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Improving Flavonoid Extraction from *Ginkgo biloba* Leaves by Prefermentation Processing

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ABSTRACT: This paper presents a prefermentation treatment method involving fungi to improve flavonoid extraction from the leaves of *Ginkgo biloba*. The fungi employed for this treatment were screened from the soil present under an ancient ginkgo tree. Seventy-six strains belonging to 23 genera were isolated and identified by a molecular identification method employing 18S rDNA sequences. Thirty-three strains grew well using ginkgo leaves as the growth medium. One strain, Gyx086, with higher extracted yield of flavonoids and more similar to the control, was finally selected for prefermentation processing. The major fermentation factors were optimized by response surface methodology. The optimal conditions for the highest total falvonoid yield were 27.8 °C for temperature, 64.2% for moisture content, and 61 h for fermentation time. Under the optimal condition, a actual total flavonoid yield of 27.59 ± 0.52 mg/g dry weight culture sample was obtained, which was about 70% higher than that of unfermented gingko leaf samples.

KEYWORDS: *fungus, Ginkgo biloba leaves, solid-state fermentation (SSF), flavonoids, response surface methodology (RSM)*

INTRODUCTION

Ginkgo biloba is one of the most popular plants, containing some functional components, such as flavonoids and anthocyanins; as a result, it was used for the treatment of disease in ancient China. The first mention of the use of ginkgo leaf for medical purposes dates back to 1505 A.D. in the text "Ben Cao Pin Hui Jing Yao" by Liu Wen-Tai. Leaf extracts used for improvement of the blood circulation, both peripherally and centrally, started in the 1960s in Germany.[1,2] Leaf extracts have two types, namely, full extracts and standardized extracts. The former is usually prepared with alcohol extraction and contains all constituents soluble in it. The latter is the more common one encountered and contains 6% of terpene trilactones (TTLs), 24% of flavonol glycosides, and <5 ppm of ginkgolic acids.[3]

Flavonoids in ginkgo leaves are one of the most popular herbal supplements.[1] The leaves are rich in all kinds of flavonoids including flavonol glycosides, biflavones, proanthocyanidins, and isoflavonoids of them; the flavonol glycosides are most prevalent in *G. biloba* leaves and extracts.[4] Hydrolysates of flavonol glycosides contain three predominant flavonol aglycones, that is, quercetin, kaempferol, and isorhamnetin, which are present in only small amounts in leaves.[5,6] Flavonoid components are believed to act as protectants against capillary fragility, antioxidants, and anti-inflammatory agents, in reducing edema caused by tissue injury, and as free radical scavengers.[7−9] Therefore, it is widely used for medicaments, foods, and cosmetics by using *G. biloba* extract (EGb).

Ethanol/water and acetone/water in different ratios and pure methanol are the most common solvents for flavonoid extraction. However, only ethanol/water is generally used in commercial processes in that it is safe and economical. Chiu et al.[10] have reported supercritical fluid extraction of ginkgolides and flavonoids. Although supercritical carbon dioxide offers safety, dry supercritical fluids cannot extract terpene lactones and flavonoids, and thus ethanol addition as a cosolvent is needed. To facilitate rapid extraction of flavonoids from the cells, it is beneficial to break open the intact cells by employing some physical or biochemical techniques. Physical techniques such as ultrasonication and microwave-assisted technology assist flavonoid extraction from *G. biloba* or other plants, leading to higher extraction yield.[11,12] Enzyme-assisted extraction of plant flavonoids has also been used by several groups: Landbo and Meyer[13] employed pectinase and protease for anthocyanin extraction from black currant juice press residues; Fu et al.[14] employed pectinase, cellulase, and *β*-glucosidase for the extraction of luteolin and apigenin from pigeonpea leaves; Chen et al.[1] improved flavonoid extraction yield from *G. biloba* leaves by using the cellulase produced by *Penicillium decumbens*.

Most fungi are known to be capable of degrading lignocelluloses, and their ability to facilitate rapid lignocellulose degradation has attracted more attention from scientists.[15] Microbial biodegradation of lignocellulose is dependent not only on the type of microbe alone but also on its growth conditions.[16] This ability mainly originates from the concerted activity of its enzymatic system. Accordingly, we propose a prefermentation treatment method involving fungi to improve the extraction yield of flavonoids from *G. biloba*. The fungi used for prefermentation were screened from the soil under an ancient *G. biboba* tree, and pure gingko leaves were used as cultures to facilitate flavonoid release. By optimizing the prefermentation conditions, the extraction content of total flavonoid from gingko leaves was obviously improved. The new method involving fungi to improve extraction yield of

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flavonoids from *G. biloba* was demonstratively feasible. To the best of our knowledge, this paper is the first to report the extraction of gingko flavonoids by a fermentation method.

MATERIALS AND METHODS

***G. biloba* Leaves.** The *G. biloba* leaves used in this study were bought from Pizhou Ginkgo Institute (Jiangsu, China). Dry leaves were ground into powder, followed by sieving through an 840 μm screen to control the particle size.

Soil Samples. Soil samples were collected from the area under ancient ginkgo trees near An'lu city (Hubei, China) in May 2011. Samples were drawn from a depth of 0−25 cm below the surface of decomposed leaf litter, crushed by a wooden hammer, sieved through a 2000 μm screen, and transferred into polythene bags. The bags were sealed and stored under moist conditions with a relative humidity value 65% at 4 °C until analysis.

The characteristics of the soil samples were studied as follows: The moisture content of the soil samples was determined by an M35 infrared moisture analyzer (Sartorius, Germany), consisting of a 60 min long dry program at 105 °C. The organic matter content was analyzed by burning at 550 °C for 4 h in an electric muffle furnace, and the pH value of the soil sample was measured in 1:1 soil/water by using a pH-meter (Shanghai "SSS" reagent Co., Ltd., China).

Isolation and Identification of the Fungal Strains. One gram soil samples were suspended in 100 mL of sterile tap water (autoclaved at 121 °C for 30 min) and stirred at 200 rpm for 15 min to establish a dilution series as described by Ulrich et al.[17] After the suspension had rested for 15 min, it was serially diluted from 10^{-3} to 10^{-6}. Aliquots (0.5 mL) were collected and spread on 11 cm plates containing Martin's agar for enumerating the colony-forming units (CFUs) of aerobic fungi. This was performed in replicates. All plates were incubated at 28 °C for 5 days. Under a 10× magnifying glass, the mycelium of a single colony was further purified to obtain a pure strain.

We tried to identify these strains by using 18S rDNA sequencing. The fungal hyphae were harvested from the agar plates. Hyphae that could not be immediately processed were frozen at −80 °C prior to extraction. Liquid nitrogen was added to 0.1 g of hyphae in a 1.5 mL microcentrifuge tube, and the cells were finely ground with a pestle. Isolation and purification of fungal DNA were carried by a specification program provided by Fungal DNA Kit (Omega, USA). 18S rDNA PCR amplification was carried out using the fungal-specific primers, namely, EF4f (5′-GGA AGG G[G/A]T GTA TTT ATT AG-3′) and Fung5r (5′-GTA AAA GTC CTG GTT CCC-3′), designed by Smit et al.[18] and commercially synthesized by Shanghai Generay Biotech Co., Ltd., China. The 50 μL reaction mixture contained 1× PCR buffer, 5 mM $MgCl_2$, 2 mM dNTPs, 2.5 U of Taq polymerase from Fermentas, 10 μM primers (each), and 1 μL of DNA template. The thermal cycler was heated to 94 °C for 3 min; then, 35 cycles were run at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1.5 min; and finally, it was settled at 72 °C for 7 min. The product was examined by 2% agarose gel electrophoresis, and the DNA sequence was determined by Sangong Biotech (Shanghai) Co., Ltd. The sequences were then analyzed using the NCBI Blast and RDP Chimera Check programs. The GenBank sequences most similar to those of the clones, along with their morphological features, were used for classifying the fungal strains to specific genera.

