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*Full Length Research Paper*

# Production of emodin from *Aspergillus ochraceus* at preparative scale

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**In order to study the chemical constituents in the pigmented culture produced from *Aspergillus ochraceus,* solid phase extraction method was employed to isolate the pigment molecules from the primary culture, followed by fractionation on preparative liquid chromatography. Structural characterization confirmed that one of the two major pigment components in the culture was emodin (1,3,8 -trihydroxy-6-methyl-anthraquinone). It was observed that production of emodin started 2 days after the culture had reached the stationary phase. The culture conditions were subsequently optimized to improve the yield of the emodin production. It was found that optimal production of emodin was achieved when fermentation was carried out at 32°C with the pH value of the culture medium at 7.0. Other conditions were also optimized, leading to the yield reaching as high as 0.8% of dry mass of *A. ochraceus*. The method described here offers an efficient approach for large scale production of emodin.**

**Key words:** Preparative liquid chromatography, solid phase extraction, emodin, *Aspergillus ochraceus.*

## INTRODUCTION

Emodin (1,3,8 -trihydroxy-6-methyl-anthraquinone) (Figure 1) is a naturally occurring pigment found in many plants (Izhaki, 2002), molds and lichens, exhibiting diverse biological activities including anticancer functions (Lin et al., 2009; Muto et al., 2007, Srinvas et al., 2007) and anti-inflammatory functions (Ding et al., 2008). Early studies on its therapeutic benefits were mainly focused on its laxative functions as it is abundant in traditional Chinese medicinal herbs used for laxative formulation, such as rhubarb, the root and rhizome of *Rheum palmatum* L. (Wang et al., 2008).

Recently, the health benefit of emodin has been linked to its involvement in many cellular processes, such as the suppression of tumor-associated angiogenesis through the inhibition of extracellular signal-regulated kinases (Kaneshiro et al., 2006). There was also a report that emodin can sensitize certain types of breast cancer cells as it is involved in the treatment of paclitaxel (Zhang et al., 1999). Its inhibitory effect on tumorogenesis-associated cell signaling pathways has made emodin an interesting molecular entity for antineoplastic studies and formulations.

Emodin can be isolated and purified from plants, such as the roots of *Rheum officinale* Baill (Yang et al., 1999; Zhang and Liu, 2004) and *Polygonum cuspidatum* (Wang et al., 2008). The methods used for its isolation and purification vary depending on the plant species. Although many methods have been attempted to improve the production yield of emodin, consistency and efficiency of its isolation from herbs are always hampered by the inconsistency of the contents of emodin found in various types of herbs, even in the same plants harvested at different times or locations. The methods for emodin extraction from herbs have included reflux extraction, stirring extraction, ultrasonic extraction (Wang et al., 2008),

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**Abbreviations: SPE,** Solid phase extraction; **HPLC,** high performance liquid chromatography; **PrepHPLC**, preparative high liquid chromatography; **DEPT,** distortionless enhancement by polarization transfer; **TMS,** tetramethylsilane; **ESI,** electrosprayionization.

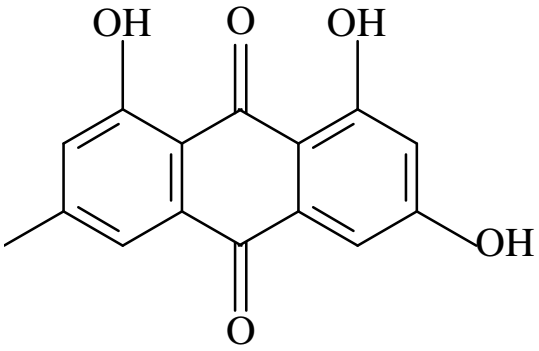


**Figure 1.** Structure of 1, 3, 8-trihydroxy-6-methyl anthraquinone (emodin).

microwave-assisted extraction (Wang et al., 2008) and supercritical carbon dioxide extraction (Lu et al., 2006), while the separation of emodin from other contaminating materials is predominantly based on chromatography using various types of stationery phase or mobile phase (Yang et al., 1999; Zhang and Liu, 2004; Wang et al., 2008).

