, respectively. Sylopute, HP-20 and a mixture of sylopute and HP-20 (1:1, w/w), which are the most effective adsorbents for removing tar/waxy compounds under the optimal conditions and for the filtration rate after adsorbent treatment, were used for the simultaneous process [23]. In addition, the effect of the ratio between adsorbent and biomass (0.04:1, 0.06:1, 0.08:1, 0.10:1, 0.12:1, 0.14:1, w/w) used for extraction under the optimal conditions, and the ratio between extractant and biomass (1:1, 2:1, 3:1, 4:1, v/w) on extraction efficiency was investigated. After extraction, the filtrate of extracted paclitaxel was recovered by vacuum filtration with a filter paper (150 mm, Whatman). Extracted filtrate was concentrated in a concentrator (CCA-1100, EYELA, Japan) and dried in a vacuum oven (UP-2000, EYELA, Japan) for 24 h. Then, it was dissolved/filtered in methanol for analysis by HPLC. The paclitaxel yield in the simultaneous process was expressed as a relative value to the yield in the CSE (control) process.

RESULTS AND DISCUSSION

To investigate the effect of critical process parameters in the simultaneous MAE and adsorbent treatment process under acidic conditions, extraction was conducted under the conditions of extractant pH, 2.2, extraction number, 1, extraction time, 6 min, ratio between extractant and biomass, 1:1, v/w, and extraction temperature, 40 °C, as optimized in our previous study [18]. In addition, the extraction efficiency was compared based on the paclitaxel yield obtained by the CSE method (four extractions using methanol as the extractant).

In the simultaneous MAE and adsorbent treatment process under acidic conditions, the paclitaxel extraction efficiency and the trend in removal of biomass-derived tar compounds were investigated according to the type of adsorbent. Each adsorbent (sylopute, HP-20, sylopute+HP-20) was added at the optimal ratio between adsorbent and biomass (0.08:1, w/w) for the simultaneous process. As shown in Fig. 1(a), the paclitaxel recovery using sylopute, HP-20 and sylopute+HP-20 as the adsorbent was 197%, 120% and 153%, respectively. In the simultaneous process using sylopute as the adsorbent, the paclitaxel recovery was highest. In addition, it was confirmed that 2-picoline, o-xylene, 2,5-xyleneol, 1-methylnaphthalene and acenaphthene, which are biomass-derived tar compounds [21],