Solid-State Fermentation Pretreatment. Dry ginkgo leaves and water were mixed using an SM-25 screw mixer (Sanyo, Japan) with a shred/water mass ratio of 5:6 for 20 min. The mixture was rationed to 250 mL triangular flasks with 85 g of the mixture in each bottle, consuming about 50% of the bottle's volume. These bottles were sealed with a hydrophobic fluoropore (PTFE) membrane (Tianke, Shanghai Jiafeng Horticulture Commodity Co., China) and sterilized at 115 °C for 15 min without significant loss of flavonols from the raw material ($p < 0.05$).

The mycelia of all pure strains were cultivated at 28 °C for 5 days; 0.03 g of mycelia was inoculated into a 250 mL triangular flask on a BCM-100A sterile console (Airtech, Suzhou Antai Air Tech Co., Ltd., China). The experiment was performed in triplicates. After sealing with the PTFE membrane, the flask was inverted 10 times to spread the mycelium. These flasks were tilted on the splint of the incubator at 26 °C for 7 days. The solid-state fermented samples (SSFS) were prepared for the analysis of flavonoids and cellulase. Noninoculated cultures with the same cultivated process were used as control/non-SSFS.

Extraction of Flavonoids. For extraction of flavonoids, 70% (v/v) aqueous ethanol was used as extraction solvent. Ten grams of SSFS or non-SSFS was placed in a 250 mL conical flask with a cover, 200 mL of the solution was added, and the sample was immersed in the solution by gentle shaking. The extraction was executed in the ultrasonic facilities (XO-5200DTNSN, Nanjing Sinotech Co., Ltd., China) with an optimized ultrasonic-assisted program at a power of 100 W, an ultrasonic frequency of 25 kMz, a time period of 45 min, and a temperature of 60 °C. After filtration, the filtrate was collected into a clean receiving flask, and the filtrate cake was subjected to the above extraction process once again. Twice-filtered liquid samples were combined for determining the total flavonoid content (TF).

Determination of TF. The UV−vis spectrophotometry method described by Chang et al.[19] was used to determine the total flavonoid content. Half a milliliter of the double-dilute extract (from SSFS or non-SSFS) or the quercetin standard solution was mixed with 3 mL of methanol, 0.2 mL of 10% aluminum chloride (substituted by distilled water for use as blank), 0.2 mL of 1 M potassium acetate, and 5.6 mL of distilled water. After 60 min of incubation, the absorbance at 415 nm was determined against a distilled water blank on a UV mini-1240 spectrophotometer (Shimadzu, Japan). All samples were prepared in triplicates, and the mean values of the crude flavonoid content are expressed as milligrams of quercetin equivalents per gram of dry weight (dw), calculated according to the standard calibration curve.

Analysis of Cellulase Activity. A filter paper assay method was used to determine total cellulase activity by referring to the Chinese national standard GB/T 23881-2009. Five grams of SSFS or non-SSFS was extracted with 200 mL of citrate buffer (0.05 mol/L, pH 5.5). The suspension was mixed by a magnetic mixer for 30 min at room temperature and followed by 24 h of incubation at 4 °C. After filtration, the filtrate was collected for analysis. Fifty milligrams of quantitative paper (Hangzhou Xinhua Paper Industry Co., Ltd.) was soaked with 1.0 mL of citrate buffer in a 25 mL test tube, mixed with 0.5 mL of the enzyme (0.04−0.18 U/mL), and incubated at 37 °C for 60 min. The reaction was terminated by adding 2.0 mL of dinitrosalicylic acid (DNS). The control tube was synchronously incubated with additional 2.0 mL of DNS. The reducing sugar content was estimated by the DNS method.[20] One unit of activity was defined as the amount of enzyme that released 1 μmol of cellobiose reducing equivalent per minute. The filter paper activity (FPA) of cellulase is expressed as the units released per minute per gram of dry weight SSFS.

HPLC Analysis of the Flavonoids. Flavonoids in the SSFS and non-SSFS were analyzed by a HPLC method; HPLC was carried out using a Waters HPLC 2695 system (Waters, USA) equipped with a Waters XBridge C18 column (5 mm, 150 mm × 4.6 mm; Waters Corp., USA) and a 2489 UV detector at 370 nm. Mobile phase A was 1% phosphoric acid solution, and B was methanol; the column temperature was maintained at 40 °C, and the flow rate was set at 1.0 mL/min with 5 μL as the injection volume. HPLC fingerprints were obtained by following a linear gradient program: from 5% of mobile phase B to 30% of mobile phase B in the 9th min, to 40% in the 21st min, to 45% in the 40th min, to 60% in the 45th min, to 70% in the 51st min, to 90% in the 60th min, and with the termination routine of 90% of mobile phase B running for 20 min. The HPLC fingerprints were used for comparing the flavonoid component in SSFS and non-SFFS. Chromatographic peaks of the flavonoids extracted from the control were set as target peaks. The chromatographic peaks of the SSFS flavonoids were one-to-one correspondence of the target peaks with ±3% of retention time deviation. If all of the peaks could be one-to-one to the target peaks, the peak similarity was considered to be 100%.

The standards of quercetin, kaempferol, and isorhamnetin (Shanghai Generay Biotech Co., Ltd., China) were used for analysis of the predominant flavonol aglycones of SSFS. As the peak areas of kaempferol and isorhamnetin are too small to calculate, the analysis process was performed with an injection volume of 10 μL and with a different elution program, in which the mobile phase was kept constant at 55% of mobile phase B and the flow rate was 0.8 mL/min for 30 min.

Box−Behnken Design (BBD) and Statistical Analysis. Response surface methodology (RSM) was used to optimize the pretreatment conditions for the total flavonoids yield of SSFS by Gyx086. A BBD with three factors and three levels including three replicates at the center point was used for fitting a second-order response surface. The factors and levels were determined on the basis of the preliminary results. Actual ranges and levels of the independent variables investigated in this study are shown in Table 2. The BBD was produced by SYSTAT 12 (Systat Software, Inc., Washington, USA), and the software was used to analyze the data, describe the response surface, and generate the response surface plots. The goodness of fit of the second-order polynomial model equation was indicated by an F test at the 5% level of significance, the determination coefficient R^2, and the lack of fit. Response surface contour plots were generated to indicate the possibility of increasing the yield of total flavonoid in SFFS by Gyx086.

The statistical analysis used for TF and FPA was carried out by the one-way ANOVA program of the SAS system for Windows 8.02 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was selected as the comparison method in the program, and the significance level was set at 0.05.

RESULTS AND DISCUSSION

Soil Characterization and Screening Fungal Strains. Breaking the intact cell of ginkgo leaf is a prerequisite for improving flavonoid extraction. The biodegradation of plant cell wall (mainly composed of lignocellulose) depends on the degradative capacity of the microorganism.[16] Thus, screening of the fungal strains is the key factor for flavonoid extraction. The strains used in this study were isolated from uncultivated soil under ancient ginkgo trees with over 200 years of tree age. The existence of fungi in this soil is due to long-time natural selection and the presence of deciduous ginkgo leaves. These strains are considered to possess strong adaptability and to cause efficient degradation of *G. biloba* leaves.

Moisture content, organic matter content, and the pH value of the soil under the ancient ginkgo tree were 18.53%, 10.58%, and 5.4, respectively. Such soil with high plant organic matter content and a pH value toward the acid extreme is suitable for fungal growth, particularly for molds.[21] According to the method described under Isolation and Identification of the Fungal Strains, 107 pure fungal isolates were obtained from the soil samples with respect to their morphological characters, including 5 yeast strains and 102 mold strains. Only 76 isolates were finally preserved in 50% glycerin under cryogenic storage after repeated morphological observation.

