JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Production of 8-Prenylnaringenin from Isoxanthohumol through Biotransformation by Fungi Cells

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ABSTRACT: 8-Prenylnaringenin (8PN), which presents in hop, enjoys fame as the most potential phytoestrogen. Although a number of health effects are attributed to 8PN, few reports are available about the production of it. In this work, screening of fungi to efficiently transform isoxanthohumol (IXN) into 8PN was designed. The biotransformation of IXN was significantly observed in *Eupenicillium javanicum*, *Cunninghamella blakesleana*, and *Ceriporiopsis subvermispora* under five kinds of transformation conditions. As a comparative result of IXN transformation, *E. javanicum* was the optimal biocatalyst to produce 8PN. Transformation caused by growing precultured fungal mycelia, a process designated as G2, was a favorable condition for IXN transformation in view of the yield of 8PN. The possible transformation pathway of 8PN bioproduction is postulated in this work. The construction of fungus and transformation mode derived from the current work is viable and an alternative procedure for 8PN formation.

KEYWORDS: 8-Prenylnaringenin, isoxanthohumol, biotransformation, identification

INTRODUCTION

Hops (*Humulus lupulus* L.) are a commonly used material in the brewing industry to provide flavor and bitterness to beer. In recent years, hops have proved to be a very rich source of prenylated-flavonoids and derivatives. The most important prenylated flavonoids in hops are xanthohumol (XN), isoxanthohumol (IXN), and 8-prenylnaringenin (8PN) (Figure 1), and they are now receiving more and more attention because of their well-known biological and pharmacological activities. XN, the main prenylflavonoid of hops (0.1−1.0% on dry weight), is a broad-spectrum cancer chemopreventive agent. It shows different inhibition mechanisms at the initiation, promotion, and progression stages of carcinogenesis.[1] Although XN is the major prenlflavonoid in hops, its content is rather low in beer due to the thermal isomerization during the brewing process. The product of thermal isomerization is IXN, a prenylatedflavonoid that has a lower anticancer activity than XN. 8PN in hops shows a potential estrogenic activity,[2] which enjoys fame as the most potential phytoestrogen isolated until now. In vivo and in vitro studies show that 8PN has a considerably higher activity than some other famous phytoestrogens, such as soy isoflavones and genistein.[3,4] Heyerick's research shows that at a low dosage (100 μg daily), 8PN still has an alleviation effect on women who suffer menopausal discomforts.[5] Because of its strong estrogenic activity, 8PN is considered a more natural alternative to hormone replacement therapy (HRT).[6] Additionally, 8PN shows some other bioactivities, such as anticancer,[7,8] osteoporosis prevention,[9] inhibiting angiogenesis,[10] and antioxidant.[11]

At present, 8PN can be easily synthesized by chemical ways from IXN or naringenin,[12,13] but some issues still remain, including complex operation, safety, pollution, and production cost. As compared with chemical methods, biotransformation is a useful tool to produce biologically active compounds with advantages such as high stereo- and regioselectivity, as well as milder reaction conditions, simple operation procedures, and environmental safety.[14,15] In the earlier study, IXN can be converted into 8PN by intestinal microbiota, and *Eubacterium limosum* was identified to be capable of this bioconversion process.[16] However, *E. limosum* is a kind of rigid anaerobic bacterium, and it is rather inconvenient to use anaerobic bacteria in the biotransformation. In some literature, some fungi were found to possess demethylation enzmyes, such as *Aspergillus* sp.,[17] *Mortierella* sp.,[18] *Cunninghamella echinulata*,[19] and *Eupenicillium* sp.[20] It was indicated that the demethylation activity may have a relationship with cytochrome P450.[17,21] Therefore, the aim of this work is to isolate a suitable fungus to produce 8PN from IXN, so as to search for more possible and efficient ways to obtain 8PN.

MATERIALS AND METHODS

Reagents. Standard IXN was purchased from J&K Scientific Ltd. (Shanghai, China). 8PN was purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Acetonitrile and methanol used in high-performance liquid chromatography (HPLC) analysis was chromatographic grade. Water was prepared in a Milli-Q water purification system (Millipore, Bedford, MA). Other chemicals used in the study were analytical grade.

