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Preparation of crystal flakes of *p*-coumaric and ferulic acids from *Erianthus arundinaceus* culms and leaves as a side process of cellulosic-ethanol production

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Abstract

We developed a simple process for the preparation of crystal flakes of *p*-coumaric and ferulic acids from an extract obtained after the alkali pretreatment of *Erianthus arundinaceus* culms and leaves for the calcium-capturing-by-carbonation (CaCCO) process for cellulosic-ethanol production. These phenolic acids were moderately extracted by calcium hydroxide and purified by adsorption to the synthetic adsorbent SP700 with subsequent elution by 60% (v/v) ethanol solution. After concentrating the eluate by evaporation and allowing the resultant murky solution to settle, yellow crystal flakes mainly consisting of *p*-coumaric acid (82.0% of the total weight) and ferulic acid (7.4 % of the total weight) were obtained, corresponding to recoveries of 46.3 % and 13.1 %, respectively. Thus, we demonstrated a new side process for phenolic acid recovery by simple purification steps. The integration of this side process into the main cellulosic-ethanol production stream could not only save eluent (ethanol) but also enable the ethanol in the eluate to be reused with the aid of a distillation facility in the main stream, thereby strengthening the ability of the bio-refinery industry to produce cost-competitive valuables.

Key words: *p*-coumaric acid; ferulic acid; adsorption; cellulosic-ethanol; *Erianthus arundinaceus*

Introduction

Second-generation fuel ethanol (bioethanol) production using lignocellulosic biomass, such as agricultural residues and energy crops, is extremely important to avoid conflict with food supplies and provide a possible solution for global warming.[1)] However, it is becoming clear that the barriers (especially the high manufacturing costs) to its commercialization are greater than the initial, optimistic forecast. Recently, the USA down-regulated its target values for cellulosic ethanol productive capacity, indicating that the future for this technology will be challenging.[2) i)] Consequently, the recovery of high-value by-products from the feedstock during the process of bioethanol production has recently emerged as a research focus,[3)~5)] in addition

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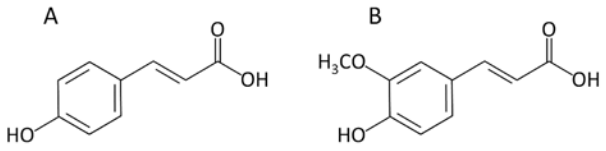

Fig.1. Structures of *p*-coumaric acid (A) and ferulic acid (B).

to other issues, such as enzyme cost reduction and process optimization for the feedstock.[6) 7)]

Phenolic acids, which occur in both free form and covalently linked forms to other components in plant cell walls, are considered to be valuable by-products.[8)] *p*-Coumaric (4-hydroxycinnamic) acid (Fig. 1A) and ferulic (4-hydroxy-3-methoxycinnamic) acid (Fig. 1B) are the major phenolic acids that exist in ester-linked and ether-linked forms.[9) 10)] These phenolic acids also exhibit beneficial physiological effects, such as antimicrobial, anti-thrombosis, and anticancer activities,[11) 12)] and ferulic acid has been extracted from rice bran and used as a food ingredient with antioxidant activity at a price of 10,000 yen/kg.[ii)] Some phenolic acids, especially those that are ester linked, are readily extracted by alkali hydrolysis; however, their separation and purification from alkaline hydrolysis solution remains problematic, although many methods have been tested.[13)~16)] The extraction and purification of these phenolic acids from lignocellulosic biomass has not yet been commercialized. Indeed, the process remains affected by problems, such as the low contents of these acids in herbaceous feedstock (no more than 2% of the dry weight, in our personal experience); the high cost of the extraction reagents (e.g., alkali and organic solvent); the generation of large amounts of wet, alkaline, fibrous residues after the phenolic-acid extraction process; and the unsatisfactory purity of the isolated product.[12) 16)] However, as part of an integrated fraction scheme, the extraction of these phenolic acids would be expected to be economically feasible.

