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# Production of Bioethanol with Simultaneous Extraction of Flavonoids from *Chamaecyparis obtusa*

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A new strategy was developed for producing bioethanol from the residue of *Chamaecyparis obtusa* with simultaneously extraction of flavonoids from *Chamaecyparis obtusa*. By optimizing the extraction conditions, it is concluded that the highest amount of flavonoids in the extract was obtained through using methanol with heating method (60 ºC) at a solid/solvent ratio of 1:10 (g/mL) for 90 min. The total extracted amount of quercetin, myricetin and amentoflavone were 3.57, 0.05 and 0.9806 mg/g, respectively. The maximum ethanol obtained from the *Chamaecyparis obtusa* waste (1 g) was 0.14 g/g after 24 h of fermentation. This study exhibits an approach to make full use of plants.

**Key Words: *Chamaecyparis obtusa*, Flavonoids, Bioethanol, Saccharification, Fermentation.**

## INTRODUCTION

Fossil fuel depletion is great concern as the world population expands and the demand for basic human needs has increased rapidly[1]. Bioethanol is an attractive alternative to gasoline and it is produced from renewable biomass, such as sugar and starch materials[2-4]. The development of ethanol as a fuel, beyond its current role as a fuel oxygenate, will require the development of biomass as a feedstock because of its relative abundance and low cost[5,6]. In this case, leaves were considered as a potential source for production of bioethanol.

*Chamaecyparis obtusa* (*C. obtusa*) is a member of the genus Chamaecyparis and is distributed mainly in Korea, Japan and Taiwan. Essential oils and flavonoids with multiple biological activities, such as antibacterial[7], antifungal[8], antimite[9], antitermite[10] and acaricidal activities[11] have been extracted from the leaves of *C. obtusa*. Quercetin, myricetin and amentoflavone are the main flavonoids in *C. obtusa* (Fig. 1), which have antioxidant and anticancer activity[12]. When these bioactive compounds were extracted, the residue of *C. obtusa* can be reused as the source of fermentation for producing bioethanol.

In order to produce bioehanol from leaves of *C. obtusa*, the bioactive compounds, especially for flavonoids, should be extracted due to their inhibition of yeast. Moreover, the extracted flavonoids were quite valuable for treating certain diseases[12]. Extraction of flavonoids from leaves, is an important industrial process that can be done by a number of methods,

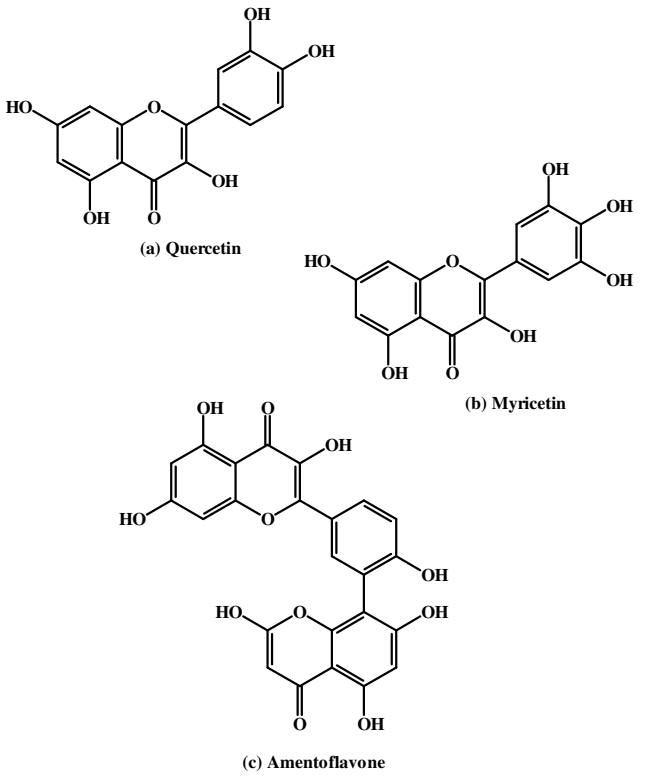


Fig. 1. Chemical structures of quercetin (a), myricetin (b) and amentoflavone (c)