These isolates were identified by 18S rDNA sequencing. They were classified into 23 genera: *Aspergillus* spp. (16 strains), *Penicillium* spp. (19 strains), *Hypocrea* spp. (5 strains), *Bionectria* spp. (4 strains), *Cladosporium* spp. (3 strains), *Mucor* spp. (3 strains), *Plectosphaerella* spp. (3 strains), *Eupenicillium* spp. (3 strains), *Gibberella* spp. (2 strains), *Alternaria* spp. (2 strains), *Stachybotrys* spp. (2 strains), *Torulaspora* spp. (2 strains), *Fusarium* spp. (2 strains), *Pleosporales*spp. (1 strain), *Geomyces* spp. (1 strain), *Ulospora* spp. (1 strain), *Candida* spp. (1 strain), *Verticillium* spp. (1 strain), *Chaetomium* spp. (1 strain), *Cryptococcus* spp. (1 strain), *Rhodotorula* spp. (1 strain), *Acrostalagmus* spp. (1 strain), and *Ascomycetes* spp. (1 strain).

TF of SSFS. Each fungal strain was inoculated in ginkgo leaf powder medium for SSF pretreatment. However, not all strains could grow well in this medium. Forty-three strains could not survive in this medium, and their survival in soil possibly depended on coexistence with fungi, bacteria, or other organisms.[22,23] Another 33 strains (10 *Aspergillus* spp. strains, 10 *Penicillium* spp. strains, 4 *Hypocrea* spp. strains, 3 *Mucor* spp. strains, 2 *Fusarium* spp. strains, 1 *Eupenicillium* spp. strain, 1 *Gibberella* spp. strain, 1 *Alternaria* spp. strain, and 1 *Pleosporales* spp. strain) grew well in the medium, with their mycelia covering the medium after 7 days of incubation, which showed that ginkgo leaves could meet their growth requirements. To clarify if these fungi were beneficial for flavonoid extraction, TF of the SSFS fermented by each strain was determined (Table 1). At the same time, the cellulase activity of the SSFS was also determined to analyze its relationship to the TF.

TF in the SSFS ranged from 13.83 ± 0.41 to 19.39 ± 0.34 mg/g dw. Thirteen SSFS had higher TF, ranging from 16.18 ± 0.26 to 19.39 ± 0.34 mg/g dw, than that of the control (15.87 ± 0.11 mg/g dw) by the variance analysis of means ($p < 0.05$). Sixteen SSFS had identical TF to the control, and another 4 SSFS had lower TF than the control. Cellulase degraded lignocellulose to enhance cell wall breakdown,[1] which might be beneficial to flavonoid release. All SSFS had significantly higher FPA than that of the control ($p < 0.001$), but only 13 SSFS showed higher TF. In an attempt to correlate cellulase activity with TF released, the measured values of FPA and TF in all SSFS were analyzed using the "regression-liner-least squares" program of the SYSTAT software. PFA (X) and TF (Y) were selected as independent and dependent, for fitting a line model. The mathematical equation obtained was $Y= 15.074 + 0.619X$. The F ratio of the line model was 37.437 with a low probability p value ($p < 0.001$) by analysis of variance, indicating high significance of the model. The positive coefficient indicated FPA was positively correlated to TF. However, the adjusted squared multiple R (R^2) was only 0.109, which suggested the goodness of fit was unsatisfactory and the observed values could be very little predicted by the equation. An analogous correlation between FPA and TF was applied to identical genera (*Aspergillus* app., *Penicillium* spp., *Hypocrea* spp., and *Mucor* spp.). The adjusted R^2 values were 0.059, 0.243, 0.437, and 0.400, respectively. In fact, FPA might only be one of the important factors correlating to the TF in the SSFS. The TF of the SSFS might be affected by (a) enzyme content, enzyme activity, and enzymatic composition (such as cellulase, pectinase, and protease);[13,14,24] (b) flavonoids produced by some endophytes;[25] and (c) flavonoids consumed by microbial growth.[26,27] In our other experiments, none of the fungi could produce flavonoids on Martin's agar (date not shown). So (a) and/or (c) might mainly influence TF of SSFS fermented by different fungi. The SSFS of Gyx017 had the highest flavonoid extraction yield of 19.39 ± 0.34 mg/g dw, but a low FPA of 1.25 ± 0.24 U/min·g dw. This could be explained as other enzymes (such as protease) producing by Gyx017 synergistically decomposed ginkgo leaf with cellulase, or the dissimilar structures or compositions between the ginkgo leaf and the quantitative paper (used for the FPA determination), which resulted in the enzymatic composition effectively hydrolyzing the cell of ginkgo leaf to release flavonoids, but inefficiency to the filter paper. Some SSFS with high FPA but common TF (such as Gyx048) might be due to inefficiency degradation of the leaf by its inappropriate enzyme composition or flavonoid

Table 1. TF and FPA of the Control and the SSF Samples

serial no.	name of strain	fungal genus of strains	FPA[a] (U/min·g dw)	TF[a] (mg/g dw)
1	control		0.38 ± 0.14 t	15.87 ± 0.11 e
2	Gyx003	*Aspergillus niger*	2.16 ± 0.47 j	16.24 ± 1.44 e
3	Gyx017	*Aspergillus awamori*	1.25 ± 0.24 p	19.39 ± 0.34 a
4	Gyx026	*Aspergillus versicolor*	1.56 ± 0.29 m	17.03 ± 0.30 c
5	Gyx027	*Aspergillus niger*	1.18 ± 0.38 q	17.15 ± 0.28 c
6	Gyx067	*Aspergillus clavatus*	1.01 ± 0.37 r	16.60 ± 0.63 d
7	Gyx082	*Aspergillus versicolor*	0.98 ± 0.25 r	14.70 ± 0.98 f
8	Gyx066	*Aspergillus ustus*	2.84 ± 0.56 d	15.62 ± 0.60 e
9	Gyx086	*Aspergillus niger*	3.40 ± 0.27 a	18.51 ± 1.17 b
10	Gyx091	*Aspergillus niger*	1.88 ± 0.43 j	16.18 ± 0.26 d
11	Gyx105	*Aspergillus awamori*	1.72 ± 0.50 l	16.08 ± 0.26 e
12	Gyx110	*Eupenicillium*	1.19 ± 0.18 q	13.83 ± 0.41 g
13	Gyx024	*Penicillium purpurogenum*	1.36 ± 0.22 o	15.79 ± 0.93 e
14	Gyx032	*Penicillium purpurogenum*	1.93 ± 0.26 j	15.60 ± 0.49 e
15	Gyx051	*Penicillium chrysogenum*	1.16 ± 0.03 q	15.71 ± 0.75 e
16	Gyx057	*Penicillium camemberti*	2.22 ± 0.67 i	16.70 ± 1.81 d
17	Gyx070	*Penicillium purpurogenum*	1.14 ± 0.29 q	14.63 ± 1.16 f
18	Gyx077	*Penicillium purpurogenum*	2.49 ± 0.09 f	15.90 ± 0.39 e
19	Gyx096	*Penicillium glabrum*	1.24 ± 0.10 p	15.58 ± 1.22 e
20	Gyx100	*Penicillium javanicum*	1.73 ± 0.59 k	15.45 ± 1.42 e
21	Gyx114	*Penicillium*	0.66 ± 0.02 s	16.05 ± 0.12 e
22	Gyx106	*Penicillium chrysogenum*	1.98 ± 0.01 j	15.36 ± 1.09 f
23	Gyx016	*Hypocrea koningii*	3.06 ± 0.64 b	16.49 ± 0.95 d
24	Gyx019	*Hypocrea jecorina*	1.61 ± 0.20 m	15.91 ± 1.13 e
25	Gyx058	*Hypocrea jecorina*	2.30 ± 0.43 h	15.75 ± 1.76 e
26	Gyx059	*Hypocrea koningii*	2.44 ± 0.58 g	17.13 ± 1.51 c
27	Gyx103	*Alternaria alternata*	1.30 ± 0.09 o	18.77 ± 0.40 b
28	Gyx028	*Verticillium dahliae*	2.06 ± 0.13 j	15.95 ± 0.36 e
29	Gyx044	*Mucor ramosissimus*	2.57 ± 0.39 e	16.93 ± 0.82 d
30	Gyx041	*Mucor ramosissimus*	2.27 ± 0.22 h	16.66 ± 1.53 d
31	Gyx050	*Mucor ramosissimus*	1.82 ± 0.19 k	16.54 ± 1.01d
32	Gyx047	*Gibberella fujikuroi*	2.03 ± 0.07 j	15.81 ± 0.12 e
33	Gyx048	*Fusarium*	2.93 ± 0.05 c	15.79 ± 0.49 e
34	Gyx071	*Fusarium oxysporum*	2.63 ± 0.56 e	16.04 ± 1.05 e