To overcome the barriers to the commercialization of second-generation bioethanol production and simultaneously achieve efficient phenolic acid recovery from lignocellulosic feedstock, we proposed a new integrated process: the simultaneous recovery of by-products (SRB)-calcium-capturing-by-carbonation (CaCCO) process [17)]. A step for by-product recovery was integrated with the bioethanol production process (the CaCCO process), in which the $Ca(OH)_2$ treatment of a lignocellulosic feedstock serves not only as a pretreatment that promotes the enzymatic saccharification of polysaccharides but also alkaline hydrolysis to facilitate phenolic acid extraction. In our previous report, we confirmed that phenolic acids could be successfully extracted by $Ca(OH)_2$ pretreatment and recovered by washing the pretreated feedstock via the SRB-CaCCO process. However, a simple and economical procedure for the separation and purification of the extracted phenolic acids has not yet been developed.

In this study, we investigated the conditions necessary for the efficient recovery of phenolic acids as mixed flakes with a synthetic adsorbent. We also adopted ethanol (the main product of the SRB-CaCCO process) as the eluent for phenolic acid purification to decrease the cost of the solvent by producing it on-site and utilizing the distillation facility in the integrated bioethanol production system. Additionally, a simple crystallization method was developed to obtain mixed flakes of phenolic acids and recover the ethanol simultaneously. *Erianthus arundinaceus*, which is one of several main cellulosic energy crops in Japan, was used as the feedstock. It has a high dry matter production potential of approximately 40 tons/ha. Furthermore, it has a relatively long harvest period from winter to spring, which supports the establishment of an intensive and stable feedstock supply. [18)]

2. Materials and Methods

(1) Materials

An *E. arundinaceus* clone was grown in our experimental field in Koshi, Kumamoto, Japan, and harvested with a forage harvester (Kemper Champion 3000, Maschinenfabrik KEMPER GmbH & Co. KG, Stadtlohn, Germany) in April, 2012. The harvester cut the *E. arundinaceus* sample (culms and leaves) into 15-mm-long pieces, which were subsequently dried in a greenhouse to a moisture content of less than 100 g/kg dry biomass. The dried pieces were further chopped into 3-mm lengths with a cutter (Zac-750, Shincowa Co., Ltd., Mishima, Shizuoka, Japan), and the resultant chips were ground with a Willey mill (AP-1, Ikeda Scientific Co., Ltd., Chiyoda, Tokyo, Japan) to pass through a 500-μm mesh sieve. The ground sample was stored in a desiccator at room temperature for further experiments and component analysis.

(2) Extraction of ester-linked *p*-coumaric and ferulic acids

The ester-linked *p*-coumaric and ferulic acids in the ground sample were extracted for quantification using the modified method by Sun et al. [19] The ground sample (50 mg) was saponified with 5 mL of 1-M NaOH and 0.5 mg of $NaHSO_3$ (to prevent phenolic acid oxidation) in a 15-mL centrifuge tube with shaking (200 rpm) for 18 h at 30 °C. The suspension was subsequently centrifuged at 10,000 × g for 5 min. After centrifugation, an aliquot of the supernatant was sampled, and the amounts of *p*-coumaric and ferulic acids were determined as described in the Analytical methods section.

(3) Extraction of *p*-coumaric and ferulic acids by $Ca(OH)_2$ treatment

p-Coumaric and ferulic acids were extracted after $Ca(OH)_2$ treatment under the extraction conditions developed in our laboratory for the CaCCO process.[17] The ground *E. arundinaceus* sample (150 g) was mixed with $Ca(OH)_2$ (15 g) and distilled water (1,350 mL) in a 2-L Duran bottle (GLS-80, Duran Group GmbH, Wertheim, Germany), and the mixture was heated at 100 °C in an autoclave for 3 h to release the *p*-coumaric and ferulic acids in free forms. After the reaction, the mixture was cooled to room temperature and then centrifuged at 10,000 × g for 5 min. The supernatant (pretreatment liquor) was collected, and the precipitate was then washed with 750 mL of distilled water using a magnetic stirrer for 10 min. The washing mixture was centrifuged again at 10,000 × g for 5 min, and the supernatant was collected and mixed with the pretreatment liquor. The mixed solution (extraction solution) was placed into a brown polypropylene bottle (Monotaro Co. Ltd, Hyogo, Japan) to shield it from light and kept at 4 °C until use. The amounts of liberated *p*-coumaric and ferulic acids in the extraction solution were measured as described in the Analytical methods section.