Emodin could be produced by various fungal species including the *A. ochraceus* from Japanese rice (Yamazaki et al., 1970) and *Aspergillus wentii* from weevil-damaged Chinese chestnuts (Wells et al., 1975) as secondary metabolites. However, there has been no report on its isolation and purification from a fungal species at an industrial scale. Here, a report is being made on the studies of a strain of *A. ochraceus* isolated from Chinese potato, which constantly produced an unknown red pigment mixture. The pigment molecules were isolated using solid phase extraction method and purified by semiproparative chromatography using silicon gel as stationery phase. Structural characterisation of the isolated yellow pigment molecule was confirmed to be emodin. Subsequently, the culture conditions were optimized to achieve a better yield for emodin production. Through the optimization of the purification procedures, emodin can be purified to 96% or better, while the yield could reach to a level comparable to that obtained from Chinese traditional medicinal herbs.

## MATERIALS AND METHODS

### Growth of culture

*A. ochraceus* strain Ipzhequ188 was initially isolated and identified from Chinese potato. This process was carried out in the Department of Life Sciences, Tianjin University of Science and Technology, Tianjin, China (unpublished data). To obtain large culture for the purification of pigment molecules, the *A. ochraceus* strain was first grown in flasks containing 100 ml of Czapek-Dox (CD) medium, which was adjusted to pH 6.8 before autoclaving. The flasks were inoculated with a conidial suspension obtained from a 5-day old culture. The fermentation process was carried out at 29 - 32°C on a rotary shaker (220 rpm).

### Preparation of primary extract

Mycelia were collected from rotary flasks after centrifugation at 5000 rpm for 20 min at 0°C. The mycelia was then filtered through a qualitative filter paper with pore size 30 - 50 µm and washed twice with distilled water. The dry cell weight (DCW, g emodin $l^{-1}$ culture) was measured by dry weight determination (kept in a drying oven for 8 h at 90°C). To a flask containing 70 g of mycelium, 200 ml chloroform was added followed by extraction in an ultrasonic bath for 30 min. The process was repeated three times (Wang et al., 2008). The primary extracts were combined, filtered and subsequently evaporated under vacuum for further purification.

### Optimization of culture conditions

Different carbon sources such as glucose, sucrose, soluble starch, maize starch and malt extract and nitrogen sources such as $(NH_4)_2SO_4$, $NaNO_3$, $NH_4Cl$, peptone and yeast extract were added in various combinations into the fermentation system. The basal growth conditions, including volume of innoculum, concentration of spores, rotary velocity, pH and temperature were modified in the fermentation process in order to achieve optimal cell growth and emodin production.

### Monitoring of cell growth and production of emodin

The contents of emodin in the mycelium samples were determined by solid phase extraction (SPE) followed by high performance liquid chromatography (HPLC) using 5 g of dried mycelium obtained after cultured at 24, 48, 72, 96, 120, 144 and 168 h, respectively. The cell growth in relation to the production of emodin was drawn on the basis of DCWs (g $l^{-1}$) and the content of emodin (percentage in dried mycelium), respectively.

### High performance liquid chromatography (HPLC)

To examine the contents of emodin in the dry cell mass after solid phase extraction, 0.1 g of pigmented mixture was reconstituted with 10 ml of methanol. 2 ml of the solution was then filtered through a 0.23 µm membrane and applied to HPLC for analysis. Separation was achieved using a Nacalai Cosmosil $C_{18}$-MS-II column (5 µm, 250 × 4.66 mm, San Diego, USA) at 20°C on an Agilent 1100 HPLC system (Ramsey, Minnesota, USA). The mobile phase consisted of organic phase A (methanol with 0.05% acetic acid) and aqueous phase B (water with 0.05% acetic acid) and operated at a flow rate of 1.0 ml $min^{-1}$ with an organic phase A gradient from 30 to 90% in 10 min. The injection volume was 5 µl. The chromatograms were recorded at 254 nm.

### Solid phase extraction (SPE)

Solid phase extraction method was employed to isolate emodin from primary extracts (Wang et al., 2008; Wang et al., 2008). The primary extract was transferred to a Lubi 24-port vacuum solid phase extraction apparatus (Shanghai, China) with a cartridge preconditioned with methanol (Sigma) and water, each at a constant pressure of 95 Kpa followed by washing with 2% acetonitrile (Sigma) in water (v/v). The pigmented molecules were eluted from the cartridges with 8% methanol in water. Fractions collected were evaporated to dryness at 85 Kpa and 60°C under a constant stream of dry nitrogen, giving rise to a red powder.