such as solid-liquid[13,14] and supercritical carbon dioxide extraction[15]. For the reason of cost, solid-liquid extraction has been preferred. Solid-liquid extraction allows soluble components to be removed from solids using solvents. Insoluble material can be separated by gravity or vacuum filtration. After extraction of flavonoids, an extensive process (pretreatment, saccharification, fermentation) was employed. Pretreatment is essential for making fermentable sugars available for the fermentation process. On the other hand, the pretreatment process contributes significantly to the production cost of bioethanol highlighting the need to determine, a more economical pretreatment protocol[16]. Then the pretreated material was fermented by *Saccharomyces cerevisiae* according to reference[17,18].

By previous considerations, this study focuses on extraction of flavonoids and pretreatment process. Different solvents were used to extract the leaves of *C. obtusa* with different time and temperature. Dipping, heating and ultrasonic methods were also involved. Alkaline was then applied for pretreatment in different concentrations. After optimizing these, the residue of leaves was fermented with variable conditions.

## EXPERIMENTAL

*Chamaecyparis obtusa* was collected from market (Jangsung, Korea). Quercetin, myricetin, amentoflavone and trifluoroacetic acid were purchased from Sigma (St Louis, MO, USA). Methanol, ethanol, acetonitrile, acetone, *n*-hexane and ethyl acetate were supplied by Duksan Pure Chemical Co., Ltd. (Ansan, Korea). All reagents were of high performance liquid chromatography (HPLC) or analytical grade. Double distilled water was filtered using a vacuum pump (Division of Millipore, Waters, USA) and filter (HA-0.45, Division of Millipore, Waters, USA) prior to use. All samples were filtered (MFS-25, 0.2 μm TF, Whatman, USA) before being injected into the HPLC system. All glassware was washed with deionized water and acetone and dried at room temperature.

**Extraction of *C. obtusa*:** In a round bottomed flask, 10.0 g dry *C. obtusa* was dissolved in 100.0 mL of one of six solvents (acetone, ethanol, ethyl acetate, methanol, *n*-hexane and water). Different extraction methods, dipping, heating and ultrasonic extraction methods, were also investigated. After extraction, the extracts were filtered and the remaining *C. obtusa* waste was oven dried.

**Optimization of dilute alkaline pretreatment:** The alkaline level was optimized by performing the extractions at sodium hydroxide concentrations (1-3 M) with a 10.0 % (w/v) solid content. The pH of the pretreated *C. obtusa* was adjusted to 5 using concentrated hydrochloric acid before simultaneous saccharification and fermentation.

**Simultaneous saccharification and fermentation:** The simultaneous saccharification and fermentation of enzymatic hydrolyzates was carried out separately in an *in situ* fermentor. After pretreatment, *C. obtusa* was used as the hydrolyzate substrate. The medium was prepared by dissolving yeast (*Saccharomyces cerevisiae*) in solution and kept at 72 h under dipping conditions. The samples were withdrawn at 12 h intervals and filtered using a centrifuge.

**Analytical methods:** All pretreatments were carried out in an oven. The HPLC system consisted of a Waters 600s Multi solvent Delivery System, Waters 616 liquid chromatograph (Waters Associates, Milford, MA, USA), Rheodyne injector (20 μL sample loop) and variable wavelength 2487 UV dual channel detector. Autochro-3000 software (Younglin Co. Ltd., Korea) was used for data acquisition. HPLC was performed using a commercial $C_{18}$ column (4.6 mm × 250 mm, 5 μm) from RStech Co. (Daejeon, Korea). The mobile phase, flow-rate, UV wavelength and injection volume was acetonitrile/water/trifluoroacetic acid (35/65/0.01, v/v/v), 0.5 mL $min^{-1}$, 372 nm and 5 μL, respectively. The concentration of bioethanol produced after fermentation was analyzed by gas chromatography (GC) (Model YL-6100GC, Younglin, Korea). The GC consisted of an manual sampler, thermal conductivity detector (TCD) and DB-1701 column, 30 m × 0.32 mm × 1.00 μm ID. The injector, detector and oven temperatures were 250, 40 and 100 ºC, respectively. Nitrogen was used as the carrier gas. The bioethanol concentration was quantified using a calibration curve prepared by injecting different concentrations of ethanol standard (0.1-10 %, v/v).