(4) Separation of *p*-coumaric and ferulic acids

(ⅰ) Adsorbent conditioning

The synthetic adsorbent (SP700, Mitsubishi Chemical Corporation, Tokyo, Japan) was soaked in sufficient distilled water overnight at room temperature to ensure wetting of the adsorbent. Subsequently, the water was removed by decantation, and the adsorbent was washed with a sufficient amount of methanol for solvent exchange. Then, the adsorbent was soaked in methanol for 1 h at room temperature. Finally, the methanol was removed by decantation, and the adsorbent was thoroughly washed to exchange the solvent with distilled water.

(ⅱ) Adsorption/elution experiments

The extraction solution was acidified to pH 2 with 5-M HCl to generate an acidified extraction solution. The conditioned adsorbent (10 mL of wet volume, corresponding to 2.25 g of dry adsorbent) and the acidified extraction solution (250 mL) were added into a 500-mL brown polypropylene bottle, and the mixture was incubated at 30 °C with shaking at 200 rpm for 1 h. This adsorbent was then used as a carrier resin for chromatography. After incubation, the adsorbent was packed into a chromatography column (Econo-Pac, 1.5 × 12 cm, Bio-Rad, California, USA) that was wrapped with aluminum foil to shield it from light. After packing, the column was washed with 5 column-bed volumes (50 mL) of distilled water at a flow rate of 1 mL/min at room temperature to remove unbound *p*-coumaric and ferulic acids, and 10 mL of eluent was collected as one fraction. Subsequently, the bound *p*-coumaric and ferulic acids were extracted by two elution methods: (1) The linear gradient elution method was designed using an ethanol solution (50 mL) with a gradually increasing ethanol concentration from 0 to 100 % (v/v) followed by washing with 100 % ethanol (30 mL) at a flow rate of 1 mL/min. A smooth gradient was achieved with the help of a gradient maker (BioLogic LP, Bio-Rad Laboratories Japan, Tokyo, Japan). Fractions were collected every two minutes, and the concentrations of *p*-coumaric and ferulic acids, total fatty acids, acetic acid, and lactic acid were estimated. (2) The isocratic elution method for the phenolic acids was designed using an ethanol solution (100 mL) with an ethanol concentration of 30, 40, 50, 60, 70, 80, or 90 % (v/v) at a flow rate of 1 mL/min. The eluate was collected every 10 min, and the concentrations of *p*-coumaric and ferulic acids in each fraction were determined as described in the Analytical methods section. These values were then applied to calculate the elution efficiency using the equation shown below.

The elution efficiency of *p*-coumaric (or ferulic) acid (%) = the amount of *p*-coumaric (or ferulic) acid in the eluate / the total amount of bound *p*-coumaric (or ferulic) acid on the adsorbent × 100.

(5) Phenolic acid crystallization

The adsorbent in the aluminum foil-wrapped chromatography column described in Materials and Methods section (4)-(ii) was washed with 5 column-bed volumes (50 mL) of distilled water, 3 column-bed volumes (30 mL) of 60 % ethanol, and 5 column-bed volumes (50 mL) of 90% ethanol in sequence at a flow rate of 1 mL/min at room temperature. After 60% ethanol was applied for elution, the eluate was collected and subsequently evaporated under vacuum at 40 °C by a rotary evaporator (EYELA N1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The evaporation was stopped when the clear eluate became murky. Then, the murky solution (30 mL) was transferred into a clean brown glass bottle. This solution was allowed to settle at room temperature, and aliquots (9.3 mL) of the liquid on the top were sampled after 2, 4, 6, 8, 12, and 24 h. The *p*-coumaric and ferulic acid content of these aliquots were analyzed as described in the Analytical methods section. Finally, the crystals precipitated from the solution were recovered by filtration using filter paper (Advantec No. 2, ADVANTEC MFS, INC., California, USA) and dried in a desiccator at room temperature.