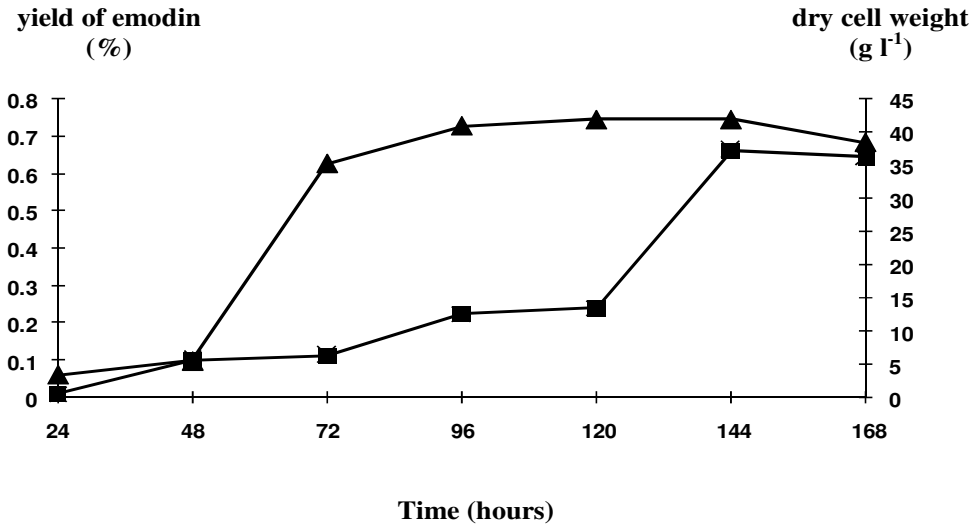


**Figure 2.** The growth curve and fermentation curve of emodin (closed square, the yield of emodin production expressed as percentage of purified emodin obtained from dry cell mass (%); closed triangle, dry cell weight expressed as gram per litre of culture (g $l^{-1}$).

**Preparative high performance liquid chromatography (PrepHPLC)**

Anlai preparative high performance liquid chromatography (Prep HPLC) system (Tianjin, China) was used to fractionate the pigmented mixture after solid phase extraction and monitored at 254 nm. The column (600 × 48 mm) was packed in the laboratory with 2.8 kg of silica gel 60 (RP-$C_{18}$, 40 – 63 µm) purchased from Merck. The mobile phase consisted of 50% methanol in water. The isocratic elution was carried out at a flowrate of 80 ml $min^{-1}$. The collected fractions corresponding to peak 7 (Figure 5) were dried at 85 Kpa and 60°C under a stream of dry nitrogen, giving rise to a yellowish powder.

**Structural analysis of emodin and other pigmented molecules**

The FT-IR spectra were recorded on a Nicolet Avatar 370 DTGS spectrometer (Madison, USA). $^{13}$C-HNMR, 1H-NMR and DEPT (Distortionless Enhancement by Polarization Transfer) spectra were acquired with a Varian Mercury AS 400 MHz spectrometer (Palo Alto, USA). Deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) was used as an internal standard in the assay. The measurements were carried out in 5 mm NMR tubes with a total sample volume of 600 µl. The probe temperature was maintained at 300 K. Mass spectrometric and mass/mass spectrometric analysis was performed using Thermo Finnigan LCQ Duo ion trap mass spectrometer (San Jose, USA) equipped with a heated capillary interface and electrospray ionization (ESI). UV-Vis absorption spectra were measured using Shimadzu UV-2401/2501 spectrophotometer. The elemental analysis data was obtained with Flash EA1112 elemental analyzer (Wigan, UK).

## RESULTS AND DISCUSSION

### The fermentation process of *A. ochraceus* and time course of emodin production

To find out the relationship between emodin production and the cell density in the fermentation process, aliquots of cultures were withdrawn from the culture vessel at designated time interval. Contents of emodin in these samples were monitored through HPLC analysis and the change of biomass in the culture was obtained by dry cell weight analysis. The time course and correlation between emodin production and changes in biomass in the culture are shown in Figure 2. It was observed that exponential cell growth took place 2 days after inoculation, which lasted more than 24 h. However, the production of emodin started only at day 5; 2 days after the cell growth entered its stationary phase. This showed that production of emodin only started when active cell growth had ceased and when the cells began the deceleration growth phase. The maximal production of emodin could be achieved when the culture was stopped at 144 h. In a typical experiment, a yield of emodin up to 0.7% of dry cell mass can be achieved.