Microorganisms. *Eupenicillium javanicum* AS 3.5706, *Mortierella ramanniana* AS 3.3413, *Mortierella isabellina* AS 3.3410, *Cunninghamella blakesleana* AS 3.910, and *Aspergillus niger* AS 3.429 were obtained from China General Microbiological Culture Collection Center (CGMCC). *Ceriporiopsis subvermispora* ACCC 31513 was bought from Agricultural

Received: March 22, 2011
Accepted: June 2, 2011
Revised: June 2, 2011
Published: June 02, 2011

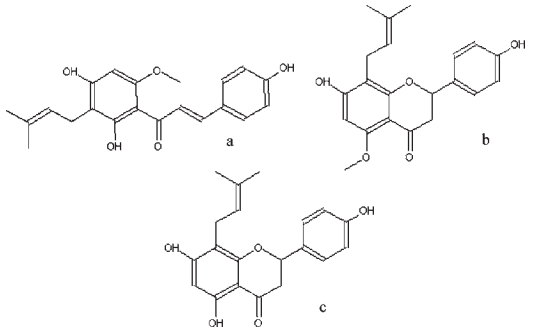

Figure 1. Structures of XN (a), IXN (b), and 8PN (c).

Culture Collection of China (ACCC). *Bjerkandera adusta* CFCC 88349 and *Gloeophyllum trabeum* CFCC 86019 were bought from China Forestry Culture Collection Center (CFCC). *Armillaria luteo-virens* Sacc QH (CGMCC 1884) was obtained from the Qingzang Plain, China, was bred after several natural mutations, and was stored in our lab. The fungi were maintained on potato dextrose agar (PDA) slants at 4 °C.

Preparation of IXN. IXN was prepared according to the reported procedure[13] with minor modification. Spent hops that were extracted by supercritical carbon dioxide were used to prepare XN. The powder (300 g) was extracted with 1.5 L of ethanol under reflux extraction (400 W and 40 °C) for 2 h. The mixture was filtered with 0.22 μm film of Type GV (Millipore), and then, the residue was repeatedly extracted twice (total 1.5 L of ethanol). The filtrate was combined, and extract solution was evaporated to dryness by rotary vaporization at 30 °C. The crude XN was purified by high-speed countercurrent chromatography (HSCCC) using *n*-hexane−ethyl acetate−methanol−water (5:5:4:3) as the solvent system. After HSCCC purification, the purity of XN was up to 96%. IXN was prepared from XN by dissolving in 1% NaOH solution and stirring at 0 °C for 2 h. Then, 50% H_2SO_4 was used to acidify the reaction mixture. It produced a light yellow precipitate, and after filtration and washing several times with water, the product was dried by Christ alpha 1−4 LD freeze dryer (Christ, Germany), and the purity of IXN was estimated to be 82.8% by means of HPLC.

Culture Media. The culture used in the biotransformation experiments was low glucose potato (LGP) medium. It was prepared as the following procedure: 200 g of potato was boiled in water for 30 min, then the solution was filtered, and the filtrates were added with water to 1 L after the addition of 10 g of glucose. The resting cells were cultured in liquid potato media, which contained 2% glucose and 20% potato boiling liquid.

Biotransformation Procedure. Biotransformation experiments were carried out in 250 mL flasks containing 30 mL of culture media. The flasks were placed on a rotary shaker at 28 °C with a shaken speed of 120 r/min. The substrate was dissolved in ethanol to reach a concentration of 6 mg/mL. In all experiments, substrate controls were composed of sterile medium with substrate (IXN) and incubated without the objective microorganism. Culture controls consisted of fermentation blanks in which fungus was grown under identical conditions without substrate.

Growing Cell Transformation System. *Type G1.* The fungi were inoculated into 30 mL of LGP medium; meanwhile, the inoculated flasks were supplemented with 0.1 mL of the prepared substrate solution. Then, the cultures were incubated and transformed for 6 days.

Type G2. The fungi were inoculated into 30 mL of LGP medium; after 3 days of preculture, 0.1 mL of substrate solution was added into each flask, and these flasks were maintained under fermentation conditions for another 6 days.

Resting Cell Transformation System. The preparation procedure of resting cells was as follows: Flasks (250 mL) containing 30 mL of liquid potato medium was inoculated with fungi cells. The cultures were incubated with a shaken speed of 120 r/min at 28 °C for 3 days. After that, culture broth was centrifuged at 4 °C at a speed of 10000 r/min for 15 min. The collected cultured cells were washed with sterilized water three times. Then, the washed wet mycelia were collected and stored at 4 °C for the following experiments.