(6) Analytical methods

p-Coumaric and ferulic acids were determined by ultra-performance liquid chromatography (UPLC, Acquity TM, Nihon Waters K.K., Tokyo, Japan) equipped with a tunable UV (TUV) detector and a photodiode array (PDA) detector (Nihon Waters K.K.). The mobile phase consisted of 50 % (v/v) acetonitrile with 0.02 % (v/v) H2SO4 (solution A) and 0.08-mM NaOH (solution B). The separation of *p*-coumaric acid from ferulic acid was performed on an Acquity UPLC BEH C18 column (2.1×100 mm, 1.7 mm, Nihon Waters K.K.) at 40 °C at a flow rate of 0.45 mL/min with a linear gradient as follows: from 10 % to 80 % A within 5 min, then back to 10 % A within 2 min, and isocratic at 10 % A for 3 min. Total fatty acids were determined by an NEFA C test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Acetic acid and lactic acid were quantified using a high-performance liquid chromatography (HPLC) system (SPD-20A, Shimadzu Corporation, Kyoto, Japan) equipped with a Aminex HPX-87H column (300×7.8 mm, Bio-Rad Laboratories Japan) with a mobile phase of 5-mmol/L H2SO4 and a flow rate of 0.6 mL/min at 50 °C.

(7) Statistical analysis

Analysis of variance (ANOVA) was conducted using Microsoft Excel (Microsoft Japan Co., Ltd., Tokyo, Japan), and the level of significance was $P<0.05$.

3. Results and Discussion

(1) Extraction of phenolic acids by $Ca(OH)_2$ treatment

The ester-linked *p*-coumaric and ferulic acids in the ground *E. arundinaceus* sample accounted for 17.8 g/kg and 4.6 g/kg dry weight biomass, respectively, according to the NaOH hydrolysis experiment. In contrast, $Ca(OH)_2$ hydrolysis at 100 °C for 3 h liberated only 12.6 g of *p*-coumaric acid and 4.1 g of ferulic acid from 1 kg of dry biomass. These lower recoveries may be attributed to the poor solubility of $Ca(OH)_2$ in water (1.73 g/L at 20 °C and 0.77 g/L at 100 °C).[20) 21)] In the SRB-CaCCO process, for which a phenolic-acid purification step is developed in this study, the main stream is bioethanol production from biomass (Fig. 2). Therefore, the severity of the $Ca(OH)_2$ treatment of the biomass should be optimized based not only on the recovery of phenolic acids but also on the efficiency of the subsequent enzymatic saccharification step and the costs of $Ca(OH)_2$, heat energy, and equipment. In our previous study, in which rice straw was used as feedstock, we confirmed that ferulic acid is rapidly liberated after a pretreatment time of 1 h but partially decomposes after prolonged reaction at 100 °C.[17)] In contrast, p-coumaric acid exhibits better heat stability and a lower liberation velocity. The same trends were observed for *E. arundinaceus*, although the cell walls were more recalcitrant to lime pretreatment for fermentable sugar recovery, indicating that more severe pretreatment conditions can be adapted with minimum loss of ferulic acid. Finally, we set the pretreatment/extraction conditions for E. arundinaceus as 100 °C for 3 h after considering the efficiencies of both treatments (data not shown).