### Optimization of carbon and nitrogen source

It is well known that carbon and nitrogen source in basal medium has a significant effect on the production of secondary metabolites in fungus. To find out how carbon and nitrogen source could affect emodin production, 5 reagents including glucose, sucrose, soluble starch, maize starch and maltose extracts were used as carbon sources and 6 reagents were chosen as the sources for nitrogen. It was observed that among the 5 types of carbon sources, emodin production level was highest when glucose was used. Further studies indicated that optimal emodin production can be achieved when glucose is present at a concentration of 45.0 g $l^{-1}$. It was also observed that peptone was a good nitrogen source for

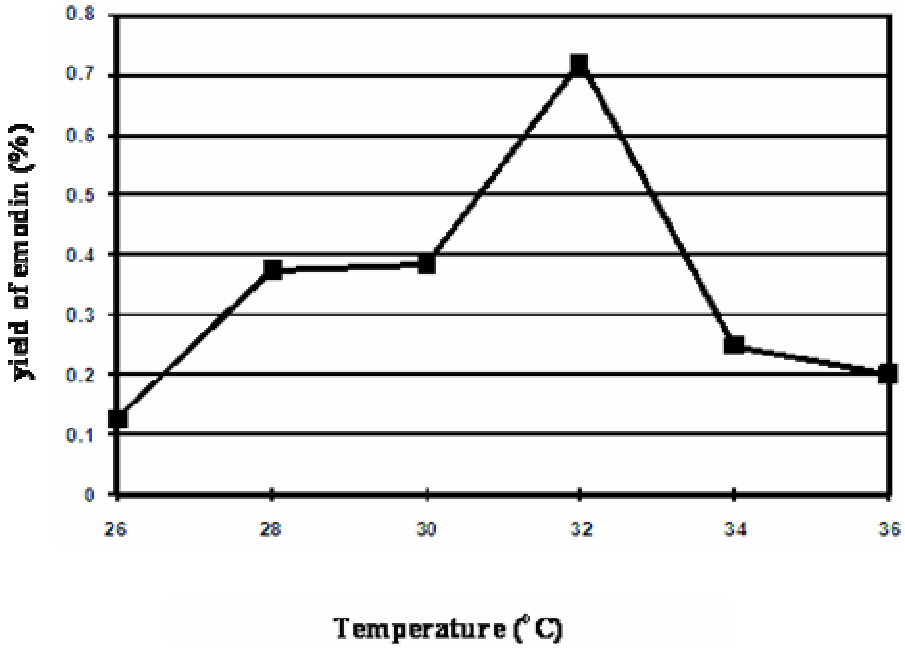


**Figure 3.** Effect of temperature on production of emodin.

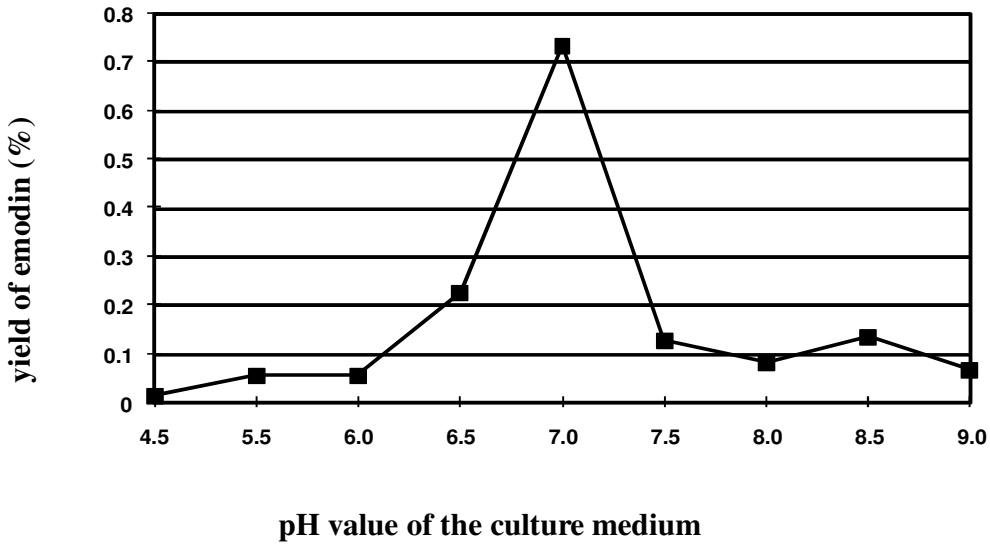


**Figure 4.** Effect of initial pH of the culture medium on the yield of emodin.

emodin production from *A. ochraceus*. By using peptone at different concentration in the medium, it was further discovered that a yield of 0.7% of dry cell weight can be achieved when peptone was included in the medium at a concentration of 3.0 g $l^{-1}$.

## Effect of temperature and pH on emodin production

To study the effect of temperature on emodin production in *A. ochraceus,* cells were grown at different temperature ranging from 26 - 36℃. It was observed that significant pigment accumulation took place at temperatures ranging from 28-34℃ and that the optimum production was obtained at 32℃ (Figure 3). Likewise, the culture medium with different pH was used to carry out the fermentation process. It was observed that emodin production only occurred in a narrow pH range of 6.5 to 7.5 and the maximal production occurred at pH 7.0 (Figure 4). This indicated that the favorable condition for emodin production