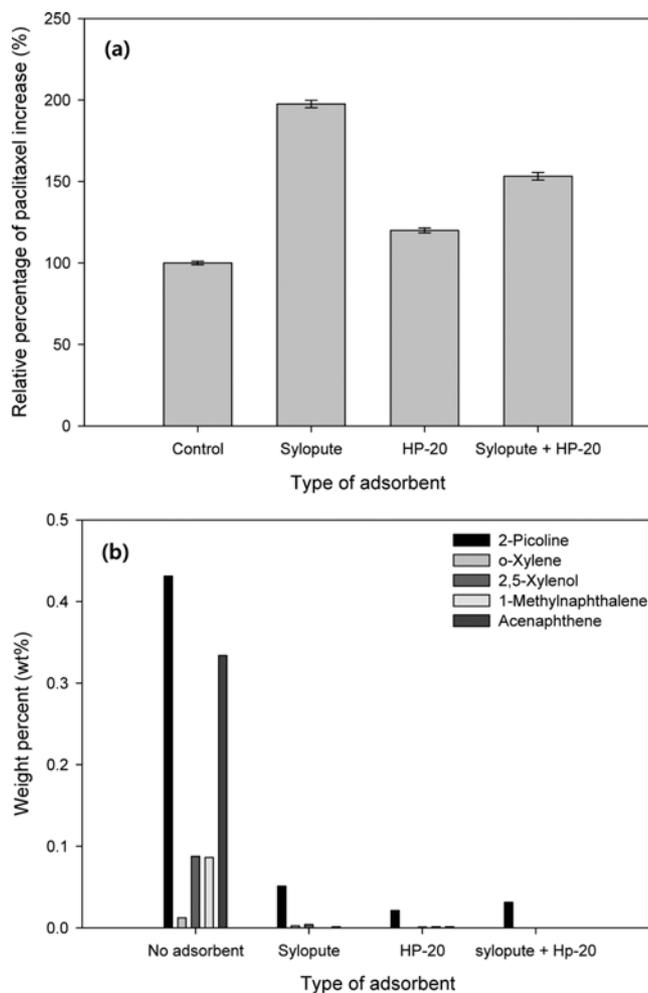


Fig. 1. Effect of adsorbent type on extraction efficiency (a) and removal of tar compounds (b). The pH of solvent, number of extractions, time, ratio of adsorbent to biomass, ratio of solvent to biomass, solvent type, and temperature were 2.2, 1, 6 min, 0.08 : 1 (w/w), 1 : 1 (v/w), 90% aqueous methanol, and 40 °C, respectively. The control represents the experimental result of CSE (four extractions using methanol as solvent) without acid hydrolysis under acidic conditions.

were eliminated by the simultaneous process. As shown in Fig. 1(b), in the simultaneous process with the addition of each adsorbent, most of the tar compounds were removed compared to the process without adsorbent (average removal >97%). Among the tar compounds, 2-picoline had a relatively low removal rate (~88%) but the 2-picoline remaining after the simultaneous process (<0.1 wt%) had little influence on the follow-up purification process (hexane precipitation) [24]. Therefore, biomass-derived tar compounds were successfully removed by the simultaneous process, and this result is comparable to the removal of tar compounds by the adsorbent treatment process alone. Furthermore, it is thought that the paclitaxel recovery in the simultaneous process under acidic conditions, compared to the CSE method, was increased because of acid hydrolysis of glycosidic bonds of glycoside (sugar-binding paclitaxel; 7-xylosyl paclitaxel) contained in biomass. This result can be confirmed by investigating changes in the content of 7-xylo-

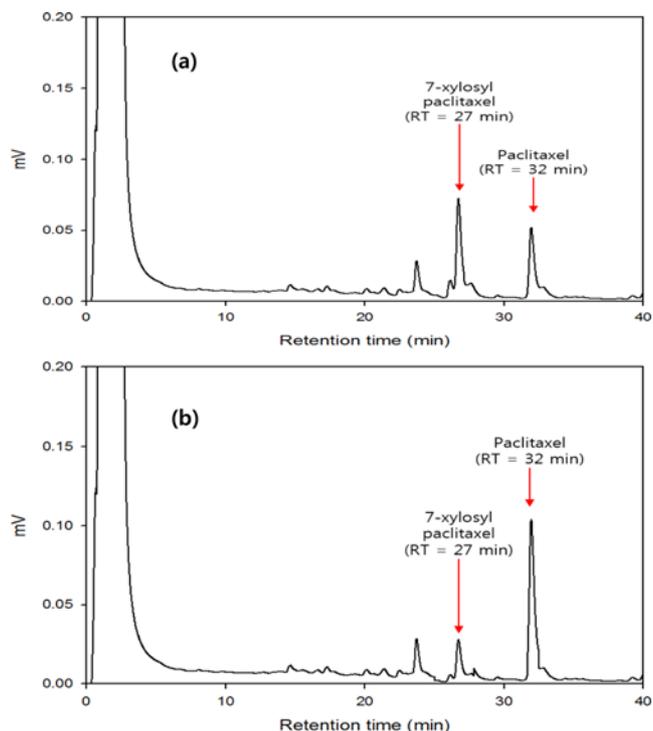


Fig. 2. Comparison of HPLC chromatograms of 7-xylosyl paclitaxel and paclitaxel in CSE method (control) (a) and simultaneous MAE/adsorbent treatment method under acidic conditions (b). 7-Xylosyl paclitaxel and paclitaxel were identified and confirmed by comparing their retention times with those of authentic compounds [paclitaxel from Sigma-Aldrich (purity: 95%) and 7-xylosyl paclitaxel from Quality Phytochemicals (purity: 99%)].

syl paclitaxel and paclitaxel in the biomass after CSE method (control) and simultaneous MAE/adsorbent treatment method under acidic conditions (Fig. 2). The content of 7-xylosyl paclitaxel in the biomass decreased after simultaneous MAE/adsorbent treatment method under acidic conditions, whereas the paclitaxel content increased. The increase of paclitaxel was perfectly proportional to the decrease of glycoside by HPLC analysis. Based on these results, sylopute was selected as the optimal adsorbent in the simultaneous process.