[a]Means with different letters in the same column are significantly different by Duncan's multiple-range test with $\alpha = 0.05$.

having been used as the carbon source by the fungus.[26−29] These interesting questions expect to be studied further.

The mean FPA of all SSFS was 1.883 U/min·g dw and that of TF was 16.240 mg/g dw. The FPA and TF of SSFS fermented by *Hypocrea* spp. and *Mucor* spp. were higher than the means. The FPA and TF of SSFS fermented by *Penicillium* spp. were lower than the means. The FPA of SSFS fermented by *Fusarium* spp. was higher than the mean, but the TF was lower than the mean. The FPA and TF of SSFS fermented by *Aspergillus* spp. were contrary to those of SSFS fermented by *Fusarium* spp. The fungi with higher TF in their SSFS were preferentially selected for the following study.

Fungi Selection for the Prefermentation Treatment Process. It is well-known that flavonoids are mainly utilized for foods and medicines, and thus selecting a safe fungal strain for the prefermentation treatment process of flavonoid extracton is very important. *Aspergillus niger*, widely used for the production of food ingredients, pharmaceuticals, and industrial enzymes,[30] is considered to be relatively safe for extraction of flavonoids (termed GRAS).[31] Gyx086 strain (an *A. niger*), with the highest FPA and higher TF in SSFS, was more suitable for the prefermentation process than other *A. niger* strains. *A. niger* var. *awamori* offered a similar level of safety[32] and was widely used in the food industry, particularly, in the wine industry. Therefore, Gyx017 strain with the highest TF in Table 3 was a potential strain for the prefermentation process. *Aspergillus versicolor* Gyx026 significantly improved the flavonoid extraction with an average yield of 17.03 mg/g dw, but it secreted the carcinogenic mycotoxin sterigmatocystin.[33] *Alternaria alternate* Gyx103 had a high flavonoid extraction yield of 18.77 mg/g dw, but it was unsafe because of the production of a toxin that could cause upper respiratory tract infections and asthma in sensitive people.[34] Controlling toxin not to be a product or separating the toxin from its fermentation product needs to be investigated before the fungus could be used for prefermentation. Strains of *Hypocrea* spp., *Mucor* spp., and *Fusarium* spp. had higher FPA, but very low TF values in the SSFS, which might be suitable for the production of cellulase instead of flavonoids. According to the discussion above, Gyx017 and Gyx086 offer better safety[30−32] and higher flavonoid extraction yields (25.6 and 16.6% higher than the control) and were more suitable for the prefermentation process and primarily selected for the following experiment.

Flavonoid Component of Non-SSFS and SSFS. HPLC chromatograms have been widely used for analysis of the flavonoids in ginkgo leaf, other plant constituents, and the quality of herbal medicines.[35−37] In this study, the compositions of flavonoids from three *G. biloba* leaf samples (non-SSFS, Gxy086' SSFS, and Gxy017' SSFS) were studied by HPLC fingerprints (Figure 1). There were 67 peaks in the control (Figure 1A), which was a non-SSFS. These peaks were taken as the target peaks for statistics of the peak similarity. The peak similarity of repeated non-SSFS was 89.6−98.5% corresponding to the target peaks; that of repeated SSFS fermented by Gyx086's was 89.3−91.5%, and that of repeated SSFS fermented by Gyx017's was 68.6−71.6%. These results demonstrated that Gyx086's SSFS had more similar flavonoid components compared with the control than the ones in Gyx017. In Figure 1C, the peaks with retention times within 8.5−20 min, which were mainly formed by the components of flavonoid glycosides, were visibly lost. This was possibly caused by the hydrolysis of flavonoid glycosides by the glycoside hydrolase such as glucosidase. The cellulolytic enzymes produced by *A. niger* on lignocellulosic substrates consisted of exoglucanase, endoglucanase, and glucosidase.[38,39] The glucosidase was found to effectively hydrolyze glycosides to their corresponding aglycones[40] and/or to flavonol transglycosylation.[41] The loss of one-to-one peaks was not significant in the Figure 1B, corresponding to the control by analysis of variance ($p > 0.05$). Aglycones had a lower water solubility than their

