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Column chromatographic extraction and preparation of cordycepin from *Cordyceps militaris* waster medium

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ABSTRACT

Large amounts of solid medium containing cordycepin, used in the industrial production of *Cordyceps militaris* through solid fermentation, are discarded as waste and contaminate the environment. We have developed a new column chromatographic extraction (CCE) method for the extraction of cordycepin from this waste and a preparation method for further separation and purification. Dried waste material was imbibed in four times its volume of water for 6 h, transferred to columns and eluted with water. Eluates were directly separated with macroporous resin DM130 columns followed by purification steps, including precipitation, crystallization, and polyamide column chromatography. Extraction rates of more than 97% were obtained with 12 volumes of water for a single column and 4 volumes of water for eluates circulated through 3 different columns designed to concentrate cordycepin. Cordycepin (98% pure) was obtained following the separation and purification processes, with an overall recovery rate of more than 90%. The CCE method has high extraction efficiency, uses a minimum volume of solvent and can be used for both quantitative analysis and large preparations of cordycepin from waste. The preparation method is simple, highly efficient, energy-saving, environmentally friendly, and has been demonstrated to be effective for large preparations of cordycepin from waste with low equipment and operating costs.

1. Introduction

Cordyceps militaris belongs to the same genus as *Cordyceps sinensis*, which is a well-known traditional Chinese medicine with multiple pharmacological functions, such as immunoregulation, anti-aging, antitumor and antimicrobial activities [1–3]. Difficulty in cultivation and limited supplies resulted in an increase in the price of *C. sinensis* to US$ 12,000 kg^{-1} in 2006 for an average quality product [2]. In contrast, *C. militaris* is easily cultivated and has been demonstrated to be the best substitute for *C. sinensis*, as both have similar chemical components and medical functions. *C. militaris* has been widely cultivated for production of fruiting bodies through solid fermentation with a reported annual production of 50–60 tons of dried fruiting bodies in the Guangdong Province of China. The rice-based, solid fermentative medium is required in a 10-fold excess to the fruiting bodies produced in order to cultivate *C. militaris*. Large amounts of used solid medium are discarded as waste and pollute the environment. The waste contains mycelium and bioactive substances with a cordycepin content of 1/10,000 to 1/1000 depending on the fungus strain and cultivating condition [4–6]. Thus, utilization of the waste media is of both economic and environmental important.

Cordycepin, a nucleoside analogue of 3′-deoxyadenosine, was first isolated from *C. militaris* [7]. It is classified as an anticancer compound [2,8–11] but also has immunoregulatory [3], antibacterial [12], antifungal [13], antiviral [14,15] and anti-infection [16] properties. It has also been shown to protect neurons from ischemic injury [17] as well having various other functions [1,2,10]. Cordycepin incorporates into RNA and causes premature termination of RNA synthesis, making it a valuable tool in the study of gene transcription [2]. Because of its cytotoxicity to terminal deoxynucleotidyl transferase positive (TdT+) leukemic cells, cordycepin is under clinical tests as a therapeutic agent for the treatment of TdT-positive acute lymphocytic leukemia (OncoVista, Inc., San Antonio, TX; granted by the US FDA in July, 2007). Cordycepin is also used in various health products worldwide and its cost has recently risen to US$ 4000 g^{-1} due to increasing demand.

Although cordycepin can be chemically synthesized, microbes like *C. militaris* are still its main source. Several extraction methods have been developed to extract cordycepin from a fermentative solution and from fruiting bodies of *C. militaris*, including ultrasound- or microwave-assisted extraction, pressurized extraction, soxhlet extraction and reflux extraction. Wang et al. [18] compared thermal reflux and ultrasound-assisted extraction with water or ethanol and found that the thermal refluxing extrac-

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tion with water was the best method for extracting cordycepin and polysaccharide from the fruiting bodies of *C. militaris*; three 90-min extractions with a sample-to-water ratio of 1:10 each is optimal. Song et al. [19] reported that the main factors affecting cordycepin extraction are the volume ratio of solvent to sample, extraction time and ethanol concentration. The optimal conditions for ultrasound-assisted extraction of cordycepin use 33 ml g^{-1} of 20% ethanol for 102 min. Yang and Li [20] compared three methods for the extraction of cordycepin and other nucleosides from *C. sinensis* and *C. militaris*. They found that extraction efficiencies are similar by liquid extraction using pressurized organic solvent or boiling water extraction. For some nucleosides, the efficiency was greatly increased using water extraction at ambient temperatures. Xia and Wen [4] tested the microwave-assisted extraction of cordycepin from solid *C. militaris* medium. The optimal conditions employed a sample to water ratio of 1:200 with a 50 W microwave treatment for 3 min. Zhong et al. [6] reported that the optimal sample-to-solvent ratio for soxhlet extraction of cordycepin from solid *C. militaris* medium was 1:16. These methods are based on macerating extraction and are not ideal because of relatively low extraction efficiency, a large volume of extraction solution and high energy consumption.

The reported separation and purification methods for cordycepin are based on ion exchange, activated carbon or silica gel column chromatography [6,21]. These methods have low adsorption amounts, low recovery rates and are not ideal for generating pure products. Chen et al. [21] successfully prepared high-purity cordycepin using preparative reverse-phase HPLC, although this method requires high equipment and production costs.