The ratio between adsorbent and biomass used for the simultaneous process was set at 0.04 : 1, 0.06 : 1, 0.08 : 1, 0.10 : 1, 0.12 : 1 and 0.14 : 1 (w/w) to investigate the extraction efficiency. The paclitaxel recovery (~197%) was almost consistent up to 0.08 : 1 (w/w) but the recovery was decreased as more adsorbent was added (Fig. 3(a)). In addition, as the amount of adsorbent was increased, the removal of tar compounds increased up to a ratio of 0.08 : 1 (w/w) above which it was consistent (Fig. 3(b)). This trend was comparable to the result of a previous study [17]. Thus, adding a larger amount of adsorbent caused adsorption/removal of more biomass-derived tar compounds, but it also caused adsorption/removal of paclitaxel, and therefore, the recovery dropped. The optimal ratio between adsorbent and biomass in the simultaneous process was thus determined to be 0.08 : 1 (w/w).

The extraction efficiency according to the ratio between extract-

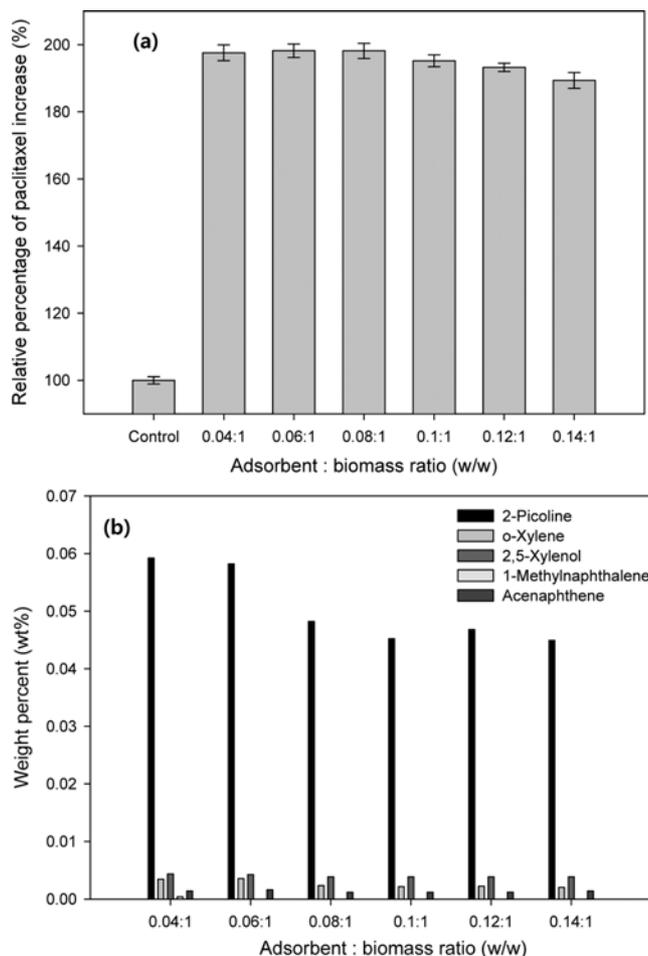


Fig. 3. Effect of ratio of adsorbent to biomass on extraction efficiency (a) and removal of tar compounds (b). The pH of solvent, type of adsorbent, number of extractions, time, ratio of solvent to biomass, solvent type, and temperature were 2.2, sylopute, 1, 6 min, 1 : 1 (v/w), 90% aqueous methanol, and 40 °C, respectively. The control represents the experimental result of CSE (four extractions using methanol as solvent) without acid hydrolysis under acidic conditions.

ant and biomass in the simultaneous process was investigated. The ratio between extractant and biomass was set at 1 : 1, 2 : 1, 3 : 1 and 4 : 1 (v/w). From the result of the simultaneous process, the paclitaxel recovery decreased as the ratio of extractant increased (Fig. 4(a)). Furthermore, as the ratio of extractant increased, a lower amount of tar compounds tended to be removed (Fig. 4(b)). This result was probably due to inadequate stirring of the solvent when the microwaves were applied to larger volumes [25]. Also, microwave energy was absorbed and dispersed by larger amounts of plant materials, an effect which is disadvantageous for the extraction process [26, 27]. Therefore, the optimal ratio between extractant and biomass in the simultaneous process was determined to be 1 : 1 (v/w).