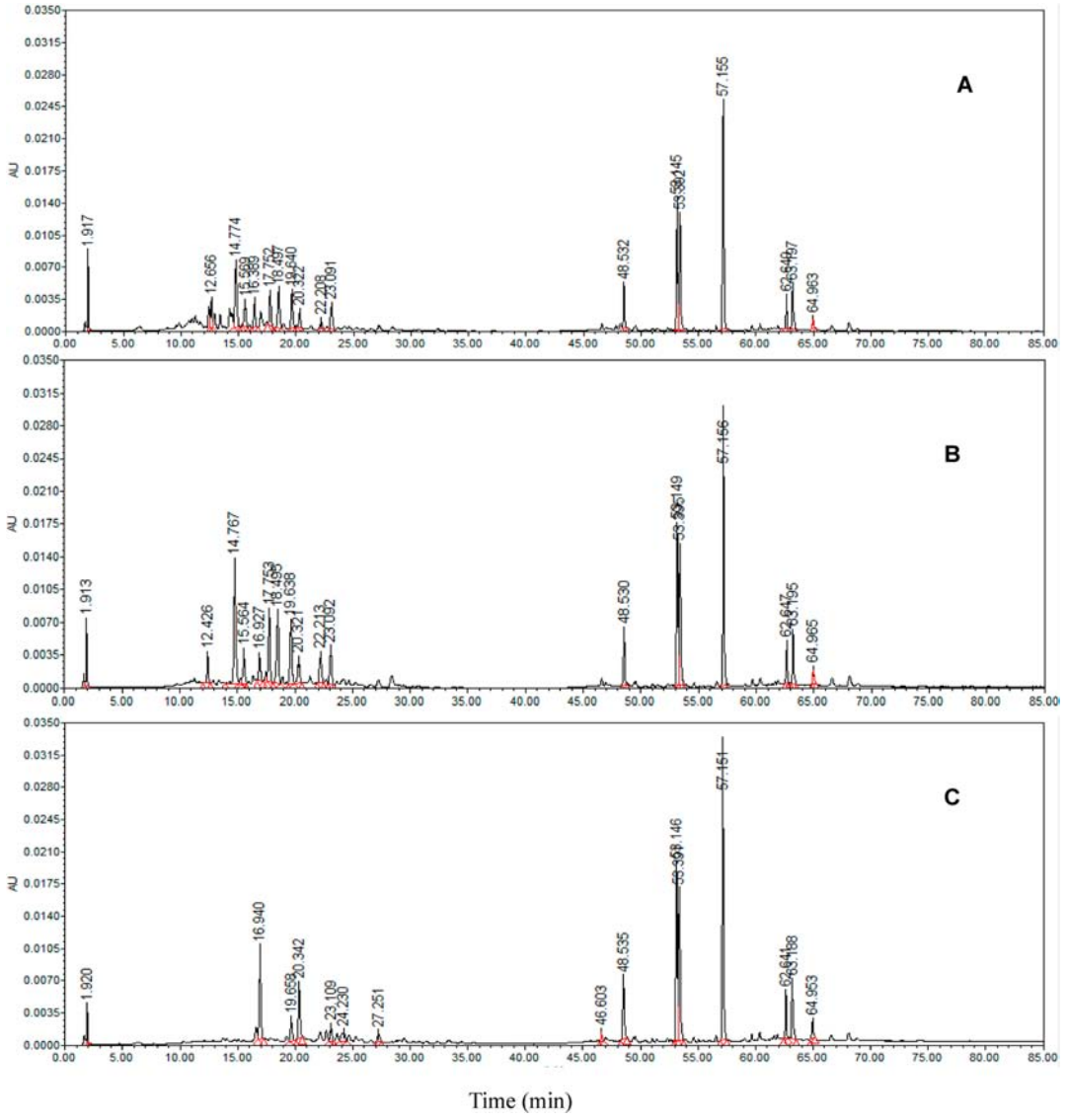

Figure 1. HPLC fingerprints of flavonoids in different *G. biloba* leaf samples: (A) control/non-SSFS; (B) SSFS of Gyx086; (C) SSFS of Gyx017.

glycosides in which glycosyl enhances their hydrophilicity. Low solubility of target compounds in the extract led to low extraction yield and required large amounts of solvents, which largely impedes the economic efficiency in industry.[1] Therefore, the flavonoid produce of SSFS fermented by Gyx086 would be more desirable than that of Gyx017. Furthermore, the areas of the homologous chromatographic peak in Figure 1 were significantly different ($p < 0.05$). The average area of main peaks in Figure 1B,C were 40.07 and 50.37% larger than those of the homologous peaks in Figure 1A, which showed that a prefermentation process evidently improved the extraction of flavonoids. The total content of three flavonol aglycones was <0.5% of TF (Figure 2), which suggested the two *Aspergillus* spp. strains had not largely transformed glycosides to their corresponding aglycones and their flavonoid products still had good water solubility by the solid-state prefermentation.

Time Course of SSF. To select the suitable extraction time for prefermentation extraction of flavonoids, the TF and FPA in the SSFS fermented by Gyx017 and Gyx086 for different times were determined (Figure 3). Two strains showed different fermentation time courses. During the early stage of prefermentation, the TF in SSFS of Gyx086 increased rapidly

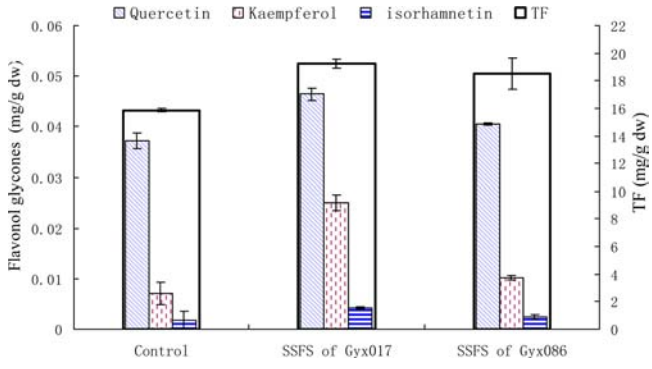

Figure 2. Flavonol glycone content and TF in the control, Gyx017's SSFS, and Gyx086's SSFS.

and attained the biggest value (25.79 mg/g dw) at the 72nd h, which was accompanied by a rapid increase in FPA that reached a peak value at the 57th h. A lower peak value of FPA in Gyx017 cultures appeared at the 72nd h in the SSF period, and the TF (22.09 mg/g dw) was highest at the 120th h. The TF peak was following the FPA peak visibly, which implied that the

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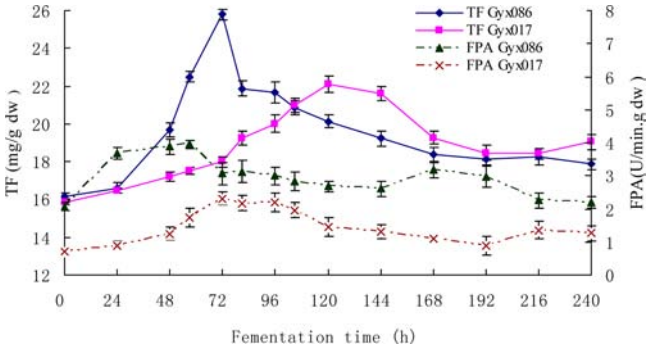

Figure 3. TF and FPA in Gyx017's and Gyx086's SSFS during SSF with solid/water ratio of 5:6 and fermentation temperature at 28 °C.

freedom of flavonoids (intracellular matter) from *G. biloba* leaves depended on cellulase degradation of lignocellulose.[1] However, the TF values of cultures were gradually decreased after the peak values, probably because part of the flavonoid substance was consumed by fungal development.[28,29] Therefore, the prefermented time for flavonoid extraction was 48−72 h for Gyx086 and 120−144 h for the Gyx017. Gyx086 was a more suitable strain for the prefermentation processing.

Effects of Temperature and Moisture Content on TF Yield. Temperature and moisture content were more important factors for improving the extraction of TF than other ones, such as the initial pH value and inoculation amount of each flask. The Gyx086 strain had a strong adaptability to the pH of the medium. Gyx086 could rapidly grow in all cultures with the initial pH series from 4.6 to 7.0. The pH value in each flask became 5.6 ± 0.12 after 2 days of fermentation, and TF values in all SSFS were not significantly different (data not shown). Different amounts of inoculum affected the fermentation time and also indistinctively influenced TF. A suitable inoculum was about 0.03 g of wet mycelium per flask.

With a constant solid/water mass ratio of 5:6, a temperature from 26 to 32 °C was adopted to investigate their effects on the Gyx086 prefermentation (as shown in Figure 4). Higher

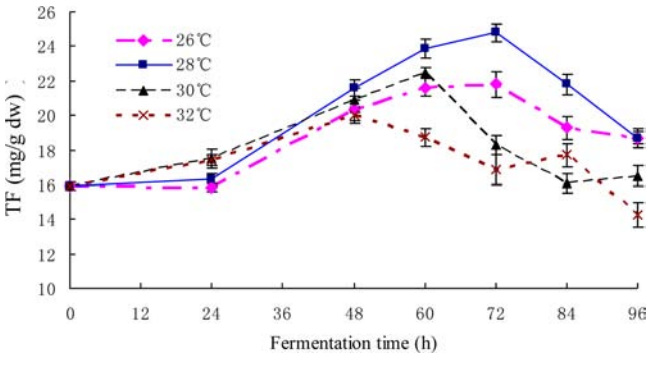

Figure 4. Temperature influence on TF in Gyx086 SSFS during SSF with a solid/water ratio of 5:6.

temperature promoted faster mycelial growth during Gyx086 SSF (data not shown), but the yield of TF did not follow the same trend. All TF broken line graphs in the figure had similar change trends, and the time dependence of the TF was obvious during the SSF. A decrease was observed after a certain period as analogously shown in Figure 3. The reason could be the same as mentioned above, and when the temperature was over 30 °C, the consumption was increased. High TF extraction yield appeared at a temperature of 28 °C. Temperature levels of 26, 28, and 30 °C were selected for the following RSM optimization.