## RESULTS AND DISCUSSION

**Optimization of extraction process:** Different extraction methods, such as heating extraction, dipping extraction and ultrasonic extraction were investigated by extracting 10.0 g of dry *C. obtusa* with 100.0 mL of methanol. In heating (stirring at 70 ºC), dipping (stirring) and ultrasonic (75 W) extraction, the powder was extracted with the solvent for 1 h. Comparing the results of these three methods, it was found that the extracted amount of flavonoids *via* heating method was the highest (Fig. 2). Thus, heating method was selected for subsequent experiments.

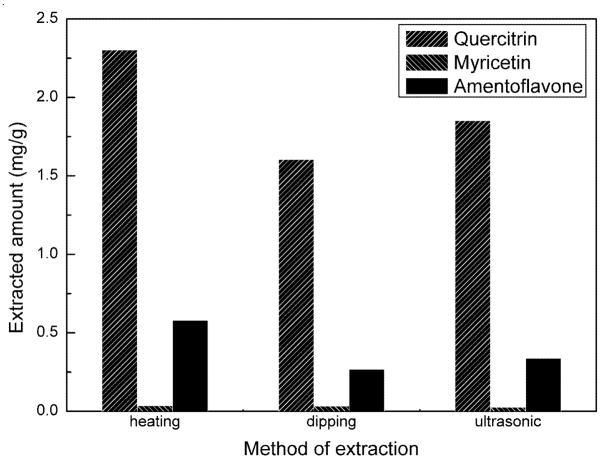


Fig. 2. Effects of different extraction method on the extraction amount of quercetin, myricetin and amentoflavone

The solvents selection was carried out using acetone, ethanol, ethyl acetate, methanol, *n*-hexane and water. 100.0 mL of solvent was used to extract 10.0 g dry *C. obtusa* for 1 h at 70 ºC. As shown in Fig. 3, methanol was proved to be the best solvent for extracting flavonoids from *C. obtusa* and, therefore, it was selected. The reason may be related to solvent polarity and the solubility of flavonoids.

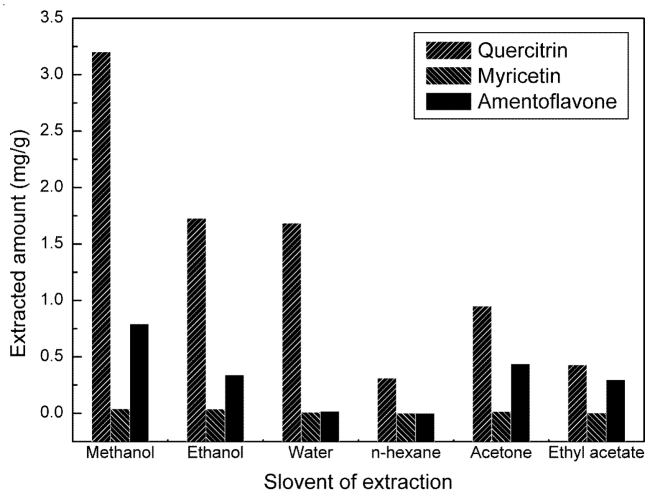


Fig. 3. Effects of different extraction solvent on the extraction amount of quercetin, myricetin and amentoflavone

The extraction was carried out at 70 ºC from 30-120 min in order to optimize the extraction time. In Fig. 4, the extracted amounts of flavonoids dramatically increased as the extraction time increased from 30-90 min. After 90 min, no obvious increase in the extracted amount of flavonoids was observed.