Three types of systems were designed in this study for the resting-cell transformation based on different liquid transformation broth. The suspension cells (3.0 g wt) were cultivated in 30 mL of 2% glucose (type R1), 0.2 M, pH 7.2, phosphate buffer (type R2), and 0.2 M, pH 7.2, phosphate buffer (0.5% Tween 80 was added, type R3), respectively. Meanwhile, 0.1 mL of prepared substrate solution was added into the flasks. Subsequently, the cultures were incubated for 6 days at 28 °C with a shaken speed of 120 r/min.

Preparation and Isolation of 8PN from Transformation Broth. After incubation, the culture broth was centrifuged at 3000 rpm for 30 min. Then, the supernatant was extracted three times by equivalent volumes of ethyl acetate, all of the organic layers were combined, and the extracted solutions were concentrated in vacuum at 35 °C. Residues were dissolved in methanol for HPLC determination of IXN and 8PN.

Simultaneous Analysis of IXN and 8PN by Means of HPLC. Samples were analyzed on a Waters 2695 HPLC system, which was equipped with two Waters 510 pumps (Waters, Milford, MA), a sample injector (Rheodyne, Cotati, CA) with a 20 μL loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millennium chromatography data system (Waters). The column used was a reversed-phase Symmetry C18 (4.6 mm × 250 mm, 4 μm). The mobile phase was acetonitrile (solvent A) and 1% formic acid (solvent B) with a linear solvent gradient, starting on injection, from 40 to 100% A over 20 min, then followed by 100% A for 5 min. The flow rate was 0.8 mL/min, and the detection wavelength was 288 nm. All of the analysis was performed at a temperature of 30 °C. The standard curve of IXN and 8PN was $y = 35494x - 5034.7$ ($R^2 = 0.9988$), and $y = 27390x - 129.15$ ($R^2 = 0.9993$), respectively. Figure 2a showed the chromatograms of standard IXN and 8PN, and the extraction samples after transformation by *E. javanicum* AS 3.5706 were also identified (Figure 3a). Routine sample calculations were made by the comparison of the peak areas with that of the standard curve. Then, the yield of 8PN was calculated using the following formula:

$$\text{yield of 8PN (\%)} = (C_{fp} - C_{sc})/C_{fs} \times 100\%$$

$$\text{the transformation rate of IXN (\%)} = (C_{fs} - C_{rs})/C_{fs} \times 100\%$$

where C_{fp} is the concentration of 8PN formed in the transformation broth, C_{sc} is the concentration of 8PN in the substrate control, C_{fs} is the concentration of IXN added in the transformation broth, and C_{rs} is the residual concentration of IXN in the transformation broth.

Mass Identification of IXN and 8PN. The mass identification of IXN and 8PN in sample was performed on a TSQ Quantum LC/MS system (Thermo Fisher Scientific, IA), which was equipped with a electrospray ionization (ESI) souce. The optimum conditions of mass identification were applied as follows: spray voltage, 3.0 kV; sheath gas pressure (N_2), 35 MPa; ion sweep gas pressure, 0.0 MPa; aux gas pressure, 12 MPa; capillary temperature 350 °C; and capillary offset, −35 V. During the mass spectra running, the eluates were recorded from m/z 0 to m/z 500, and the fragmentation experiments were carried out on eluting substances. The mass spectra of standards of IXN and 8PN are shown in Figure 2b,c, respectively, by the presence of various fragment ions. Among them, the product ions at m/z 233, 175, 119 (for IXN) and at m/z 219, 133, 119 (for 8PN) can be indicated as the main fragments. As reported in Figure 3b,c, reversed phase high-performance liquid chromatography−electrospray ionization tandem mass spectrometry (RP-HPLC-ESI-MS) mass spectra of IXN and 8PN extracted from the cultures of *E. javanicum* AS 3.5706 were characterized by the fragment ions at m/z 233, 175, 119 (for IXN) and at m/z 219, 133, 119 (for 8PN). Thus, the presence of IXN and 8PN in culture broth was confirmed by ESI-MS as well.