In this work, we report on the development of a new and highly efficient column chromatographic extraction (CCE) method for extracting cordycepin from *C. militaris* waste medium, as well as a preparation method for further separation and purification of cordycepin. Using these methods, we successfully prepared cordycepin with a purity greater than 98% in both a laboratory setting and in larger scales at costs of less than US$ 100 g^{-1}. This method offers many advantages over the current technology, including high extraction efficiencies and recovery rates, the use of minimal volumes and concentrations of solvents, energy-savings and environmental friendliness.

2. Materials and methods

2.1. Materials and reagents

Solid waste media of *C. militaris* cultures were generated by the cultivation center in our laboratory. The medium contained 1 kg of rice in 1.5 l of nutrient solution (1% glucose, 1% peptone, 0.2% KH_2PO_4, 0.1% $MgSO_4$, 0.1% ammonium citrate, 1% silkworm powder, 0.001% vitamin B1), and was autoclaved at 120 °C for 2 h before it was used for cultivation of *C. militaris*. After fermentation, fruiting bodies of *C. militaris* were harvested and the culture medium (as shown in Fig. 1) was dried in an oven at 45 °C for 2 d. Dried waste medium was ground and sieved with pore sizes of 833 μm and/or 350 μm.

Cordycepin standard was purchased from Sigma Co. (St. Louis, MO, USA). HPLC-grade solvents were purchased from Burdick & Jackson Inc. (Muskegon, MI, USA). Macroporous DM130, DM131 and DM101 resins were purchased from Shandong Chemical Co. (Shandong, China). The ethanol used in all experiments was 95%, food grade and was purchased from local suppliers. Other reagents were analytical or biochemical grade.

2.2. Ultrasound-assisted maceration extraction

As a control for the new extraction method, macerating extraction of cordycepin from the waste medium was used under optimal conditions we determined. Dried media were fully imbibed with 4 volumes of water (solvent to sample ratio, v/w, pH 6.5) for 6 h in the dark at room temperature. Water (60 °C) was then added to a final amount of 10 or 20 times the volume and extracted with ultrasound treatment (100 W) in a 60 °C water bath for 1 h. The extraction solution containing the waste medium was centrifuged at 4000 rpm for 10 min. The supernatant was used for HPLC analysis.

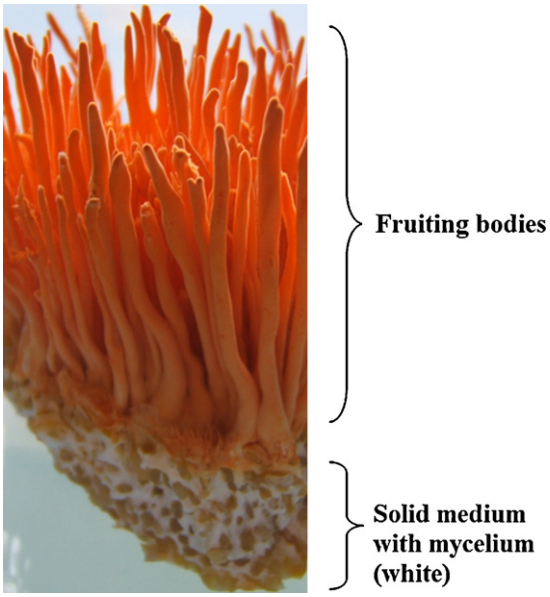

Fig. 1. Fruiting bodies of *C. militaris* cultured by solid medium fermentation in our laboratory.

2.3. Column chromatographic extraction

As solid *C. militaris* waste medium is a water swelling material, it was imbibed with 4 volumes of water (pH 6.5) for 6 h before it was transferred to chromatographic columns. The columns were loaded and eluted using common procedures. Eluates were collected in fractions, each with a volume equal to four times the sample, and were analyzed by HPLC until no cordycepin was detected in the eluates.

For cyclic CCE, only the first eluate fraction, which contains a high amount of cordycepin, was collected as a final extraction and processed to the separation steps. The second fraction was used to extract the next material and the third fraction was used for eluting the second column (Fig. 2). Through this cyclic method, the final extracting solution was only four times the volume of the dry material, while the columns were eluted three times (each with four times the volume of solvent) and almost no cordycepin remained on the columns.

2.4. Static adsorption and desorption of cordycepin with a macroporous resin

All resins were pretreated with the following procedure before use. The resin was first imbibed in de-ionized water overnight. Fully absorbed resins were soaked sequentially with 80% ethanol, 0.1 mol l^{-1} HCl, and 0.1 mol $^{-1}$ NaOH, each for 4 h or more and washed with distilled water after each soaking.