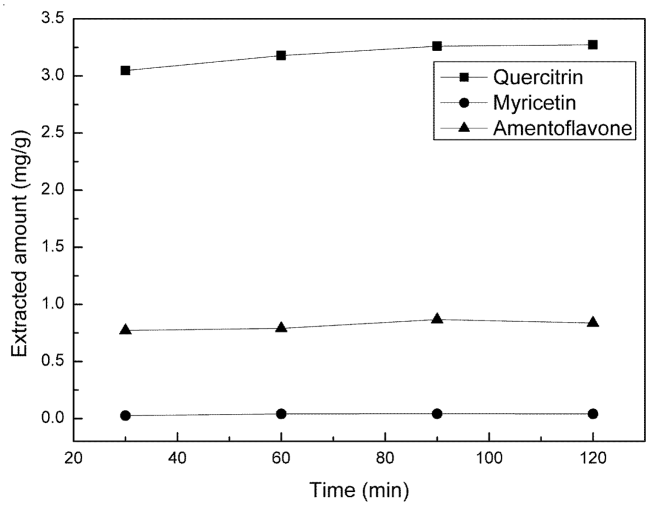


Fig. 4. Effects of different extraction time on the extraction amount of quercetin, myricetin and amentoflavone

The effects of the temperature were examined as an important impact factor. The leaves of *C. obtusa* were extracted from 30-80 ºC (Fig. 5). Generally, the amount of flavonoids increased when the extract temperature was increased. However, the temperature did not significantly influence the extracted amount of flavonoids above 60 ºC. Considering the energy consumption, 60 ºC was considered as a suitable temperature.

The effects of solid/solvent ratio were examined as an important impact factor. The amount of flavonoids increased when the solid/solvent ratio was increased from 1:5-1:50 (g/mL). It is obvious that the increase of solid/solvent ratio is useful for improving the extraction yields. This mechanism belongs to physical process. When the amount of solvent increases, the chance of bioactive components coming into contact with the solvent increases, which leads to higher leaching-

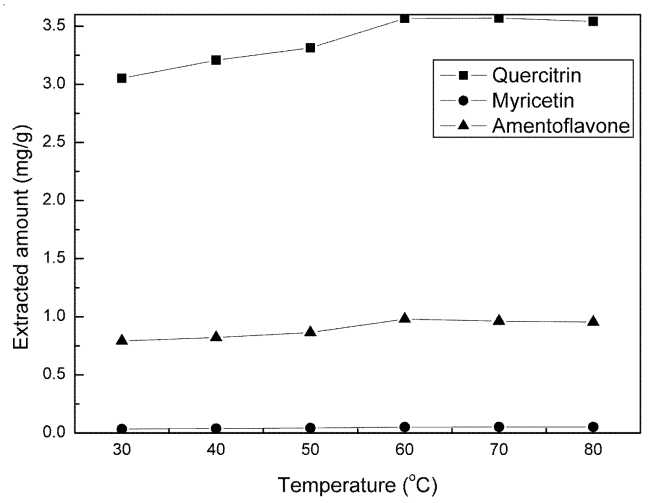


Fig. 5. Effects of different extraction temperature on the extraction amount of quercetin, myricetin and amentoflavone

out rates. A solid/solvent ratio of 1:10 (g/mL) was sufficient to reach a high extraction efficiency (Fig. 6).

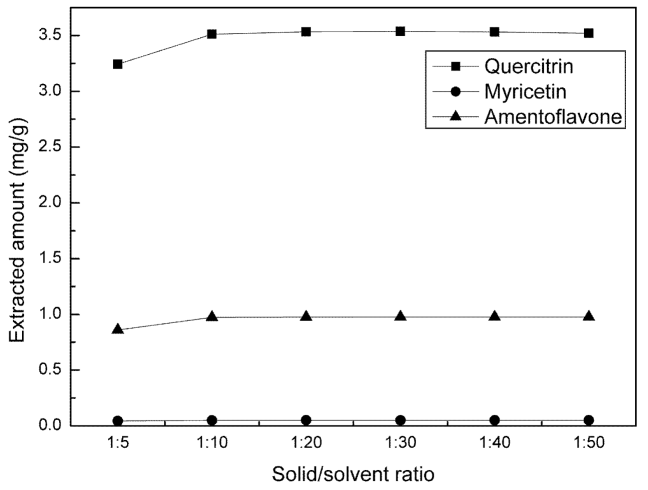


Fig. 6. Effects of different solid/solvent ratio on the extraction amount of quercetin, myricetin and amentoflavone