From the result of investigation of the effect of critical process parameters on the extraction efficiency in the simultaneous process, the paclitaxel recovery (~197%) was highest under the conditions of extractant pH, 2.2, extraction number, 1, extraction time, 6 min, ratio between extractant and biomass, 1 : 1 (v/w), extraction

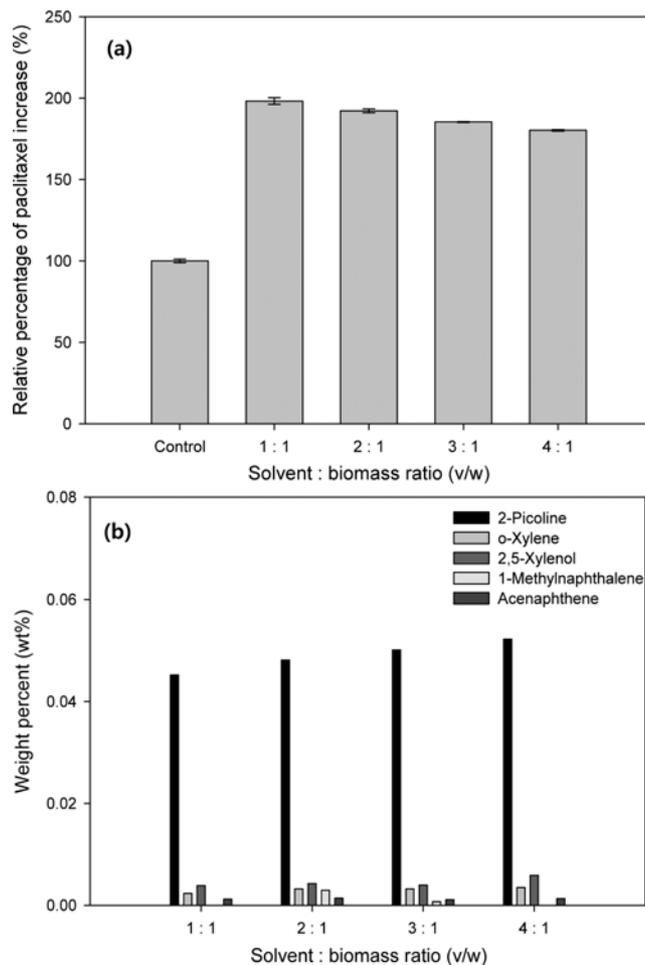


Fig. 4. Effect of ratio of solvent to biomass on extraction efficiency (a) and removal of tar compounds (b). The pH of solvent, type of adsorbent, number of extractions, time, ratio of adsorbent to biomass, solvent type, and temperature were 2.2, sylopute, 1, 6 min, 0.08 : 1 (w/w), 90% aqueous methanol, and 40 °C, respectively. The control represents the experimental result of CSE (four extractions using methanol as solvent) without acid hydrolysis under acidic conditions.

temperature, 40 °C, type of adsorbent, sylopute, and ratio between adsorbent and biomass, 0.08 : 1 (w/w). To determine the feasibility of the simultaneous process, crude extract obtained by hexane precipitation, which is the follow-up purification process in the existing process and simultaneous process, was compared (Fig. 5(a)). The purity of paclitaxel obtained through the simultaneous process and existing process was 25.6±0.5% and 26.2±0.4%, respectively, which are comparable. As shown in Fig. 5(b), most of the paclitaxel (~99%) was recovered from biomass by four extractions using methanol as the extractant in the existing process. When conducting hexane precipitation in the process, the overall recovery was approximately 90%. In contrast, the paclitaxel recovery was 197% in the simultaneous process with one extraction compared to CSE in the existing process. When conducting hexane precipitation in the follow-up process, the overall recovery was approximately 94%. Using the simultaneous process developed in the present study, the efficiency of paclitaxel extraction from biomass was im-

