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Improving the acetylcholinesterase inhibitory effect of *Illigera aromatica* by fermentation with *Clonostachys rogersoniana*

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***Illigera aromatica* was fermented by *Clonostachys rogersoniana*. The acetylcholinesterase (AChE) inhibitory effects of unfermented and fermented *I. aromatica* revealed that *C. rogersoniana*-fermented *I. aromatica* (CFIA) induced significantly more AChE inhibitory activity (IC_{50}: 35.4 ± 2.1 μg/mL). The biotransformation of actinodaphnine (1) into (4*R*,6a*S*)-4-hydroxyactinodaphnine (2) was found during the fermentation, which played an important role in the improvement of the AChE inhibitory activity of *I. aromatica*. Subsequently, the fermentation conditions—including the solid–liquid ratio, fermentation temperature, and fermentation time—were optimized. *I. aromatica* immersed in 100–200% water and fermented with *C. rogersoniana* at ambient temperature for 30 days was conducive to the biotransformation of actinodaphnine (1) and improved the AChE inhibitory activity of *I. aromatica*. The present study provides a novel approach for improving the pharmacological effect of *I. aromatica* and suggests that CFIA may be used as an alternative AChE inhibitor.**

[**Key words:** *Illigera aromatica*; *Clonostachys rogersoniana*; Acetylcholinesterase inhibitory effect; Biotransformation; Actinodaphnine]

The plants of the genus *Illigera* (Hernandiaceae), which contains approximately 30 known species, are distributed across southern Africa and Asia (1). Some of these plants are used as ethnic drugs by the locals. The main constituents of such plants include alkaloids (2–5), phenolic acids (6,7), and steroids (8,9), which have been reported to have various pharmacological effects. *Illigera aromatica* S. Z. Huang et S. L. Mo, belonging to the genus *Illigera* (Hernandiaceae), is a small liana that primarily grows in Yunnan and Guangxi Provinces of China (10). Its tubers are known as Hei-Chui-Feng in Zhuang ethnic drugs, which are frequently used by the locals to treat tuberculosis and to promote blood circulation. Previous phytochemical research has shown that its major components contain steroids (9), terpenes, alkaloids, and phenolics (11,12). Actinodaphnine (**1**) is the major component of *I. aromatica*, which exhibits weak acetylcholinesterase (AChE) inhibitory activity.

Fermentation processing of traditional Chinese medicines has been practiced for more than 4000 years in China, and it is frequently used to produce secondary metabolites from domestic plants in bulk by utilizing the metabolic mechanisms of microorganisms. Recently, fermented traditional Chinese medicines derived from pure strains have become attractive options. Qin et al. (13) reported that the fermentation of *Kadsura angustifolia* by *Penicillium* sp. SWUKD4.1850 could enhance the production of unusual triterpenoids. *Pediococcus acidilactici* KCCM11614P that was fermented with *Ginseng marc* possessed increased antioxidative and nitric oxide (NO) scavenging activity (14). *Angelica dahurica* fermented with *Eurotium cristatum* showed greater antioxidant activity compared to that of unfermented material (15). Zhang et al. (16) reported that microorganism-fermented *Diospyros lotus* possessed improved antioxidant activity and anti-α-glucosidase activity. Biotin addition to the medium during *Pichia guilliermondii* fermentation enhanced anti-oxidative activity and lignocellulosic ethanol production (17).

Although *I. aromatica* has been used as a traditional Chinese medicine, its documented pharmaceutical effect is not sufficiently strong. To improve the bioactivity of *I. aromatica*, in the present study, *I. aromatica* was fermented by *Clonostachys rogersoniana*. The AChE inhibitory effects of unfermented and fermented *I. aromatica* were evaluated and the biotransformation that occurred during fermentation was also investigated.

MATERIALS AND METHODS

Chemicals Methanol (high performance liquid chromatography (HPLC) grade) was purchased from Tedia Company, Inc. (Fairfield, OH, USA). Distilled water was obtained from Wahaha Co., Ltd. (Hangzhou, China). All of the other chemicals that were used were of analytical grade.