After $Ca(OH)_2$ treatment of the ground *E. arundinaceus* sample, 90% of the total alkaline-extracted *p*-coumaric and ferulic acids were recovered by washing the precipitate from the extraction solution. The concentrations of *p*-coumaric acid and ferulic acid in the extraction solution were 1.02 and 0.31 g/L, respectively. Additionally, some carboxy group-containing compounds, such as acetic acid (3.75 g/L), lactic acid (0.90 g/L), and unknown fatty acid(s) (0.2 mEq/L),

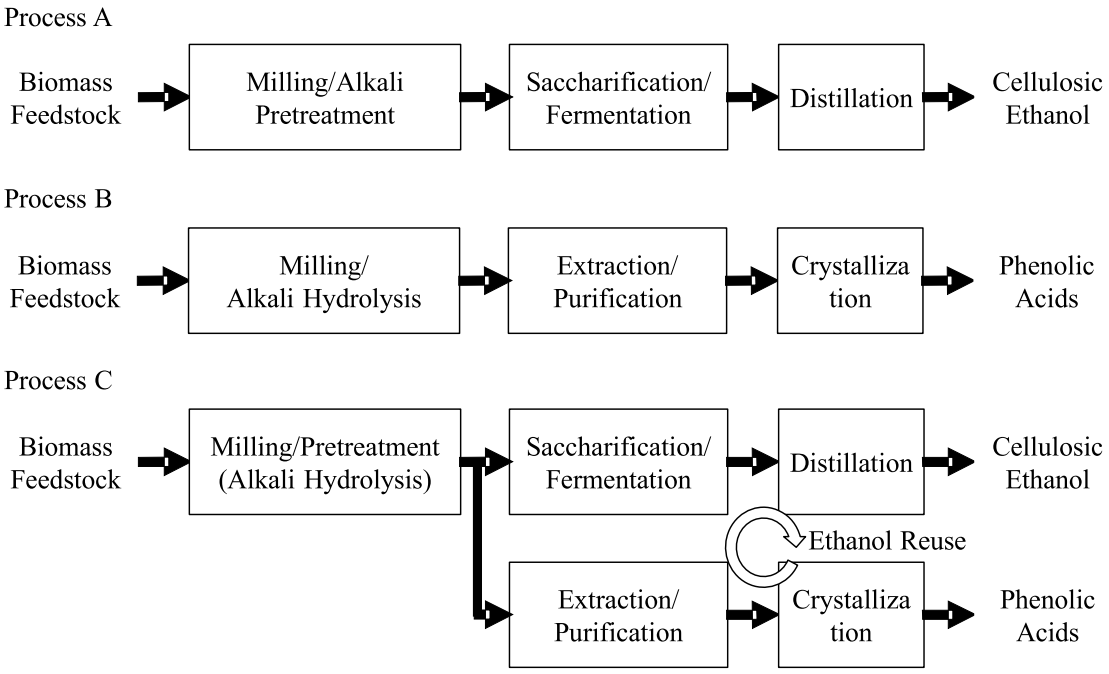

Fig.2. Flow charts of three biomass utilization processes.

Process A is mainly used to produce cellulosic ethanol by the CaCCO process. Process B is used to obtain phenolic acids by chemical extraction. Process C (i.e., the SRB-CaCCO process) is the integration of processes A and B and is used for the simultaneous recovery of cellulosic ethanol and phenolic acids.

were also detected in the extracted solution.

(2) Conditions for phenolic acid adsorption and elution

Several types of adsorbents, including activated charcoal, ion exchange resin, and synthetic adsorbent, were preliminarily evaluated for the purification of *p*-coumaric and ferulic acids from alkaline extracted solution. SP700 showed the highest adsorption capacity and has been shown to have excellent handling properties for the purification of organic compounds mainly via hydrophobic interactions.[22) 23)] In addition, the use of ethanol to elute the phenolic acids from SP700 is desirable for rapid evaporation/concentration without desalting and efficient crystallization because this solvent is more non-polar than water. Therefore, the adsorption of each phenolic acid should be ideal when both the phenol moiety and carboxy group in the molecule are protonated. The extraction solution was acidified to pH 2s, which is lower than the pK_{a1} values of both *p*-coumaric acid (pK_{a1}=4.51) and ferulic acid (pK_{a1}=4.72).[24)] In our preliminary experiments, the adsorption capacities of SP700 for *p*-coumaric and ferulic acids in the acidified extraction solution were 121 g/kg dry adsorbent and 51.0 g/kg dry adsorbent in 1 h, respectively.