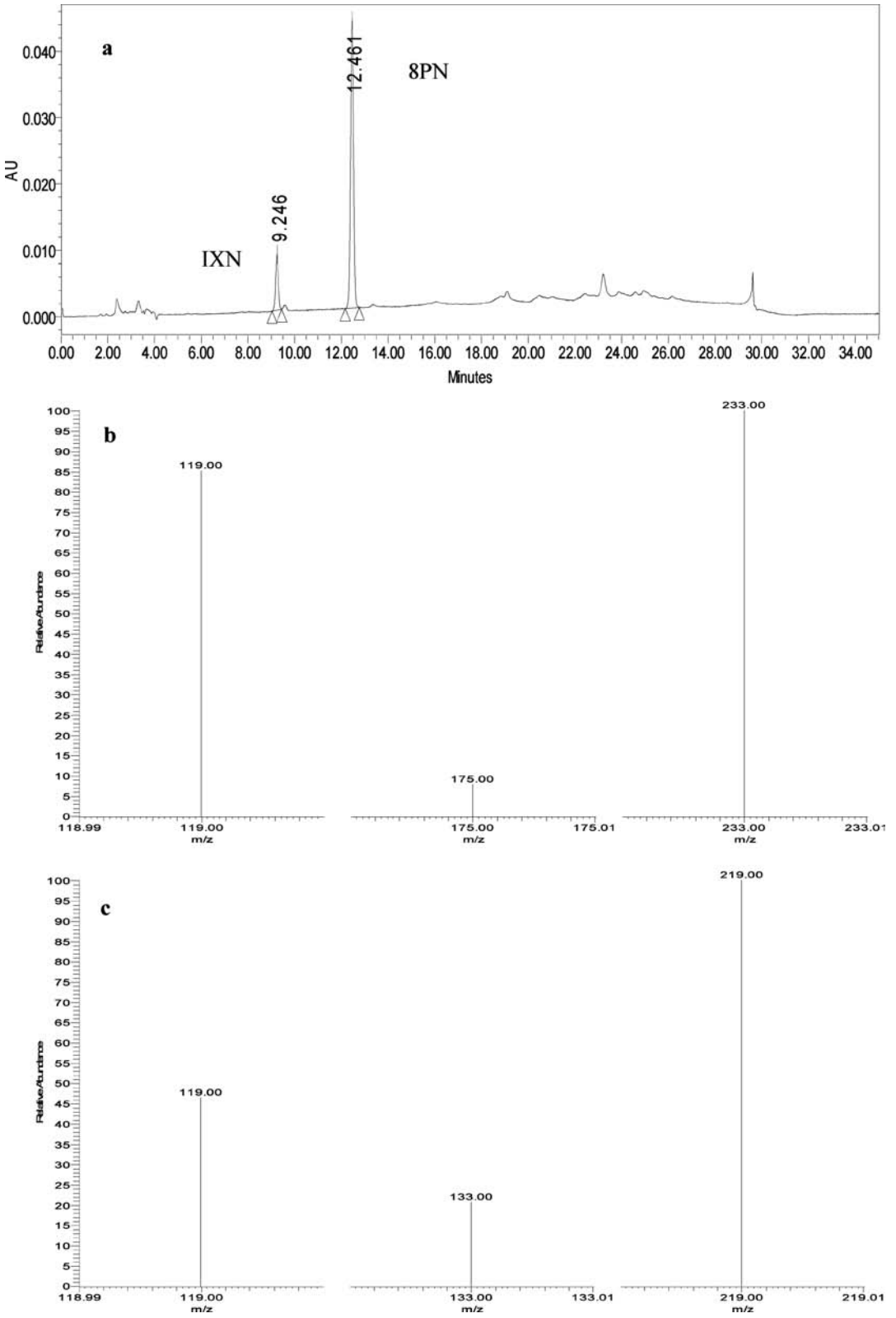

Figure 2. Representative HPLC chromatograms of standard IXN and 8PN (a) and ESI-MS spectra of standards of IXN (b) and 8PN (c).

Statistical Analysis. Data are presented as mean values ± 95% confidence intervals. Analysis of variance (ANOVA) was performed using ANOVA procedures. Significant differences between the different treatments were determined by Tukey's pairwise comparison test at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Screening and Identification of Fungi with Efficient 8PN Production. The biotransformation efficiency and possibility are