As indicated by Figure 5, the TF yield was significantly influenced by the moisture content in the media. TF increased

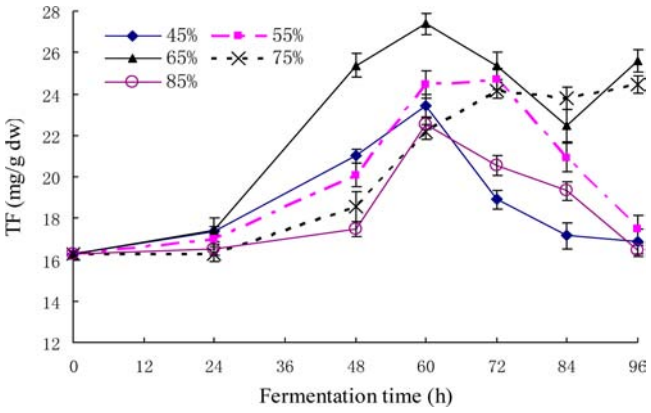

Figure 5. Influence of moisture content on TF in Gyx086 SSFS during SSF at 28 °C.

along with the enhancement of moisture content in the culture from 45 to 65%, but at >75% of moisture content, the growth of Gyx086 delayed, and the TF in the SSFS was decreased. A moisture content of near 65% was suitable for improving the TF yield, so moisture contents of 60, 65, and 70% were selected for the following RSM optimization.

Optimization of Gyx086 Prefermentation by RSM. The results generated by the BBD experiment are shown in Table 2.

Table 2. Box−Behnken Design Matrix for Optimization of the TF Yield during the Gyx086 SSF[a]

	factors (X)			responses (Y)	
run	X_1 coded (uncoded)	X_2 coded (uncoded)	X_3 coded (uncoded)	experimental value	predicted value
1	−1 (26)	−1 (60)	0 (60)	24.822	24.704
2	1 (30)	−1 (60)	0 (60)	23.897	23.734
3	−1 (26)	1 (70)	0 (60)	22.365	22.528
4	1 (30)	1 (70)	0 (60)	22.871	22.990
5	−1 (26)	0 (65)	−1 (48)	22.858	22.853
6	1 (30)	0 (65)	−1 (48)	25.075	25.113
7	−1 (26)	0 (65)	1 (72)	25.840	25.803
8	1 (30)	0 (65)	1 (72)	23.028	23.035
9	0 (28)	−1 (60)	−1 (48)	23.867	23.995
10	0 (28)	1 (70)	−1 (48)	22.809	22.653
11	0 (28)	−1 (60)	1 (72)	24.394	24.549
12	0 (28)	1 (70)	1 (72)	23.095	22.971
13	0 (28)	0 (65)	0 (60)	27.741	27.540
14	0 (28)	0 (65)	0 (60)	27.238	27.540
15	0 (28)	0 (65)	0 (60)	27.641	27.540

[a] X_1, temperature (°C); X_2, moisture content (%); X_3, fermentation time (h); Y, TF of the SSFS (mg/g dw).

The data were analyzed by the Response Surface Methods−Estimate Model program of the SYSTAT 12 software. A quadratic full model was selected as the fit model, and the results of the analysis of variance (ANOVA), lack of fit test, and regression coefficient analysis were statistically analyzed as shown in Table 3. The goodness of fit can be indicated by the value of the squared multiple R (R^2). The R^2 value is a measure

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of how much variability can be explained by the experimental parameters and their interactions. The ability of models to predict response values increases as R^2 values approach 1.0.[42,43] The R^2 was 0.994 for the fitted model (Table 3), which indicated 99.4% of observed values could be explained by the fitted model. The model including all sources (linear, quadratic, and interaction) significantly influenced Y ($p < 0.05$). Lack of fit test with a p value of 0.609 (as shown in Table 4) was not significant ($p > 0.05$), which suggested that the quadratic full model was appropriate for the experiment, and it was considered reasonable to analyze trends of the responses. As can be seen in Table 2, there was very good fitness between the observed and predicted responses. Table 5 illustrates the regression coefficients and corresponding p values for the model. The model could be mathematically expressed by using the regression coefficients for coded factors, where Y stood for the response variables of the TF yield, and X_1, X_2, and X_3 represented the coded variables. The positive coefficients of $X_1{*}X_2$ indicated a significant positive effect on the response Y ($p < 0.05$). In contrast, the quadratic terms (X_1^2, X_2^2, and X_3^2), the interaction term $X_1{*}X_3$, and the linear X_2 had a significant inverse effect on the yield of TF ($p < 0.0001$). The terms X_1, X_3,and $X_2{*}X_3$ insignificantly effected Y ($p > 0.05$).

Table 3. ANOVA for Response Surface Quadratic Model of the TF Yield by Gyx086 Prefermentation: ANOVA of Variance Analysis for the Response Surface Quadratic Model

source	df	type I SS	mean squares	F ratio	p value
regression	9	47.469	5.274	86.374	0.000
linear	3	4.776	1.592	26.070	0.002
quadratic	3	35.844	11.948	195.665	0.000
interaction	3	6.849	2.283	37.386	0.001
residual error	5	0.305	0.061		
total error	14	47.774			
squared multiple R		0.994			
adjusted squared multiple R		0.982			

Table 4. ANOVA for Response Surface Quadratic Model of the TF Yield by Gyx086 Prefermentation: ANOVA of Lack of Fit Test for the Response Surface Quadratic Model

source	df	SS	mean squares	F ratio	p value
lack of fit	3	0.163	0.054	0.765	0.609
pure error	2	0.142	0.071		
residual error	5	0.305	0.061		

Table 5. ANOVA for Response Surface Quadratic Model of the TF Yield by Gyx086 Prefermentation: ANOVA of the Regression Coefficients for the Response Surface Quadratic Model

effect	coefficient (coded)	standard error	t	p value
constant	27.540	0.143	193.032	0.000
X_1	−0.127	0.087	−1.451	0.206
X_2	−0.730	0.087	−8.358	0.000
X_3	0.218	0.087	2.499	0.055
$X_1{*}X_1$	−1.696	0.129	−13.191	0.000
$X_2{*}X_2$	−2.355	0.129	−18.310	0.000
$X_3{*}X_3$	−1.643	0.129	−12.778	0.000
$X_1{*}X_2$	0.358	0.124	2.895	0.034
$X_1{*}X_3$	−1.257	0.124	−10.176	0.000
$X_2{*}X_3$	−0.059	0.124	−0.480	0.651

Fitted 2-D contour plots for the yield of TF (Y) were generated on the basis of the model by using the SYSTAT 12 software program Response Surface Methods−Contour/Surface and are depicted in Figure 6. The contour helps to visualize the shape of the response surface. Each figure presents the effect of two variables on TF when the third variable was kept at the optimal level. All response plots showed clear optimum peaks, implying that the optimum conditions for maximum values of TF were in the design space, and also showed all variables had an arched curvilinear effect on TF of the Gyx086's SSFS. As shown in Figure 6A, with the optimal time fixed, X_1 (temperature) and X_2 (moisture content) showed an arched curvilinear effect on TF. The contour plot suggested TF significantly decreased when the moisture content was over 66.8% or below 61.7% and the temperature was over 29.05 °C or below 26.7 °C. The two variables had a nearly circular contour effect on TF, which suggested minor interaction between them. Instead of this, an elliptical contour plot indicated a significant interaction between variables,[44] as shown by the contour plot in Figure 6B with an optimal X_2 fixed. The two variables (X_1 and X_3) had an approximately line effect on TF near −1 coded level and had a line countereffect on TF near +1 coded level. When the temperature (X_1) was over 29.05 °C or below 26.7 °C, TF obviously declined, and likewise when the fermentation time (X_3) was over 68 h or below 54 h. The interaction effect of the variables (X_2 and X_3) is shown in Figure 6C with an optimal X_1 (temperature) fixed. The close circular contour also suggested TF was insignificantly affected by the interaction between X_2 and X_3. The X_2 (moisture content) and X_3 (fermentation time) had coincident curvilinear effects on TF compared with Figure 6A,B. All contour plots showed similar relationships with respect to the effect of each variable. The statistically optimal values of variables for TF located in the center circles in three contour plots were verified from canonical analysis of the response surface. The stationary point obtained by the SYSTAT 12 software program Response Surface Methods−Optimize was a maximum. The optimal values for the three factors were 27.813 °C for temperature, 64.183% for moisture content, and 61.262 h for fermentation time. Under these conditions the predicted TF was 27.617 with a 95% confidence interval (the predicted response value from 27.255 to 27.979).