Plant materials and microorganisms The tubers of *I. aromatica* were collected in Wenshan, Yunnan, China, in November 2017 and were identified by Shuda Yang (Assistant Professor) at the College of Pharmacy of Kunming Medical University, China. A voucher specimen (2017-xqt-01) was deposited at the College of Chemistry and Environmental Science of Qujing Normal University, China.

C. rogersoniana 828H2 were obtained from the Yunnan Institute of Microbiology in Yunnan Province of China. The nucleotide sequence data were deposited in GenBank (accession number KT625993).

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Fermentation procedure The strain of *C. rogersoniana* was activated in a potato dextrose agar (PDA) slant-culture medium and stored at 28°C for 7 days. The seed culture medium was prepared using cultivated *C. rogersoniana* in potato dextrose broth (PDB) medium. The fermentation culture medium consisted of 10 g of powder of *I. aromatica*, was infiltrated with 20 mL of water, and was sterilized at 121°C for 30 min. Afterward, the fermentation medium was inoculated with the mature *C. rogersoniana* and was cultivated in a constant-temperature incubator at 28°C for 30 days.

Extraction and isolation The unfermented *I. aromatica* (UFIA, 10.00 g) and *C. rogersoniana*-fermented *I. aromatica* (CFIA) were each immersed in 50 mL of methanol and were thoroughly extracted three times (30 min each) at 35°C by ultrasonic extraction. The extracts were decanted and filtered at room temperature and then concentrated in a rotary evaporator at $\leq$ 50°C to obtain two extracts: UFIA (0.986 $\pm$ 0.18 g) and CFIA (1.025 $\pm$ 0.31 g).

Next, 0.5 g of UFIA was subjected to silica-gel column chromatography [CC, $CHCl_3$—MeOH (9:1–6:1)] and purified by Sephadex LH-20 (MeOH) to yield **1** (45.3 mg). Additionally, 0.5 g of CFIA was chromatographed over silica-gel CC ($CHCl_3$: MeOH = 8:1–3:1) and purification by Sephadex LH-20 (MeOH) yielded **2** (40.5 mg).

AChE inhibitory activity The AChE inhibitory activity was determined using the Ellman method that has been described previously (11).

Thin layer chromatography analysis The thin layer chromatography (TLC) analysis was carried out using a GF_{254} silica-gel plate and chromatography was performed with dichloromethane-methanol (5:1). The plate was visualized by spraying with a modified Dragendorff's reagent.

HPLC analysis All of the samples (UFIA, CFIA, and standards) were filtered through a 0.45-μm filter before injection into an Agilent 1260 series HPLC system equipped with an Agilent G1311C quatpump, an Agilent G1315D diode array detector, an Agilent G1329B autosampler, an Agilent Zorbax SB-C18 (250 × 4.6 mm i.d., 5 μm) column, and an Agilent OpenLab ChemStation. A gradient-elution system consisting of solvents A (water containing 0.1% H_3PO_4 and 0.4% Et_3N) and B (50% methanol-acetonitrile) was used for the analysis, and the gradient program was as follows: 0–5 min, 20–40% B; 5–15 min, 40–55% B; 15–20 min, 55–85% B. The post time was set to 5 min and the flow rate was set to 1.0 mL/min. The peaks were confirmed by the retention time at 280 nm.

RESULTS AND DISCUSSION

AChE inhibitory effects of UFIA and CFIA The AChE inhibitory activities of UFIA and CFIA were measured. The results shown in Fig. 1 indicated that the IC_{50} for AChE inhibitory effect of CFIA was 35.4 $\pm$ 4.1 μg/mL, which was significantly higher than that of UFIA (IC_{50}: 47.9 $\pm$ 3.2 μg/mL). *C. rogersoniana* is a fungus isolated from the rhizosphere soil of *Panax notoginseng*. Previous studies have shown that *C. rogersoniana* fermentation improves AChE inhibitory activity of *Illigera henryi* and the biotransformation of aporphine alkaloids (18,19). The AChE inhibitory activity of CFIA in the present study was significantly higher than that of *C. rogersoniana* fermented with *I. henryi*, suggesting that fermentation of *I. aromatica* with *C. rogersoniana* improved the AChE inhibitory activity of *I. aromatica* and that CFIA is a more effective AChE inhibitor.