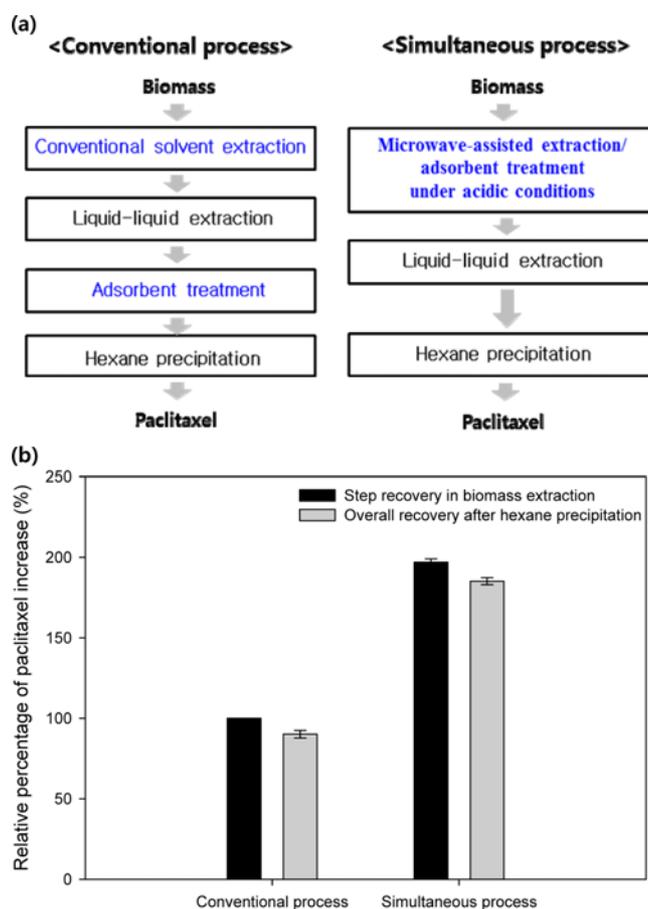


Fig. 5. Schematic diagram (a) and relative percentage of paclitaxel increase (b) of simultaneous process and conventional process for recovery and purification of paclitaxel.

proved by MAE, biomass-derived tar compounds were efficiently removed by adsorbent treatment and paclitaxel recovery was increased by acid hydrolysis of glycoside (sugar-binding paclitaxel, 7-xylosyl paclitaxel) contained in biomass. Furthermore, the existing paclitaxel separation/purification process could be simplified.

CONCLUSIONS

We have developed a simultaneous microwave-assisted extraction and adsorbent treatment process in order to increase the recovery and separation efficiency of the anticancer agent paclitaxel from plant cell culture under acidic conditions. In addition, we investigated the effect of critical process parameters in the simultaneous process, such as adsorbent type, ratio of adsorbent to biomass, and ratio of extractant to biomass. The simultaneous process under the conditions of extracting solution (90% aqueous methanol), pH 2.2, extraction time, 6 min, ratio of extracting solution to biomass, 1 : 1 (v/w), extraction temperature, 40 °C, type of adsorbent, sylopute, and ratio of adsorbent to biomass, 0.08 : 1 (w/w), facilitated 1.97-fold higher recovery of paclitaxel in a shorter extraction time than the existing process (CSE method). Biomass-derived tar compounds were successfully removed by the simultaneous process alone (average removal >97%), and this result is comparable to the rate of tar

compound removal in the adsorbent treatment process alone. From the result of hexane precipitation, which is a follow-up purification process, in the existing process and simultaneous process, the paclitaxel purity (25.6-26.2%) was similar, whereas the paclitaxel recovery increased more than two-fold using the latter. As a result, using the simultaneous process under acidic conditions, the paclitaxel recovery was improved by MAE (one extraction for 6 min), tar compounds were effectively removed by adsorbent treatment, and the paclitaxel recovery was increased by acid hydrolysis. Furthermore, it is expected that the production cost of paclitaxel would be reduced due to simplification of the paclitaxel separation/purification process.

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