Fig. 3 illustrates the elution patterns of the adsorbed components (*p*-coumaric acid, ferulic acid, acetic acid, lactic acid, and total fatty acids) in the acidified extraction solution from SP700 by the linear gradient elution method. Acetic acid and lactic acid did not interact with the adsorbent. In contrast, most of the *p*-coumaric and ferulic acids (85.7 % and 83.2 % of the total quantities, respectively) and 68.0 % of the total fatty acids in the acidified extraction solution adsorbed to SP700, and the phenolic acids started to desorb from the adsorbent when the ethanol concentration reached 30 % (v/v). The fatty acid elution peak appeared when the ethanol concentration exceeded 80 % (v/v). Under the tested conditions, 54.8 % of the adsorbed fatty acids were released into the eluent, and all of the phenolic acids were recovered from SP700. The elution patterns of *p*-coumaric and ferulic acids were quite similar, reflecting the difficulty of separating these two acids with this purification step.

Next, the isocratic elution method was adopted to investigate the sharpness of the elution of the phenolic acids from the SP700 onto which the acidified extraction solution was loaded. The influence of the ethanol concentration

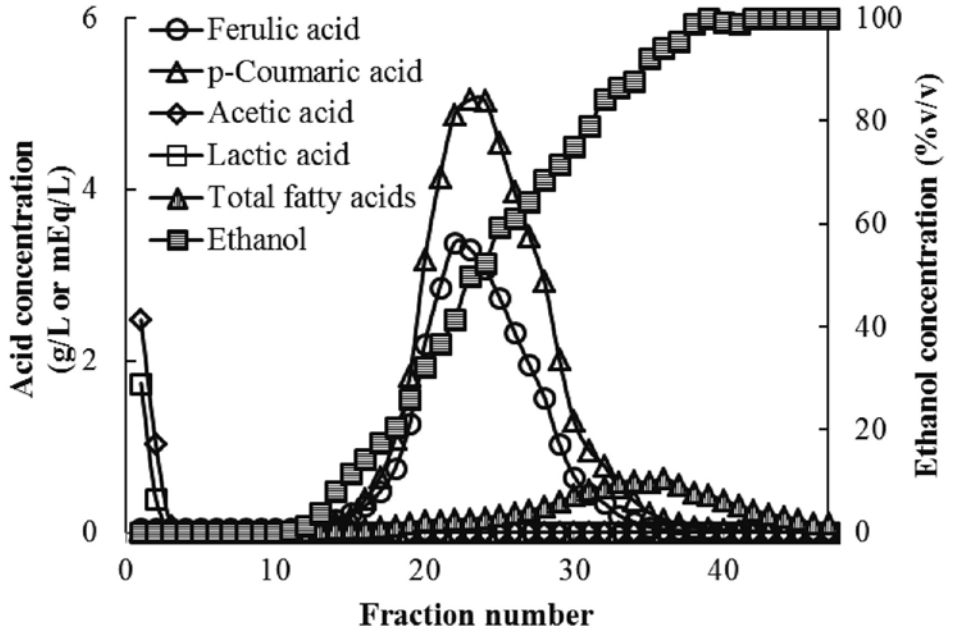

Fig.3. Elution patterns of *p*-coumaric acid, ferulic acid, and other compounds adsorbed on SP700 by a linear gradient of ethanol solution.
The concentrations of *p*-coumaric acid, ferulic acid, acetic acid, and lactic acid: g/L; the concentration of total fatty acids: mEq/L (oleic acid was used as the standard)