One gram of pretreated macroporous resin, with surface water absorbed with a paper towel, was added into 20 ml of extracts (pH adjusted with HCl or NaOH) in a 150 ml Erlenmeyer flask and incubated at room temperature with shaking (120 rpm). At 10 min, 20 min, 30 min, 40 min, 60 min, 120 min and 180 min, 0.5 ml of solution was taken and centrifuged at 4000 rpm for 5 min. The

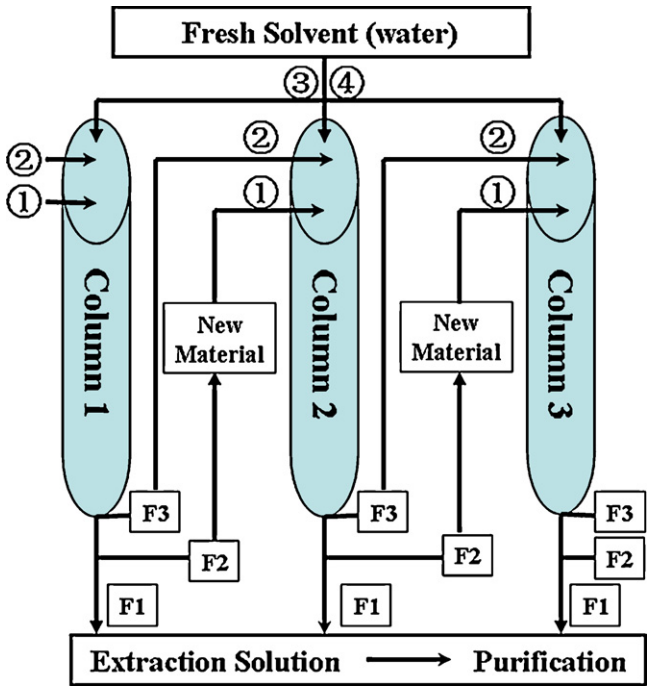

Fig. 2. Cyclic chromatography extraction of bioactive substances from biological materials. F1, F2 and F3 refer to Fraction 1, 2, and 3, respectively of the collected eluates. Each fraction contained four times the volume to the dry weight of materials. Only F1 was collected as an extraction solution.

cordycepin contents in the supernatants were determined by HPLC. The adsorption capacity of macroporous resins for cordycepin was calculated.

Once fully adsorbed for 3 h in extraction solution, the solution containing resins was vacuum-filtered. Resins were desorbed in 20 ml of 80% ethanol for 30 min. The cordycepin content in the desorption solutions was determined by HPLC and the desorption rates were calculated.

2.5. Column separation and purification of cordycepin

For separation of cordycepin with macroporous resin, pretreated resins were loaded onto columns. The columns were washed with distilled water until their pH was neutral, and then washed a final time with 2 bed volumes (BVs) of distilled water (pH 9.0) before the samples were loaded. Eluates were collected in fractions and analyzed by HPLC.

For purification of cordycepin with polyamide resin, cordycepin crystals were dissolved in distilled water. The aqueous solution flowed through a prepared polyamide column at a rate of 2 BVs h^{-1}. After the sample was loaded, the column was eluted with 3 BVs of distilled water. All eluates were collected together and vacuum-evaporated to 1/4 the original volume. The concentrated solution was then crystallized at 4 °C.

2.6. HPLC determination of cordycepin

An HPLC system (Dalian Elite Analytical Instrument Ltd., Dailian, China) with dual P230 pumps, a UV230+ detector and analytical software was used for the detection and analysis of cordycepin. HPLC conditions were as follows: column, YMC-packed C18 (250 mm × 4.6 mm, 5 μm); mobile phase, methanol:water (20:80, v/v); flow rate, 1.0 ml min^{-1}; UV detection at 260 nm; and injection amount, 10 μl. The samples were filtered through a 0.45 μm membrane filter before injection. Quantitative analysis of cordycepin and other substances was determined by their peak area based on their standard curves. Peaks for cordycepin and other compounds in the samples were identified by their retention times and co-injection tests with their corresponding standard compounds.

3. Results and discussion

3.1. Column chromatographic extraction of cordycepin from C. militaris waste medium

The extraction process was separated into two steps. The first step involved dissolving the target substances with a minimum volume of their ideal solvents. As the dried waste medium began to expand after imbibition, the materials were fully imbibed in open containers with 4 volumes of water for 6 h until the cordycepin was dissolved to its maximum amount (data not shown) and was then loaded onto columns. The second step was to elute the target substances from the columns with water. As shown in Fig. 3, the extraction rates of cordycepin from *C. militaris* waste medium by the ultrasound-assisted water maceration were 87.3% and 91.6% with 10 and 20 volumes of water, respectively, while the extraction rates by the CCE method were 77.5%, 89.1%, 95.8% and 98.8% for 4, 6, 8 and 10 volumes of water, respectively. The extraction rate for the CCE method was 11.5% higher than that of the ultrasound-assisted extraction with, both using 10 volumes of water, and was even 7.2% higher than that of the latter method using 20 volumes of water.

To optimize the conditions for the column chromatographic extraction of cordycepin from *C. militaris* waste medium, several factors were tested, including imbibition time, the particle size of the material, extraction temperatures, flow rates of eluate and height to diameter ratio (*H*/*D*) of the loaded columns. The results indicated that 6 h was needed for the dried waste medium to be fully imbibed (data not shown). Imbibition times longer than 6 h (Fig. 4A), the particle size of the material (either ≥833 μm or between 350 μm and 833 μm, Fig. 4B) and extraction temperatures (either 30 °C or 60 °C, Fig. 4C) did not have significant effects on cordycepin extraction. The flow rates of eluates (2 BV h^{-1}, 6 BV h^{-1} and 30 BV h^{-1}) had limited but consistent effects on the extraction of cordycepin, in favor of a low flow rate (2 BV h^{-1}) (Fig. 4D). The height to diameter ratio (*H*/*D*) of material loaded onto columns had significant effects on the extraction of cordycepin. The extraction efficiency of cordycepin increased about 5% when the *H*/*D* ratios were increased two-fold. Cordycepin was not completely extracted with 12 volumes of water at a *H*/*D* of 2.5:1. The extraction efficiency reached 98% and 100% when the *H*/*D* was increased to 5:1 and 10:1, respectively. These results demonstrate that a minimum *H*/*D* of 5:1 is necessary for efficient extraction of cordycepin from the material.