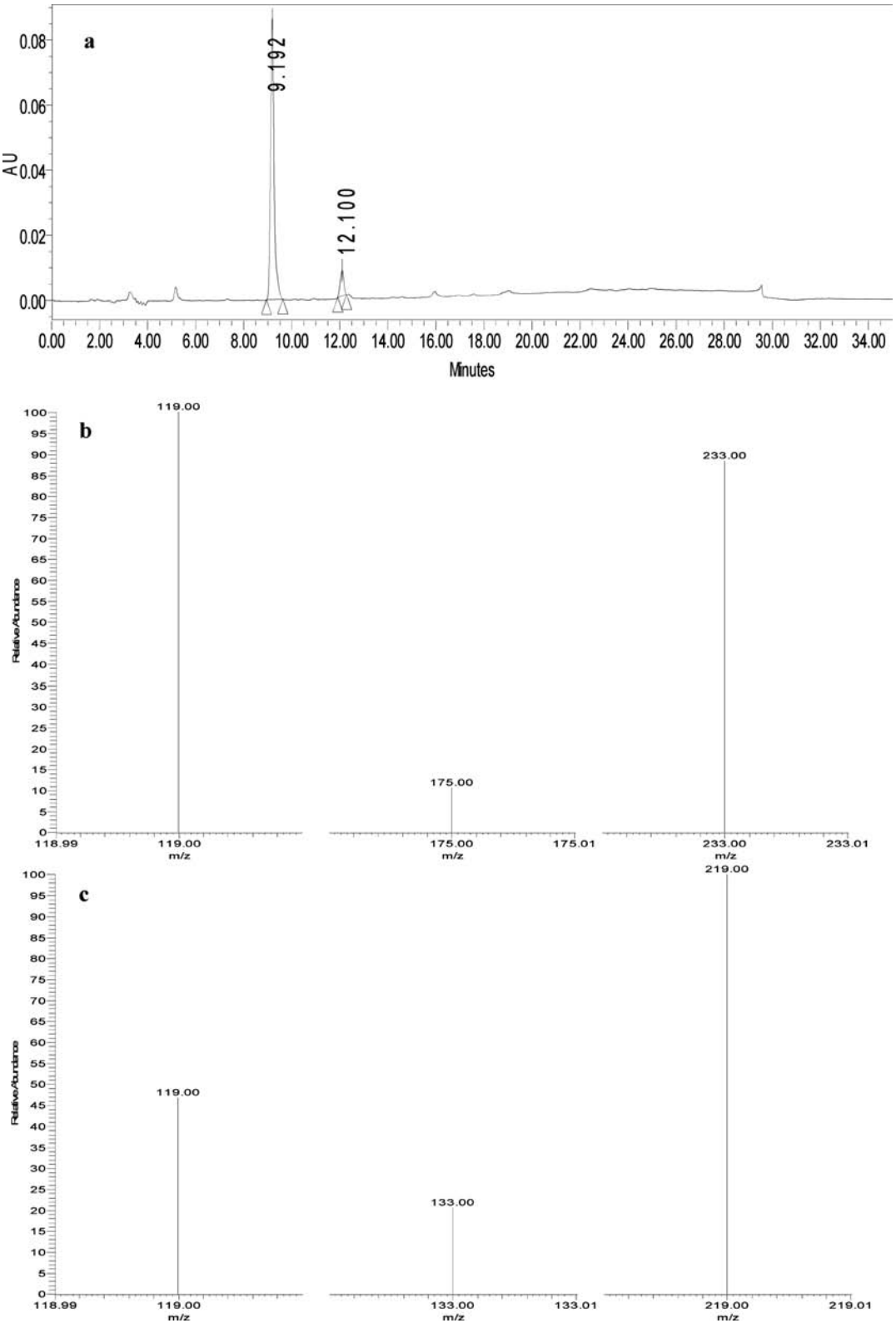

Figure 3. Representative HPLC and ESI-MS data for sample extracted from the cultures of *E. javanicum* AS 3.5706: (a) HPLC chromatograms of sample, (b) mass spectrum for IXN, and (c) mass spectrum for 8PN.

regulated by different biocatalysts under normal culture conditions.[14,15] In this study, the isolation of fungi with 8PN production was taken out. As shown in Table 1, nine kinds of fungi were used to compare 8PN yield from IXN. Among the fungi examined, *E. javanicum* AS 3.5706 showed an outstanding capability of 8PN production (7.25 ± 0.88%, $P < 0.05$)

Table 1. Screened Fungi for 8PN Production from IXN[a]

no.	fungi name	yield of 8PN (%)	transformation rate of IXN (%)
1	*A. luteo-virens* Sacc QH	0.20 ± 0.02 c	93.45 ± 6.72 a
2	*A. niger*	0.91 ± 0.01 c	87.17 ± 1.48 a
3	*E. javanicum*	7.25 ± 0.88 a	88.93 ± 2.07 a
4	*C. subvermispora*	1.49 ± 0.15 b	97.39 ± 0.25 a
5	*M. ramanniana*		97.72 ± 0.11 a
6	*M. isabellina*	0.85 ± 0.14 c	95.86 ± 1.25 a
7	*C. blakesleana*	2.41 ± 0.83 b	96.54 ± 0.45 a
8	*B. adusta*		79.61 ± 17.77 b
9	*G. trabeum*		95.43 ± 2.31 a

[a] Blank cells indicate no detected level. Values in the same column followed by the same letter are not significantly different at $p < 0.05$.