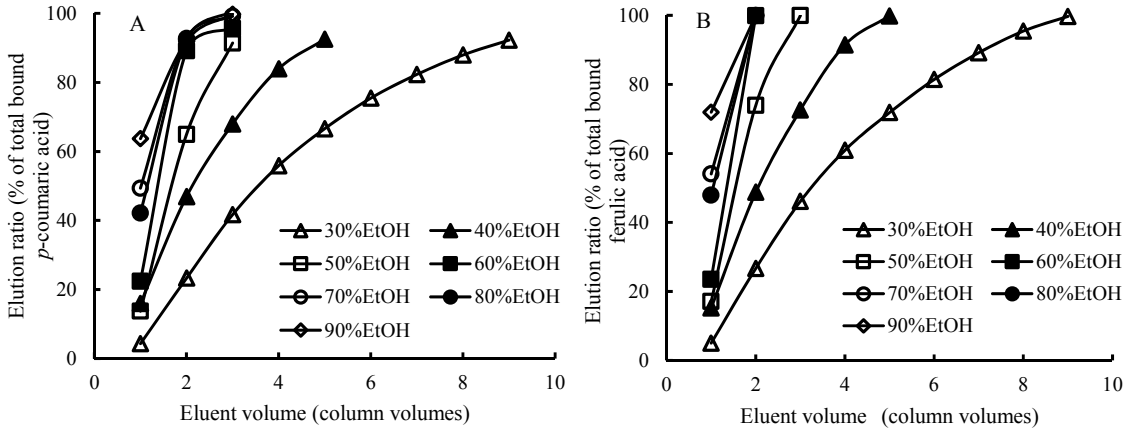

Fig.4. Effect of ethanol concentration (v/v) on the elution of *p*-coumaric acid and ferulic acid from SP700.

on the desorption patterns of *p*-coumaric acid and ferulic acid from the adsorbent is shown in Figs. 4A and 4B, respectively. The elution of both acids became more rapid as the ethanol concentration increased, and when the ethanol concentration exceeded 60 % (v/v), almost all of the bound acids were recovered using 3 column-bed volumes of eluent. To minimize the eluent volume, ethanol usage, and elution time, isocratic elution with 60% ethanol solution was adopted for the recrystallization experiment. In this test, the concentrations of *p*-coumaric acid and ferulic acid in the eluate increased by 5-fold and 8-fold, respectively, compared to those in the acidified extraction solution. SP700 purification recovered 85.5 % of the *p*-coumaric acid and 83.2 % of the ferulic acid in the acidified extraction solution.

(3) Phenolic acid crystallization

After purification by SP700, *p*-coumaric and ferulic acids in the eluate were crystallized based on their lower solubility in water than in ethanol solution. Buranov and Mazza reported that the solubility of ferulic acid is highly dependent on the ethanol concentration and that this compound is nearly insoluble in 30 % (v/v) ethanol solution.[11] At the end of the evaporation of ethanol from the eluate, the ethanol concentration decreased to 0.2% (v/v), and the concentrations of *p*-coumaric and ferulic acids reached 13.7 g/L and 4.6 g/L, respectively. After transferring the murky solution obtained in the evaporation step to a glass bottle, yellow crystals started to precipitate out of the solution. The concentration of *p*-coumaric acid in the murky solution rapidly decreased in the first 4 h of crystallization ($P < 0.05$), and 60 % of the total *p*-coumaric acid precipitated after 24 h (Fig. 5). In contrast, only 15 % of the total ferulic acid in the solution crystallized within 24 h. The different behaviors of the two acids may be attributable to their different initial concentrations in the solution; the final concentrations after 24 h of crystallization were very similar: 4 g/L. To maximize the yield of high-purity crystals, the crystallization process was allowed to proceed for 6 h. After filtrating and drying the precipitates, slightly brown crystals were obtained. The recoveries from the acidified extraction solution were 46.3 % for *p*-coumaric acid and 13.1 % for ferulic acid, and the purities reached 82.0 % and 7.4 % of the total weights of the crystals, respectively. The residual solution, which contained approximately 8-g/L phenolic acids, could be re-concentrated for recrystallization to increase the yields (data not shown).