From the results above, extraction conditions had limited effects on the extraction of cordycepin in the CCE method. We tested the effects of temperatures, flow rates of eluates and *H*/*D* of the column

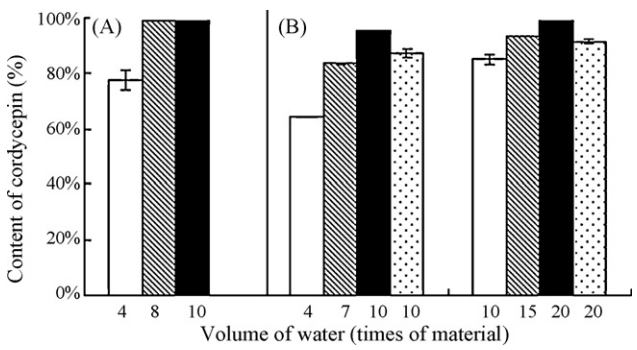

Fig. 3. Comparison of column chromatographic extraction (A) and ultrasound-assisted extraction of cordycepin (B) from *C. militaris* waster medium. □, ▨, ■ represent sequential extraction, ⬚ represents one-step extraction.

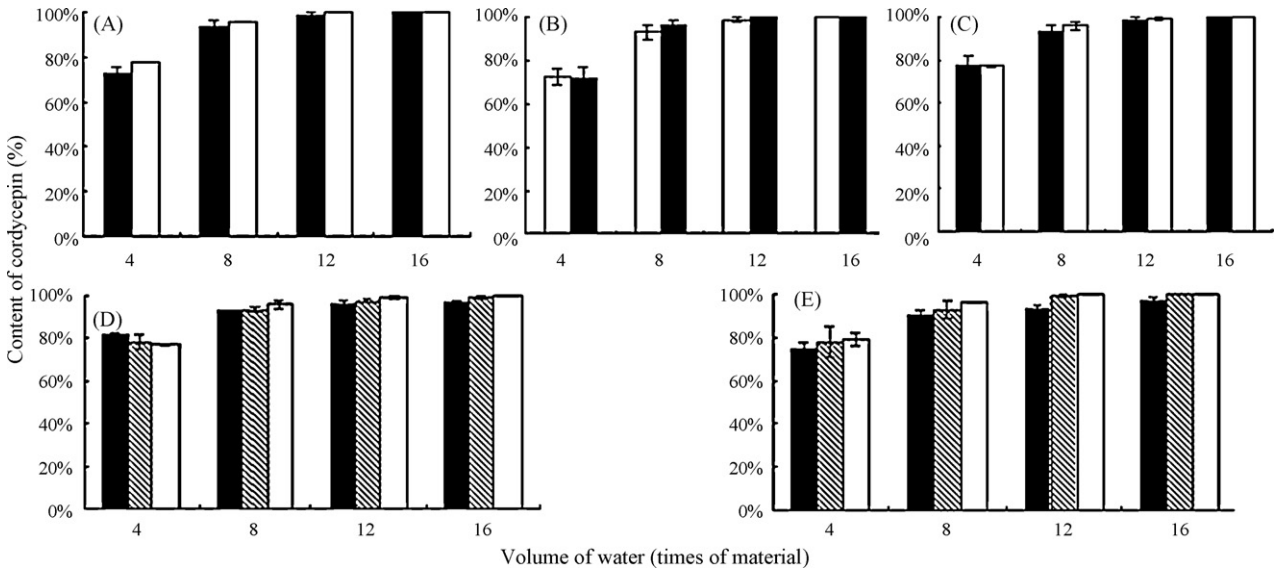

Fig. 4. Effects of column chromatographic conditions on cordycepin extraction from *C. militaris* waste medium. (A) Imbibition time: 6 h (black), and 12 h (white). (B) Material thickness: 833 μm (black), and 350–833 μm (white). (C) Temperatures: 30 °C (black) and 60 °C (white). (D) Flow rates: 30 BV h^{-1} (black), 6 BV h^{-1} (striation) and 2 BV h^{-1} (white). (E) Height to diameter ratio (*H*/*D*) of extracting material in columns: 2.5:1 (black), 5:1 (striation), and 10:1 (white).

on extraction efficiency of adenosine from the same material of *C. militaris* waster medium. These three factors showed more and significant effects on the extraction of adenosine than on that of cordycepin (data not shown). The main reason may be that cordycepin is a component outside of cells, while adenosine is within cells.

Based on these results, the optimal conditions for the column chromatographic extraction of cordycepin were determined as follows. Dried waste medium from *C. militaris* was ground to sizes of 833 μm, imbibed with 4 volumes of water for 6 h at room temperature (30 °C), and then loaded onto chromatographic columns with an *H*/*D* ratio of 10:1. The columns were eluted with water at a flow rate of 2 BV h^{-1}. The eluates were collected for analysis and further processing. Repeated experiments under these conditions showed that more than 97% of cordycepin and more than 96% of polysaccharides (data not shown) were extracted from the waste medium.