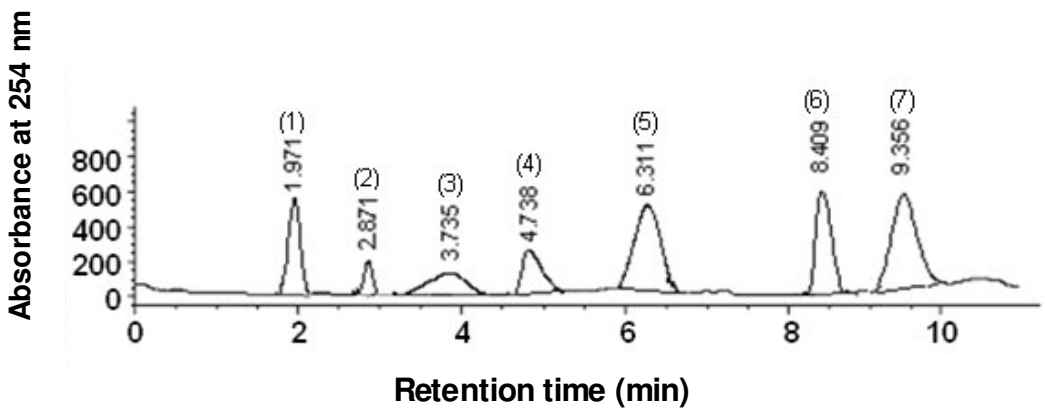


**Figure 5.** Separation chromatogram of the primary extract from *A. ochraceus* on preparative high performance liquid chromatography.

is in a neutral medium and that the yield would be severely affected if the medium were either too basic or too acidic.

## Separation and purification of emodin

There were 7 major peaks observed in the HPLC chromatogram of the samples obtained after solid phase extraction. Among the fractions collected from these peaks, two of them were pigmented, which were from peaks 6 and 7, respectively (Figure 5). The factions from peak 7 were yellowish, which contained emodin after extensive structural analysis. Initially, peaks 6 and 7 overlapped, indicating a poor separation among these two pigmented molecular entities. The chromatographic conditions including the composition and concentration of mobile phase, flow rate and injection volume were subsequently optimized in order to gain better separation. This was done by using various types of organic solvents including acetonitrile and methanol. Experimental results suggested that either 50% acetylnitrile or 50% methanol in water could result in a better separation between emodin and the other pigmented molecules in the preparative chromatography. However, methanol is a better choice when cost is factored in the production process. Furthermore, it was found that the flowrate in the chromatography did not have a significant effect on the separation. Typically, separation was carried out at room temperature with the flowrate set at 80 ml $min^{-1}$ and injection volume at 40 ml in a purification run. Under these experimental conditions for purification, the purity of emodin obtained was about 96% (Figure 6) and the yield of 0.7% or higher of emodin from dry mycelium was consistently achieved.