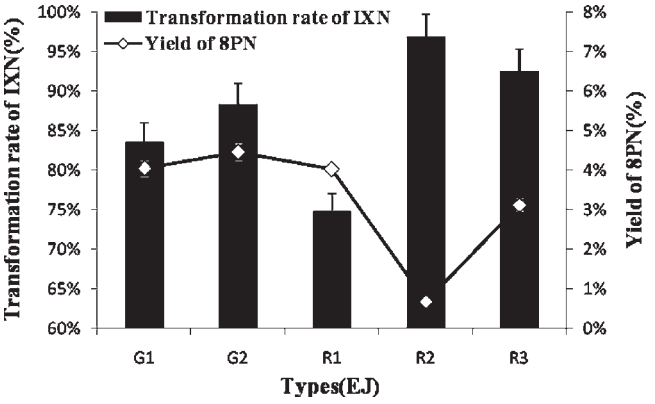

Figure 4. Effect of transformation conditions on IXN conversion and 8PN production by *E. javanicum* AS 3.5706.

transformed from IXN. *C. blakesleana* and *C. subvermispora* were the second choice for 8PN production. Other tested fungi have insignificantly increased effects on 8PN production. Thus, these three fungi with efficient 8PN formation were determined to compare their capabilities under the designed transformation platforms.

As revealed in Table 1, no direct correlation was observed between IXN transformation and 8PN production. The complex structure of IXN and versatile enzymes derived from biocatalysts likely determine biotransformation directions of IXN.

Screening of Biotransformation Conditions for 8PN Production. The transformation efficiency is affected by several factors, such as culture system, transformation conditions, and the type of fungus used. In the present study, thus, the transformation conditions examined are compared with enhanced 8PN production from IXN. The results of screening experiment for biotransformation systems are shown in Table 1. *E. javanicum* AS 3.5706, *C. blakesleana*, and *C. subvermispora* were selected to evaluate the effect of different bioconversion conditions on 8PN production (Figures 4−6).

E. javanicum AS 3.5706 was used to evaluate the effect of designed conditions on IXN conversion. The rate of IXN transformation and yield of 8PN were as well as compared (Figure 4). The growing cell transformation conditions (G1 and G2) had a better increasing effect on 8PN than those of the resting cell transformation. In contrast, the transformation of IXN in the resting cell systems (R1, R2, and R3) was generally

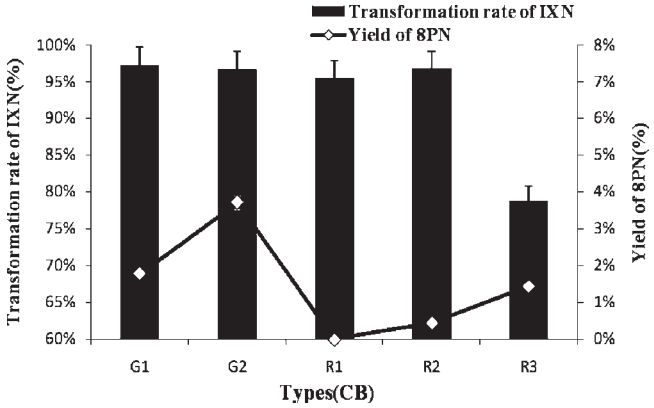

Figure 5. Effect of transformation conditions on IXN conversion and 8PN production by *C. blakesleana* AS 3.910.

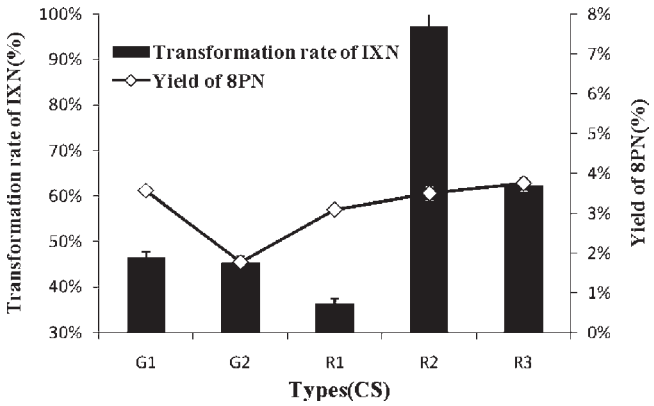

Figure 6. Effect of transformation conditions on IXN conversion and 8PN production by *C. subvermispora* ACCC 31513.

better than the growing cell transformation conditions (G1 and G2). As clearly shown, the G2 condition was better than G1 when 8PN was evaluated. In this work, the results herein imply that the preculture of this fungus before IXN added was beneficial for 8PN formation.