Our results indicate that this chromatographic extraction method has a higher extraction efficiency compared to those obtained with ultrasound-assisted water extraction or to previously reported results [4,6]; this increased efficiency is accomplished with less solvent and simple equipment and conditions. This method can also be used for the extraction of active substances from the fruiting bodies of *C. militaris* (data not shown), in which dried fruiting bodies can be loaded directly onto columns with extracting solvent (water) without a separate imbibition step. This method was tested for the extraction of bioactive substances from several plant materials, including polyphenols, caffeine and theanine from green tea and camptothecin from the dried leaves of *Camptotheca acuminate* Decne. With ideal solvents, target substances can be completely extracted (until substances in eluates cannot detected by HPLC) from their biological materials with 12 (4 × 3) volumes of 30–60% ethanol for polyphenols, caffeine and theanine from green tea or 20 (5 × 4) volumes of 60% ethanol for camptothecin from *C. acuminate* Decne (unpublished data of our laboratory). The method was demonstrated to be particularly useful for the accurate extraction and quantitative determination of substances in biological materials.

3.2. Cyclic chromatographic extraction of cordycepin from *C. militaris* waste medium and scale-up tests

As shown in Fig. 5, a total of 12 volumes of extraction solution from the chromatographic column extraction were divided into three fractions, each with 4 volumes of the materials. The cordycepin content was much higher in the first fraction (77.3%) than in the second (18.6%) or third (3.4%) faction. To eliminate the volume of extraction solution, a cyclic chromatographic extraction, which circulated the extraction solution three times through different columns from low to high contents of cordycepin, was designed (Fig. 2). Through the cyclic chromatographic extraction, the extraction rate of cordycepin, with a total of 4 volumes of water, was 97.24%, a value 20% higher than with the same volume of water (four times) or only 2% lower than with 12 volumes of water in the non-cyclic single column extraction. These results indicate that cyclic chromatographic extraction can extensively reduce the volumes of the solvent and the final extraction solution with high extraction efficiency.

The cyclic chromatographic extraction was tested in gradually amplified scales with similar results. Fig. 6 shows the results from columns with diameters of 20 cm and heights of 200 cm for 20 kg of dried waste medium from each extraction. Compared to the small-scale extraction, the extraction rates of cordycepin in the amplified tests were 7% lower in the first fraction, 2% lower in the second two fractions, but reached the same levels of 97–98% in all three fractions. Repeated tests demonstrated that the extraction rates achieved with the cyclic chromatographic extraction method for cordycepin can reach the same level (more than 97%) in amplified experiments with only 4 volumes of extracting solvent. This method has high extraction rates with a minimum amount of sol-

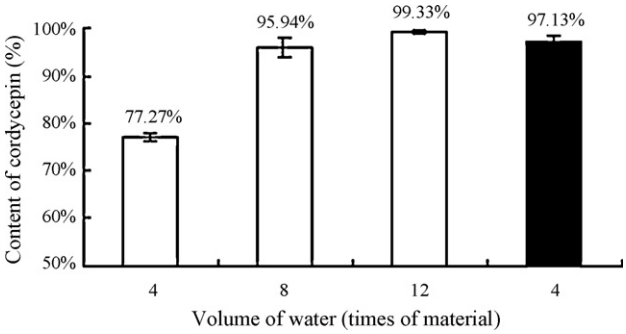

Fig. 5. Comparison of the extraction efficiencies of non-cyclic chromatographic extraction (white) and cyclic chromatographic extraction (black).

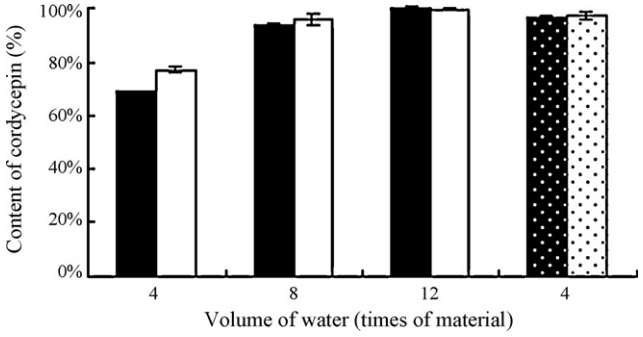

Fig. 6. Comparison of the extraction efficiencies of cordycepin between small-scale experiments (white) and large-scale tests (black).

vent, good reproducibility and should be particularly useful for large scale and continuous extraction of cordycepin from waste medium.

3.3. Separation of cordycepin by macroporous resin

Macroporous resins had good performances in adsorption and separation of cordycepin in our pre-screening tests of different types of resins. Three resins, DM130, DM131 and DM101, were further tested for their adsorption and desorption of cordycepin at different pH conditions. As shown in Fig. 7A, the static adsorption and desorption tests demonstrated that the adsorption of cordycepin by the resins was dependent on pH. All of them had high adsorption at pH 9.0 or pH 6.5 (with distilled water without adjustment of pH), and low adsorption at pH 3.0. DM130 was found to be the best resin for adsorption of cordycepin at pH 9.0 with an adsorption capacity of 662.1 $\mu g\,g^{-1}$ resin. Its adsorption reached 90% saturation within 10 min (data not shown). The desorption of the three resins to cordycepin was also dependent on pH. The desorption rates were high at pH 3.0 and pH 9.0, but low at pH 6.5 for all three resins. DM130 and DM101 showed a similar desorption of about 96%. In conclusion, DM130 was the best resin for adsorption and desorption of cordycepin.