Verification of the Model. To verify the model, the predicted optimum levels of the variables (fermentation temperature, 27.8 °C; solid/water ratio, 64.2%; and fermentation time, 61 h) were used for prefermentation. Five batches of prefermentation experiment were performed. The average TF yield was 27.59 ± 0.52 mg/g dry weight SSFS, which was very close to the predicted value 27.617 and showed insignificant difference ($p > 0.05$) by one-way ANOVA program of the SAS software. The model could be considered quite reliable for predicting the yield of TF by a prefermentation process using the Gyx086 strain.

In summary, the goal of this study was to provide a new prefermentation treatment method involving fungi to improve flavonoid extraction from the leaves of plants (as *G. biloba* in the study). Fungi employed for this treatment were screened from the soil present under an ancient ginkgo tree. One strain, Gyx086, was finally investigated for prefermentation processing.

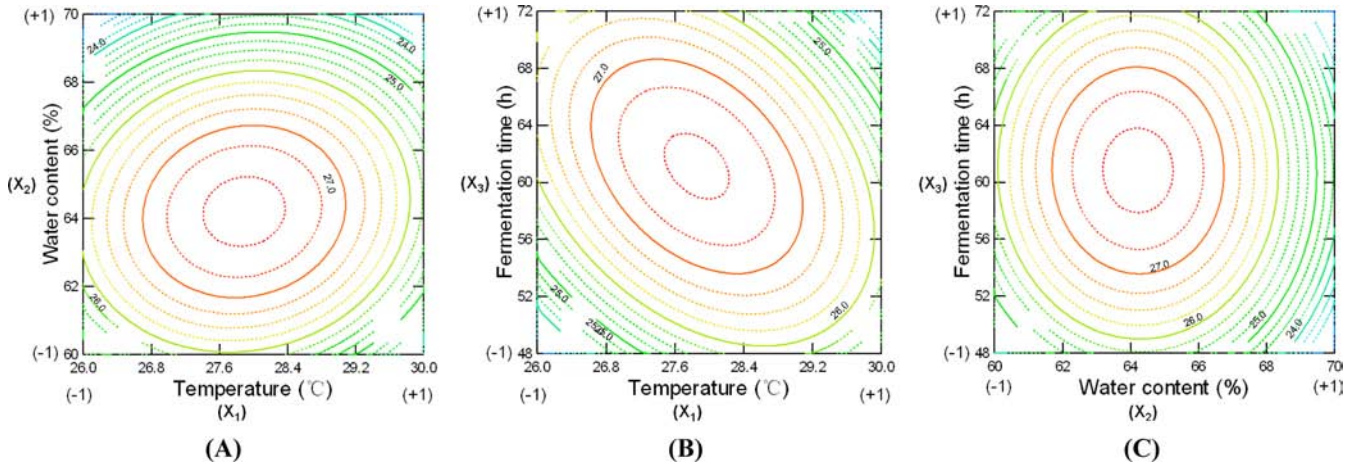

Figure 6. (A) Interactive effects of temperature (X_1) and moisture content (X_2) on TF of Gyx086's SSFS (as predicted by 2-D contour plots). (B) Interactive effects of temperature (X_1) and fermentation time (X_3) on TF of Gyx086's SSFS (as predicted by 2-D contour plots). (C) Interactive effects of moisture content (X_2) and fermentation time (X_3) on TF of Gyx086's SSFS (as predicted by 2-D contour plots).

The individual and interactive roles of the major fermentation factors were optimized by a single-factor test and Box–Behnken design. The optimal value of the quadratic regression model was confirmed by a verification test, which indicated that high yields of TF were obtained when the optimized fermentation conditions were used. Therefore, the prefermentation processing for the extraction of flavonoid from the leaves of *G. biloba* will be a new approach for further production. Additional studies are now required for large-scale production.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

SSF, solid-state fermentation; SSFS, solid-state fermented samples; TF, flavonoid extraction content; FPA, filter paper activity; RSM, response surface methodology

REFERENCES

(1) Chen, S.; Xing, X. H.; Huang, J. J.; Xu, M. S. Enzyme-assisted extraction of flavonoids from *Ginkgo biloba* leaves: improvement effect of flavonol transglycosylation catalyzed by Penicillium decumbens cellulase. *Enzyme Microb. Technol.* **2011**, *48*, 100–105.

(2) Liang, L. X. Medicinal history of ginkgo in China. *Res. Inf. Chin. Med.* **1993**, *3*, 44–45.

(3) van Beek, T. A.; Montoro, P. Chemical analysis and quality control of *Ginkgo biloba* leaves, extracts, and phytopharmaceuticals. *J. Chromatogr., A* **2009**, *1216*, 2002–2032.

(4) Wang, F.; Jiang, K.; Li, Z. Purification and identification of genistein in *Ginkgo biloba* leaf extract. *Chin. J. Chromatogr.* **2007**, *25*, 509–513.

(5) Ju, X. R.; Wang, H. Determination of flavonoids in extract of *Ginkgo biloba* L. by high performance liquid chromatography. *Chin. Food Sci.* **2001**, *22*, 66–68.

(6) Lai, Y. H.; Tong, H. Z.; Xian, Y. F.; Zhu, H. R.; Yu, Y. Flavonoids hydrolysis conditions in EGb quantification. *J. China Pharm. Univ.* **2007**, *38*, 569–571.

(7) Robak, J.; Gryglewski, R. J. Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **1988**, *37*, 837–841.

(8) Yamakoshi, J.; Piskula, M. K.; Izumi, T.; Tobe, K.; Saito, M.; Kataoka, S.; Obata, A.; Kikuchi, M. Isoflavone aglycone–rich extract without soy protein attenuates atherosclerosis development in cholesterol-fed rabbits. *J. Nutr.* **2000**, *130*, 1887–1893.

(9) Chan, P. C.; Xia, Q.; Fu, P. P. *Ginkgo biloba* leaf extract: biological, medicinal, and toxicological effects. *Environ. Carcinog. Ecotoxicol. Rev.* **2007**, *25*, 211–244.

(10) Chiu, K. L.; Cheng, Y. C.; Chen, J. H.; Chang, C. J.; Yang, P. W. Supercritical fluids extraction of *Ginkgo* ginkgolides and flavonoids. *J. Supercrit. Fluids* **2002**, *24*, 77–78.

(11) Cho, Y. J.; Hong, J. Y.; Chun, H. S.; Lee, S. K.; Min, H. Y. Ultrasonication-assisted extraction of resveratrol from grapes. *J. Food Eng.* **2006**, *77*, 725–730.