The influence of *C. blakesleana* on IXN was compared under different transformation conditions (Figure 5). On the basis of the results of Figure 5, it showed that G1 and G2 are both favorable for 8PN production in comparison to the resting cell systems. The high yield of 8PN obtained was observed in G2 conditions. No significant differences for IXN transformation rate were observed under the five designed conditions.

As well, the effect of *C. subvermispora* on IXN transformation was evaluated using five designed systems (Figure 6). Clearly, R2 showed the best transforming ability among the designed experiments (98.8%, $P < 0.05$). Although the transformation degree of IXN by *C. subvermispora* was generally lower than 90%, the yield of 8PN (>3%) was higher than *E. javanicum* AS 3.5706. The present results suggest that there is no direct correlation between IXN transformation and 8PN formation. That is because besides the demethylation, there are also many complex metabolic pathways that likely exist for IXN biotransformation by fungi, which may include oxidation, degradation, glucosylation, and so on.

The comparisons of five transformation conditions using three designed biocatalysts were further carried out. As presented in

Figures 4−6, G2 is the favorable condition as the yield of 8PN is evaluated. Thus, the transformation condition has a great influence on conversion process. The form of substrate in the presence of developed mycelia and the addition time both affect the bioconversion yield. The presence of Tween 80 in the transformation broth is useful for 8PN formation. The solubility degree of precursor in the transformation broth not only affects the contact between fungal cells and precursor, due to its toxic effect, but also decreases mycelia viability.[15] In contrast, in the present work, the effect of the precursor on fungal cells was not elucidated.

Comparison of Fungi with 8PN Production from IXN. The development of biocatalysts with efficient productivity of 8PN is the main objective in this study. In considering of the comparative results of Figure 7, *E. javanicum* AS 3.5706 had the higher yield of 8PN (generally above 4%) under five kinds of the designed conditions, that of which was considered the desirable fungus to converse IXN in the future study. *E. limosum* was identified to be capable of this conversion (O-demethylation) of IXN into 8PN from 12 fecal cultures.[16] In this investigation, *E. javanicum* is another fungus identified with an efficient yield of 8PN. More importantly, this fungus can be aerobically grown and easily operated. IXN seems to be an ideal substrate for 8PN biosynthesis. As reported previously, human intestinal microbiota may activate up to 4 mg/L IXN in beer into 8PN by means of the simulator of the human intestinal microbial ecosystem (SHIME).[6]

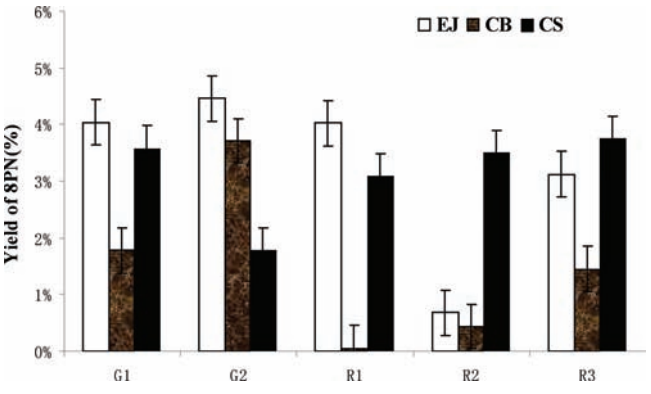

Figure 7. Comparison of *E. javanicum* AS 3.5706, *C. blakesleana* AS 3.910, and *C. subvermispora* ACCC 31513 for 8PN production under the designed transformation conditions.

8PN is one of the strongest phytoestrogens known in nature. This compound and its precursors, XN and IXN, may also have antibreast cancer activity.[22] XN is readily accessible from carbon dioxideextracted hops where its content ranges to up to 1% of dry matter. Although IXN is present in this source with low concentrations, it can be easily obtained from XN by dissolving in 1% NaOH and acidification of the reaction mixture.[13] Because of the interesting biological properties, several synthesis methods have been speculated, which included synthesis from naringenin, phloroacetophenone with low yield, or XN.[13] Among them, the demethylation of XN can form 8PN by means of chemical methods. However, this procedure results in the formation of a mixture of epimers that are difficult to separate. Thus, the chemical protections in the special groups are necessary, that of which lead to problems including operating safety and production costs.