Fig. 8 shows the elution efficiency of cordycepin from DM130 columns with different concentrations of ethanol at different pHs. Similar to the results from the static adsorption tests, cordycepin

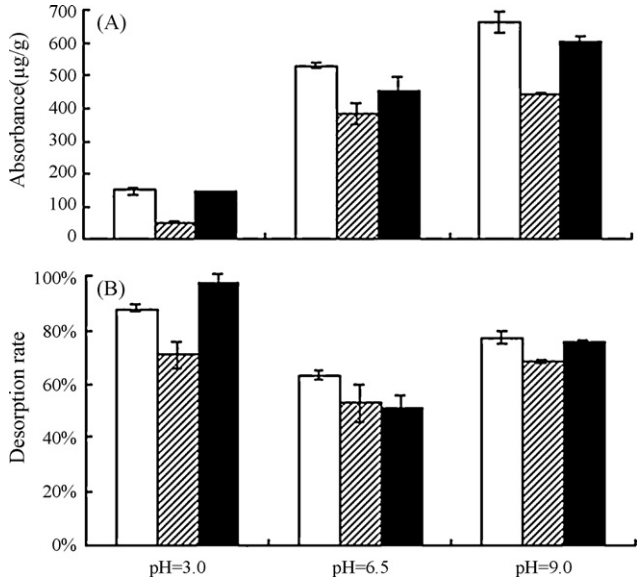

Fig. 7. Adsorption (A) and desorption (B) of cordycepin by three macroporous resins at different pHs. Black is for DM101, striation for DM131 and white for DM130.

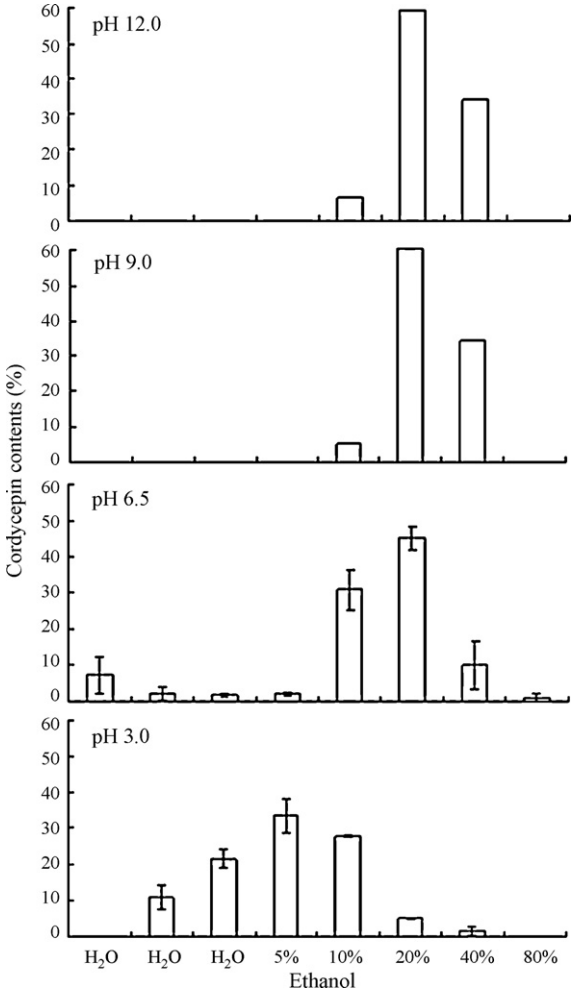

Fig. 8. Elution conditions for cordycepin adsorbed on DM130 resin.

was adsorbed well by DM130 resin in basic conditions (pH 9.0 and pH 12.0) without any cordycepin leakage during the sample loading and water washing steps. There were no significant differences in the results obtained at pH 9.0 or pH 12. At pH 6.5, there were 8% and 2% leakages of cordycepin during the sample loading and washing steps, respectively. However, under acidic conditions (pH 3.0), cordycepin could not be adsorbed effectively by the resin and could be washed out by water. Experiments also showed that flow rates of the eluates (1, 2 and 4 BVs h^{-1}) did not have a significant effect on the adsorption and desorption of cordycepin by the resin (data not shown).