## Structural analysis of the purified emodin

The pigmented fractions from peak 7 were further purified by preparative liquid chromatography. After chromatography, the final product was obtained as orange amorphous powder with a melting point of 256.1 - 256.9°C. Elemental analysis indicated that the contents of carbon, hydrogen and oxygen were 66.67, 3.71 and 29.62%, respectively. The proportion of carbon, hydrogen and oxygen was 3:2:1, respectively. According to the chemical shift δ (ppm) in the $^{13}C$ NMR spectrum (δ164.41, 107.86, 165.58, 108.82, 120.39, 148.16, 124.03, 161.36, 189.58, 181.21, 132.69, 113.25, 108.76, 134.97, 21.47), this compound had 15 carbons. The quasi-molecular ion at m/z 269.2 $[M-H]^+$ was observed in the ESI-MS. Therefore, the molecular formula was determined to be $C_{15}H_{10}O_5$.

The $^{13}C$-NMR and DEPT spectral data showed four methine carbon atoms (107.86, 108.82, 120.39, 124.03, =CH-), ten quaternary carbon atoms (164.41, 165.58, 148.16, 161.36, 189.58, 181.21, 132.69, 113.25, 108.76, 134.97) and one methylene carbon (δ21.47, $-CH_3$). Among the quaternary carbons, two were from ketones revealed from the chemical shift at 189.58 and 181.21, respectively. The ultraviolet spectra of the purified emodin exhibited a maximum absorbance at 434 nm, typical of an anthraquinone derivative. The $^1H$-NMR spectral data of δ7.405, δ7.089, δ7.0535 (δ7.056, δ7.051, J=2.0), δ6.542 (δ6.545, δ6.539, J=2.4) indicated the three hydroxyl groups were positioned at 1 C, 3 C, and 8 C, respectively and the methyl group was attached to 6 C in anthraquinone. Two signals at low field were observed at δ12.159 and 12.032 for the hydroxyl proton vicinal to oxygen atoms of the ketone groups in anthraquinone. Therefore, the purified compound was confirmed to be 1, 3,8-trihydroxy-6-methyl anthraquinone or emodin (Figure 1).

## Conclusion

In summary, emodin was produced in large quantity from a strain of *A. ochraceus.* Using solid phase extraction followed by preparative liquid chromatography, emodin

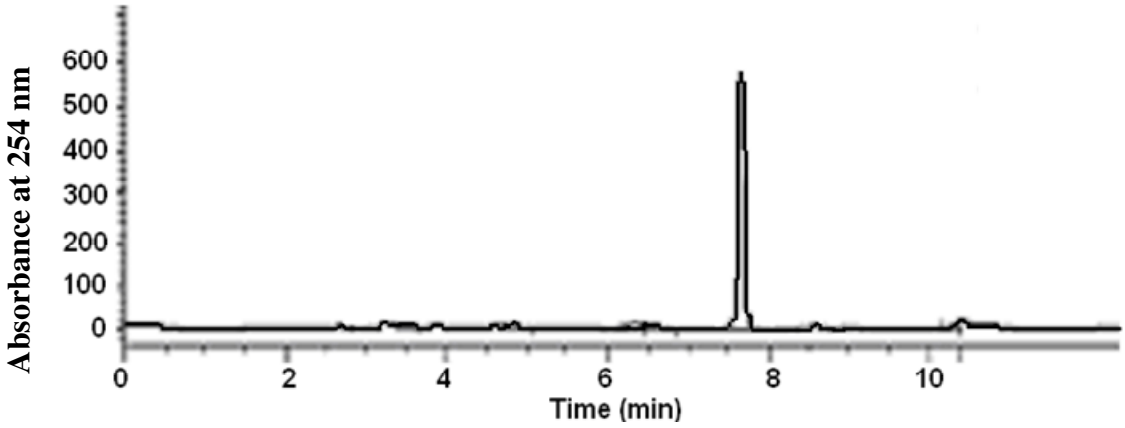


**Figure 6.** Chromatogram of purified emodin after rechromatography on HPLC.

with a purity of 96% or higher can be obtained. The structure of emodin was analyzed and confirmed using various types of analytical techniques. Under optimal conditions, the yield of emodin can reach as high as 0.8% of dry mass of *A. ochraceus*. To the best of the present knowledge, the yield of emodin reported here is higher than those relied on for isolation from traditional Chinese medicinal herbs. Compared to those methods, fermentation of *A. ochraceus* culture for emodin production is more controllable and more amenable to standardization.

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