Proposed Biotransformation Pathway of 8PN. Until now, little has been known about transformations of prenylflavonoids by aerobic microorganisms or intestinal microflora. The importance of this microbial community in the metabolism of phytoestrogens has been clearly established. Decroos et al.[23] recently isolated a microbial consortium capable of transforming soy phytoestrogen daidzein into equol, and Wang et al.[24] found two bacteria responsible for the conversion of lignans. Microbial O-demethylation (8PN from IXN) is known for a number of anaerobic bacteria that can even use methyl ethers for respiratory growth. These bacteria either use fumarate as an electron acceptor[25] or are so-called homoacetogens converting CO_2 into acetate.[26] *E. limosum* is a homoacetogenic bacterium, which is frequently isolated from human feces.[27] It is known to be involved in the biotransformation of specific isoflavonoids.[28] As the results obtained from the present work, it showed that 8PN can be produced through biotransformation by a suitable fungus, *E. javanicum*. Obviously, the demethylation of IXN was a well-known pathway, and others involved in 8PN formation are a prenylnation process using naringenin as the precursor. As demonstrated in Figure 8, the possible pathways of 8PN production were postulated. Sasaki et al.[29] identified a prenyltransferase gene from *Sophora flavescens* SfN8DT-1, responsible for the prenylation of the flavonoid naringenin at the 8-position, which may form 8PN. In addition, a demethylation process is possibly one other step for 8PN production. Although demethylase is not still identified in the cultured cells, this process may play an important role in 8PN biosynthesis. Thus, as for 8PN

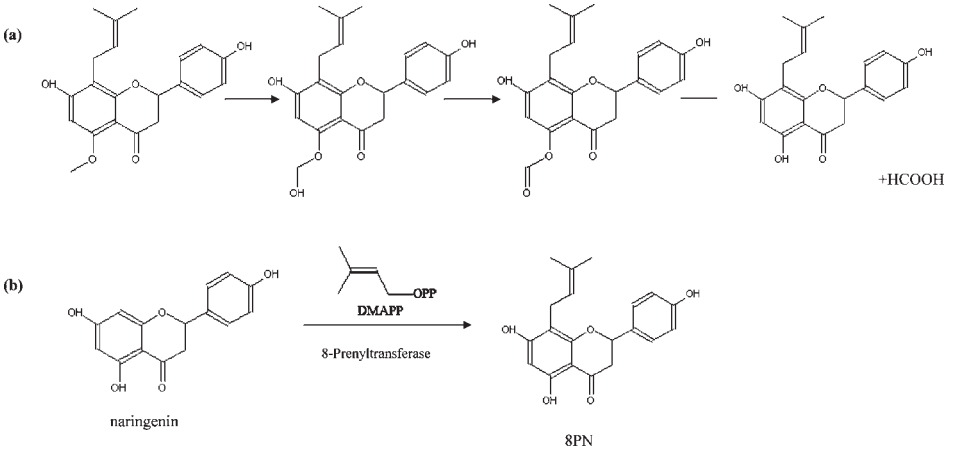

Figure 8. Proposed possible metabolic pathway of 8PN from IXN (a)[17] and naringenin (b).[29,30]

production, the detailed mechanism under the designed bioconversion condition remains to be elucidated in the future, importantly focused on the identification of demethylation enzymes involved.

In summary, as started from XN a byproduct of hops extraction, 8PN can be formed via IXN using the culturing fungi cells. In this study, *E. javanicum* was found to be an optimal fungus for 8PN biosynthesis. The optimal biotransformation condition was derived from the comparative results of five different procedures. This study further shows that 8PN can be produced through biotransformation strategies; among them, thhe demethylation process may be a crucial step for 8PN formation. However, the characterization and genetic engineering of enzymes that regulate 8PN biosysnthesis remain to be deeply uncovered.

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ABBREVIATIONS USED

8PN, 8-prenylnaringenin; IXN, isoxanthohumol; XN, xanthohumol; HRT, hormone replacement therapy; HPLC, high-performance liquid chromatography; ACCC, Agricultural Culture Collection of China; CFCC, China Forestry Culture Collection Center; CGMCC, China General Microbiological Culture Collection Center; HSCCC, high-speed countercurrent chromatography; LGP, low glucose potato medium; ESI, electrospray ionization; RP-HPLC-ESI-MS, reversed phase high-performance liquid chromatography−electrospray ionization tandem mass spectrometry; ANOVA, analysis of variance; SHIME, simulator of the human intestinal microbial ecosystem

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