Repeated experiments indicated that the following chromatographic conditions provided reasonably good adsorption and separation for cordycepin in DM130 column chromatography with more than 95% recovery rates. The extraction solution from the cyclic column chromatographic extraction was adjusted to pH 9.0 and was loaded directly onto prepared DM 130 columns with a volume of no more than 4 BVs. The column was washed with 4 BVs of water (pH 9.0) to remove large amounts of impurities. The column was then eluted with 0.5 BV (monitored by HPLC) to 1.5 BV (not monitored) of 40% ethanol (pH 3.0). More than 95% of the cordycepin could be found in this fraction. The flow rates in all these processes were 2 BVs h^{-1}. The column was regenerated with 1–2 BVs of 95% ethanol followed by 4 BVs of distilled water. The columns could be used more than five times without a significant decrease in their adsorption performance, but not from the fruiting bodies of *C. militaris,* which have many more impurities and

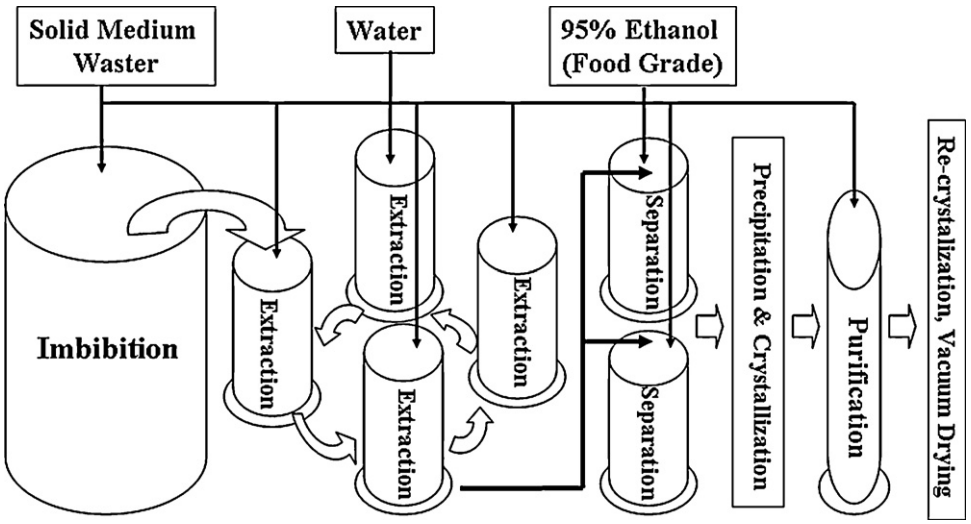

Fig. 9. Diagram of cordycepin extraction and preparation from *C. militaris* waste medium.

colored substances that make the column difficult to regenerate. Cordycepin, a weak alkaline substance, is in the form of neutral or positive ion in alkaline conditions and can be adsorbed by DM130 resin, while it is in negative ion at acidic conditions and cannot be effectively adsorbed by the resin.

3.4. Purification of cordycepin by precipitation, crystallization and polyamide resin column chromatography

The 40% ethanol fraction, which contained more than 95% of the cordycepin from the DM130 columns, was vacuum-concentrated 20-fold at 65 °C. Four volumes of 95% ethanol (food grade) were added to the concentrates (to a final ethanol concentration of 78%). The solution was mixed thoroughly, incubated at room temperature overnight and then vacuum-filtered. The pellet was washed with 1/10 of a volume of 80% ethanol and was then filtered again. This step removed polysaccharides, proteins and most colored substances adsorbed by the DM130 resin. Less than 1% of the cordycepin remained in the pellet. The supernatants were combined and vacuum-concentrated at 65 °C until the ethanol was completely removed. The water fraction was cooled down to room tempera-