**Production of ethanol:** In the pretreatment process of dry *C. obtusa* residue, three sodium hydroxide concentrations (1, 2 and 3 M) were compared with 10 % (w/v) solid content. The pH value of the pretreated *C. obtusa* was then adjusted to 5.0. After fermentation, the highest amount of ethanol was produced at a sodium hydroxide concentration of 2 M (Table-1).

After pretreatment, the *C. obtusa*/yeast ratios were monitored during the fermentation process in order to investigate their relationship on production of ethanol. The results are shown in Table-2 and the *C. obtusa*/yeast ratio of 1/1.5 (2 M NaOH, 24 h fermentation) was proved to be best.

The alkaline pretreated *C. obtusa* waste was simultaneously saccharified and fermented by *Saccharomyces cerevisiae* after diluting the sodium hydroxide solution to a low concentration. Tables 1 and 2 shows that the maximum ethanol obtained from the *C. obtusa* waste (1 g) was 0.14 g/g after 24 h of fermentation. Further increase of fermentation time cannot increase the yield of ethanol, or even, the concen-

TABLE-1
EFFECT OF SODIUM HYDROXIDE CONCENTRATION AND FERMENTATION TIME ON PRODUCTION OF ETHANOL

| Sodium hydroxide concentration (M) | Fermentation time (h) | Amount of ethanol (g/100 g of *C. obtusa*) |
|---|---|---|
| 1 | 12 | – |
| | 24 | 10.84 |
| | 48 | 11.25 |
| | 72 | 8.53 |
| 2 | 12 | 10.88 |
| | 24 | 14.40 |
| | 48 | 12.71 |
| | 72 | 6.14 |
| 3 | 12 | 12.34 |
| | 24 | 14.47 |
| | 48 | 11.94 |
| | 72 | 6.06 |

TABLE-2
EFFECT OF *C. obtusa*/YEAST RATIO ON PRODUCTION OF ETHANOL

| Sodium hydroxide concentration (M) | *C. obtusa*/yeast ratio(g $g^{-1}$) | Fermentation time (h) | Amount of ethanol (g/100 g of *C. obtusa*) |
|---|---|---|---|
| 2 | 1:0.5 | 24 | 12.82 |
| | | 48 | 12.94 |
| | 1:1 | 24 | 13.97 |
| | | 48 | 12.80 |
| | 1:1.5 | 24 | 14.22 |
| | | 48 | 11.96 |

tration of ethanol reduced to 0.06 ± 0.01 g/g at 72 h. This phenomenon can be explained that the activity of yeast decreased with increasing fermentation time. Moreover, the yeast was inhibited by increased concentration of ethanol and ethanol may be converted to other compounds with further fermentation.

In our best of knowledge this is the first report of the conversion of *C. obtusa* to fermentable sugars by a sodium hydroxide pretreatment with subsequent fermentation to ethanol. Simultaneously, flavonoids in *C. obtusa* were extracted.

## Conclusion

By investigation of the extraction method, solvent, temperature, extraction time and solid/solvent ratio, it is concluded that the highest amount of flavonoids in the extract that was obtained through using methanol with heating method (60 ºC) at a solid/solvent ratio of 1:10 (g/mL) for 90 min. The total extracted amount of quercetin, myricetin and amentoflavone were 3.57, 0.05 and 0.9806 mg/g, respectively. Residue of *C. obtusa* was then use for producing bioethanol by subsequent pretreatment and fermentation. The production of bioethanol from *C. obtusa* would have the dual advantage of producing energy and serving as an effective method of weed management. The present study demonstrates a process which can obtains the flavonoids and bioethanol from *C. obtusa* simultaneously.

## ACKNOWLEDGEMENTS

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