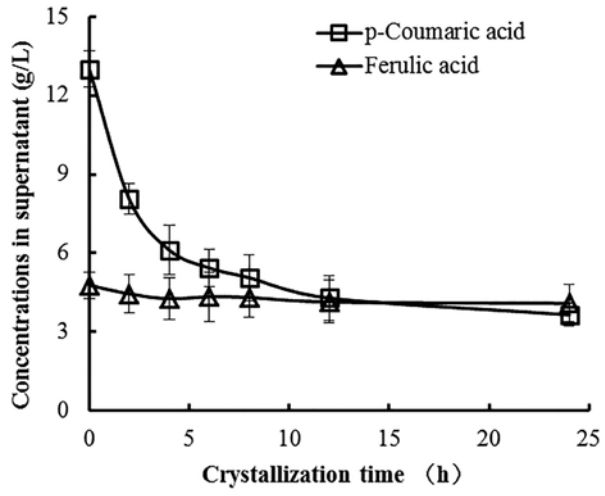

Fig.5. Changes of *p*-coumaric acid and ferulic acid concentrations in the supernatant during the crystallization process.

This simple purification method provides crystals of a mixture of *p*-coumaric and ferulic acids. However, whether the co-crystallization of *p*-coumaric acid and ferulic acid occurs remains unclear. Although the moderate purity and mixed form of these acids would restrict their direct application in industry, the recovery of these compounds as crystals is desirable for concentration, stabilization, storage, and transportation. Depending on the intended applications of these phenolic acids, the necessity of and detailed processes for further purification would likely differ. For example, Salgado et al. used a crude product of ferulic acid and *p*-coumaric acid extracted from agro-industrial waste to produce styrene derivatives via microorganisms.[16] Additionally, hydrophobic adsorption chromatography can be used to separate and purify these two phenolic acids,[13) 25] which would facilitate their application as individual phenolic acids based on their unique functionalities.

The purification process of *p*-coumaric and ferulic acids can be integrated as a side process in the CaCCO process for the conversion of herbaceous feedstock to cellulosic bioethanol.[17] Assuming that 1 L of bioethanol can be prepared from 4 kg of dry *E. arundinaceus* culms and leaves, 24 g of phenolic acid flakes could be simultaneously obtained as a by-product. In addition to phenolic acids, plant culms and leaves contain valuable natural compounds, such as unique lipids, bio-functional carbohydrates, and various secondary metabolites.[26] In the course of maximizing the feedstock value via the bio-refinery approach, the side process described in this study could be further adapted for the extraction and purification of valuables other than phenolic acids, thereby strengthening the cost-competitiveness of the main process.[27]

4. Conclusion

In this study, we developed a simple process for preparing crystal flakes of phenolic acids. Integrating this process into the CaCCO process is expected to save costs for feedstock, reagents, and equipment for conversion and to strengthen the whole process by producing multiple valuables instead of a single valuable, bioethanol. The flakes of phenolic acids possess fundamental value as

an environmentally friendly, natural product, and the commercial value of these flakes will vary based on their purities and the prices of the compounds. Therefore, this side process should be further improved by increasing the purity, recovery, and production efficiency.

Acknowledgements

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セルロース系エタノール製造時の副工程としての*Erianthus arundinaceus* 茎葉部からの*p*-クマル酸及びフェルラ酸の結晶粉末の調整

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要　旨

CaCCO (Calcium Capturing by Carbonation)法を用いたエリアンサス茎葉からのエタノール製造プロセスにおいて、副生するフェノール酸（*p*－クマル酸及びフェルラ酸）を沈殿固形物として回収するための工程を検討した。CaCCO法での水酸化カルシウム前処理時に可溶化した両フェノール酸は、合成吸着剤に吸着後、60 % (v/v)エタノールにより脱離した。溶離液を回収・濃縮した後、室温に6時間静置し再結晶させることで淡黄色の沈殿物が析出した。本沈殿物の中の*p*－クマル酸とフェルラ酸の含有量は、全沈殿物重量に対してそれぞれ82.0 %と7.4 %であり、回収効率はそれぞれ46.3 % と13.1 %であった。このように、エタノール製造工程において可溶化する副産物を簡素な工程で回収・精製できることを実証した。それに加えて、本工程では、主産物となるエタノールをフェノール酸精製時の溶離液及び溶媒として使用し、エタノール精製工程を併用してこれを効率的に再生することで、バイオリファイナリー工程全体の最適化が可能となるものと期待される。