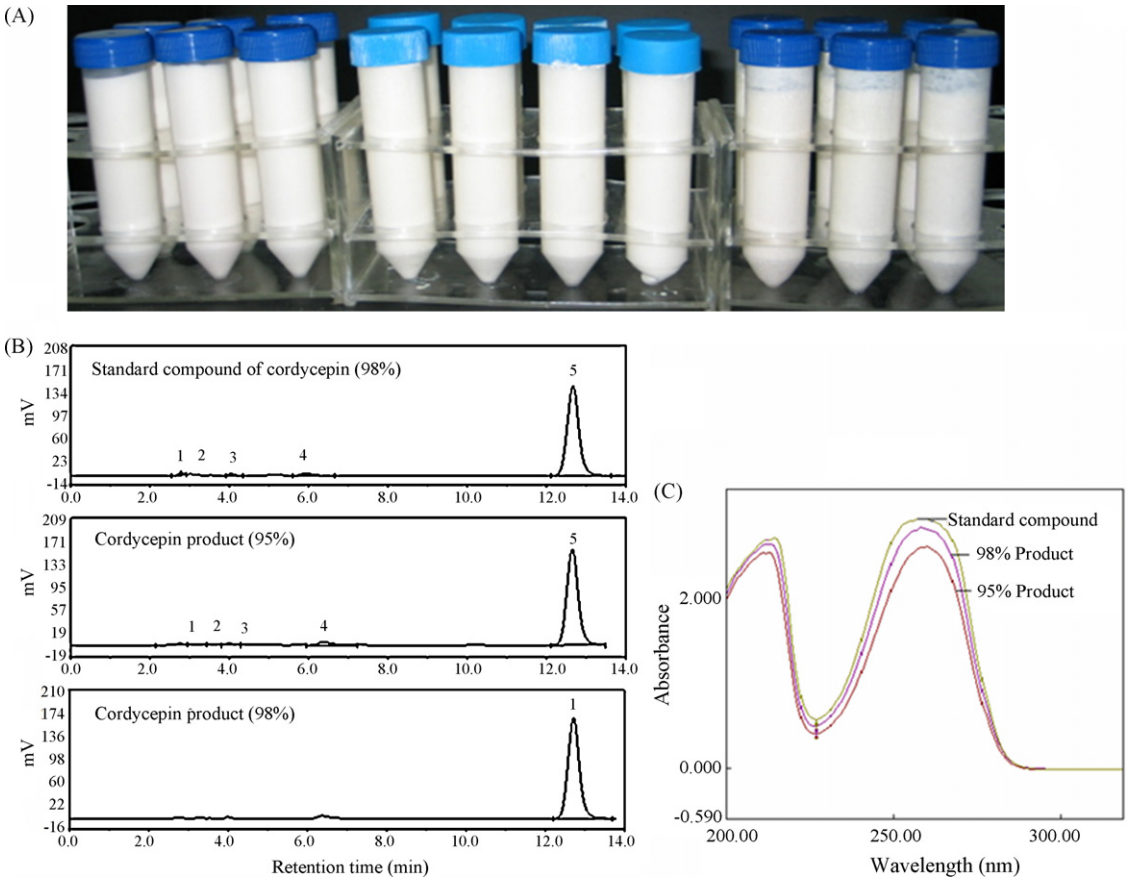

Fig. 10. Cordycepin crystals derived from a large-scale preparation and their spectral analysis. (A) Cordycepin crystals resulting from large-scale preparation. (B) HPLC analysis of the prepared cordycepin and its standard. (C) The UV spectra of the prepared products with purities of 95% and 98% and the standard compound.

ture and crystallized at 4 °C overnight. Crystals were separated by centrifugation at 4000 rpm for 30 min, re-dissolved (at 70 °C) and re-crystallized in 4 volumes of water three or four times until the solution was colorless. After the crystals were vacuum-dried, 95% pure product was obtained.

Even after the crystallization was repeated until the solution became colorless and the crystals become pure white, the cordycepin still contained trace amounts of a colored substance (visible after dried) and its purity could not reach 98% or higher. Polyamide resin, activated carbon and H_2O_2 were tested for their ability to remove the trace amount of colored substance in the cordycepin crystal (data not shown). Polyamide resin was effective at removing the colored substance without adsorption to cordycepin and was used to remove the trace amount of impurity. Activated carbon adsorbs both the colored substance and the cordycepin and therefore could not be used. H_2O_2 did not have good decolorization performance.

To accomplish polyamide column chromatography, cordycepin crystals containing trace amounts of the colored substance were fully dissolved in distilled water at room temperature, and then the solution was passed through a polyamide column at 2 BVs h^{-1}. After the sample was loaded, the column was eluted with 2–3 BVs of water. Because the colored impurity was adsorbed by the polyamide resin and the cordycepin was not, pure cordycepin was collected in the water fraction. All eluates were combined together, vacuum-concentrated to 1/4 or 1/2 the volume and re-crystallized one or two more times. The crystals were vacuum-dried, and a cordycepin product with greater than 98% purity was obtained. The supernatant in all of the crystallization steps was concentrated and re-crystallized or put back into extraction solution for recovery of the remaining cordycepin.

Based on the methods and procedures above, a production line was designed for cordycepin preparation (Fig. 9) through chromatographic extraction, separation and purification with combinations of precipitation, crystallization, vacuum concentration and drying. The line mainly consists of three chromatographic units. Unit one has four columns (20 cm diameter × 200 cm height) for continuous extraction through cyclic chromatographic extraction. Unit two has two columns (15 cm diameter × 160 cm height) for continuous separation of cordycepin with DM130 resin. Unit three has one column (10 cm diameter × 100 cm height) for the final purification of cordycepin. Using this production line, continuous preparation of highly purified cordycepin can be easily reproduced in a laboratory with an overall recovery rate greater than 90% at low cost and low energy consumption. More than 100 g of purified cordycepin can be obtained each time. The products and their HPLC analysis are shown in Fig. 10.

4. Conclusions

We report for the first time a new CCE method for the extraction of cordycepin from solid *C. militaris* waste medium, as well a new method for its separation and purification using macroporous resins and polyamide resin. Extraction rates of more than 97% were obtained with 12 volumes of water for a non-cyclic single CCE and 4 volumes of water for cyclic CCE which circulated eulates through 3 different columns. The extraction solution, with minimal volume, high content of cordycepin and few impurities, was loaded directly onto macroporous DM130 resin columns for separation without filtration or centrifugation and concentration. The method used simple equipment and minimized both the use and concentration (which consumes energy) of solvents. The non-cyclic CCE method was particularly useful for quantitative determination of substances in biological materials, while the cyclic CCE method was better for large scales and continuous extraction.

The best conditions for separation of cordycepin by DM130 resins were found to utilize an adsorption or loading at pH 9.0, washing with water at pH 9.0 and desorbing or eluting with 40% ethanol at pH 3.0. The eluates from the 40% ethanol fraction were precipitated, crystallized and purified through polyamide resin columns. Through this separation and purification method, cordycepin products with 98% purity can be made with an overall recovery rate of more than 90%. Furthermore, the whole process uses common equipment and water and food grade 95% ethanol as solvents, which is environmentally safe. The method has been demonstrated to be a simple and highly efficient method for both laboratory study and large scale and continuous preparation of cordycepin from *C. militaris* waste medium at both low cost and low energy consumption.

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