(12) Wang, Y. L.; Xi, G. S.; Zheng, Y. C.; Miao, F. S. Microwave-assisted extraction of flavonoids from Chinese herb *Radix puerariae* (Ge Gen). *J. Med. Plants Res.* **2010**, *4*, 304–308.

(13) Landbo, A. K.; Meyer, A. S. Enzyme-assisted extraction of antioxidative phenols from black currant juice press residues (*Ribes nigrum*). *J. Agric. Food Chem.* **2001**, *49*, 3169–3177.

(14) Fu, Y. J.; Liu, W.; Zu, Y. G.; Tong, M. H.; Li, S. M.; Yan, M. M.; Efferth, T.; Luo, H. Enzyme assisted extraction of luteolin and apigenin from pigeonpea [*Cajanuscajan* (L.) Millsp.] leaves. *Food Chem.* **2008**, *111*, 508–512.

(15) Malherbe, S.; Cloete, T. E. Lignocellulose biodegradation: fundamentals and applications. *Rev. Environ. Sci. Biotechnol.* **2002**, *1*, 105–114.

(16) Waldrop, M. P.; Balser, T. C.; Firestone, M. K. Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem.* **2000**, *32*, 1837–1846.

(17) Ulrich, A.; Wirth, S. Phylogenetic diversity and population densities of culturable cellulolytic soil bacteria across an agricultural encatchment. *Microb. Ecol.* **1999**, *37*, 238–247.

(18) Smit, E.; Leeflang, P.; Glandorf, B.; van Elsas, J. D.; Wernars, K. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* **1999**, *65*, 2614–2621.

(19) Chang, C. C.; Yang, M. H.; Wen, H. M.; Chern, J. C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **2002**, *10*, 178−182.
(20) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426−428.
(21) Fierer, N.; Schimel, J. P.; Holden, P. A. Variations in microbial community composition through two soil depth profiles. *Soil Biol Biochem.* **2003**, *35*, 167−176.
(22) Boucher, D. H.; James, S.; Keeler, K. H. The ecology of mutualism. *Annu. Rev. Ecol. Syst.* **1982**, *13*, 315−347.
(23) Lindstrom, K.; Murwira, M.; Willems, A.; Altier, N. The biodiversity of beneficial microbe-host mutualism: the case of rhizobia. *Res. Microbiol.* **2010**, *161*, 453−463.
(24) Zeng, B. Q.; Zhou, X. Q.; Xie, X. Y. Cellulase hydrolysis followed by microwave-assisted extraction for extracting hesperidin from navel orange peels. *Chin. Food Sci.* **2010**, *31*, 85−89.
(25) Tan, R. X.; Zou, W. X. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.* **2001**, *18*, 448−459.
(26) Barz, W. Isolation of rhizosphere bacterium capable of degrading flavonoids. *Phytochemistry* **1970**, *9*, 1745−1749.
(27) Coope, J. E. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *J. Appl. Microbiol.* **2007**, *103*, 1355−1365.
(28) Westlake, D. W. S.; Spencer, J. F. T. The utilization of flavonoid compounds by yeasts and yeast-like fungi. *Can. J. Microbiol.* **1966**, *12*, 165−174.
(29) Medina, M. L.; Kiernan, U. A.; Francisco, W. A. Proteomic analysis of rutin-induced secreted proteins from *Aspergillus flavus*. *Fungal Genet. Biol.* **2004**, *41*, 327−335.
(30) Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A. E.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G. J.; Debets, A. J. M.; Dekker, P.; van Dijck, P. W. M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J. M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S. P.; de Groot, P. W. J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P. T. W.; van den Hondel, C. A. M. J. J.; van der Heijden, R. T. J. M.; van der Kaaij, R. M.; Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J. E. C.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N. M. E.; Ram, A. F. J.; Rinas, U.; Roubos, J. A.; Sagt, C. M. J.; Schmoll, M.; Sun, J.; Ussery, D.; Varga, J.; Vervecken, W.; de Vondervoort, P. J. J. v.; Wedler, H.; Wosten, H. A. B.; Zeng, A.-P.; van Ooyen, A. J. J.; Visser, J.; Stam, H. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* **2007**, *25*, 221−231.
(31) Abarca, M. L.; Accensi, F.; Cano, J.; Cabanes, F. J. Taxonomy and significance of black aspergilli. *Antonie van Leeuwenhoek* **2004**, *86*, 33−49.
(32) Schuster, E.; Dunn-Coleman, N.; Frisvad, J.; van Dijck, P. On the safety of *Aspergillus niger* − a review. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 426−435.
(33) Engelhart, S.; Loock, A.; Skutlarek, D.; Sagunski, H.; Lommel, A.; Farber, H.; Exner, M. Occurrence of toxigenic *Aspergillus versicolor* isolates and sterigmatocystin in carpet dust from damp indoor environments. *Appl. Environ. Microbiol.* **2002**, *68*, 3886−3890.
(34) Wiest, P. M.; Wiese, K.; Jacobs, M. R.; Morrissey, A. B.; Abelson, T. I.; Witt, W.; Lederman, M. M. Alternaria infection in a patient with acquired immunodeficiency syndrome: case report and review of invasive *Alternaria* infections. *Clin. Infect. Dis.* **1987**, *9*, 799−803.
(35) Gan, F.; Ye, R. Y. New approach on similarity analysis of chromatographic fingerprint of herbal medicine. *J. Chromatogr., A* **2006**, *1104*, 100−105.
(36) Hasler, A.; Sticher, O.; Meier, B. Identification and determination of the flavonoids from *Ginkgo biloba* by high-performance liquid chromatography. *J. Chromatogr., A* **1992**, *605*, 41−48.
(37) Zou, H. B.; Du, A. Q.; Zhang, X. L.; Wei, P. H.; Lu, W. J.; Yang, G. S.; Hassan, Y. A. E. Quality control methodology and their application in analysis on HPLC fingerprint spectra of herbal medicines. *Chromatogr. Res. Int.* http://www.hindawi.com/journals/cri/2012/851792/.
(38) Chandra, M. S.; Viswanath, B.; Reddy, B. R. Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. *Indian J. Microbiol.* **2007**, *47*, 323−328.
(39) Kang, S. W.; Park, Y. S.; Leeb, J. S.; Hong, S. I.; Kim, S. W. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Bioresour. Technol.* **2004**, *91*, 153−156.
(40) Mandalari, G.; Bennett, R. N.; Kirby, A. R.; Lo Curto, R. B.; Bisignano, G.; Waldron, K. W.; Faulds, C. B. Enzymatic hydrolysis of flavonoids and pectic oligosaccharides from bergamot (*Citrus bergamia* Risso) peel. *J. Agric. Food Chem.* **2006**, *54*, 8307−8313.
(41) Puupponen-Pimiae, R.; Nohynek, L.; Ammann, S.; Oksman-Caldentey, K.-M.; Buchert, J. Enzyme-assisted processing increases antimicrobial and antioxidant activity of bilberry. *J. Agric. Food Chem.* **2008**, *56*, 681−688.
(42) Acikel, U.; Ersan, M.; Acikel, Y. S. Optimization of critical medium components using response surface methodology for lipase production by *Rhizopus delemar*. *Food Bioprod. Process.* **2010**, *88*, 31−39.
(43) Li, B. B.; Smith, A. B.; Hossain, M. M. Extraction of phenolics from citrus peels II. Enzyme-assisted extraction method. *Sep. Purif. Technol.* **2006**, *48*, 189−196.
(44) Wu, S. J.; Yu, X.; Hu, Z. H.; Zhang, L. L.; Chen, J. M. Optimizing aerobic biodegradation of dichloromethane using response surface methodology. *J. Environ. Sci.−China* **2009**, *21*, 1276−1283.