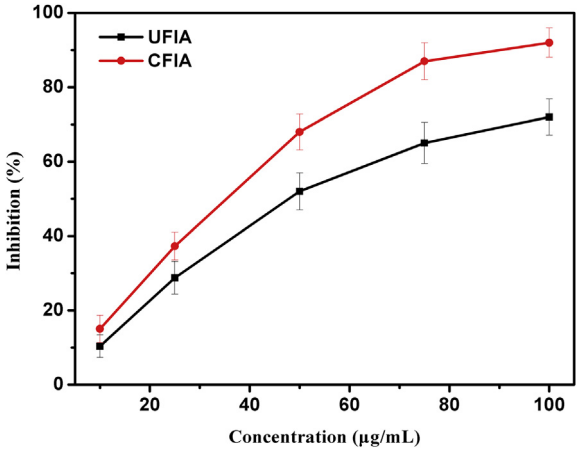

FIG. 1. AChE inhibitory activities of UFIA and CFIA at different concentrations.

TLC analysis To explore the change of chemical constituents that led to the improvement of the AChE inhibitory effect of *I. aromatica*, TLC was used to determine changes of major components. The results shown in Fig. S1 suggest the presence of an obvious biotransformation between two alkaloids.

Identification of compounds 1 and 2 The major components of UFIA and CFIA were isolated from their extracts and their structures were identified by ^1H and ^{13}C nuclear magnetic resonance (NMR) data (Table 1). Comparison of our NMR data with NMR data in the literature (11,18) revealed that **1** and **2** were identified as actinodaphnine and (4*R*,6a*S*)-4-hydroxyactinodaphnine, respectively.

HPLC analysis To determine the biotransformation during the fermentation, HPLC was employed to determine the contents of actinodaphnine (**1**) and (4*R*,6a*S*)-4-hydroxyactinodaphnine (**2**) in UFIA and CFIA. The results shown in Fig. 2 suggested that the major peak at t_R 14.2 min, corresponding to actinodaphnine (**1**), was almost completely absent after fermentation; another major peak at t_R 9.5 min, corresponding to (4*R*,6a*S*)-4-hydroxyactinodaphnine (**2**), was found after fermentation. These results imply that the biotransformation of actinodaphnine (**1**) into (4*R*,6a*S*)-4-hydroxyactinodaphnine (**2**) occurred during fermentation. The conversion rate was determined to be 93.2 $\pm$ 4.7% by their peak areas.

AChE inhibitory effects of compounds 1 and 2 The AChE inhibitory activities of compounds **1** and **2** were tested and the results are shown in Fig. 3. (4*R*,6a*S*)-4-Hydroxyactinodaphnine (**2**) possessed more inhibitory capacity against AChE, with an IC_{50} value of 21.7 $\pm$ 3.9 μM, compared to that of actinodaphnine (**1**) (IC_{50}: 49.3 $\pm$ 3.5 μM). This result suggested that the improved AChE inhibitory activity of CFIA was ascribed to the biotransformation of actinodaphnine (**1**) into the more active (4*R*,6a*S*)-4-hydroxyactinodaphnine (**2**).

Effects of fermentation time, temperature, and moisture The solid-state fermentation of traditional Chinese medicines is always affected by fermentation time, temperature, and moisture. In the present study, the conversion rates of

TABLE 1. ^1H and ^{13}C NMR spectroscopic data for **1** and **2** in MeOD.

Position	Actinodaphnine (**1**)		(4*R*,6a*S*)-4-Hydroxyactinodaphnine (**2**)	
	δ_C mult.	δ_H mult. (*J* in Hz)	δ_C mult.	δ_H mult. (*J* in Hz)
1	149.9 s		149.0 s	
1a	117.8 s		117.4 s	
1b	143.9 s		129.1 s	
2	107.6 s		144.5 s	
3	121.2 d	6.66 s	108.7 d	6.72 s
3a	121.2 s		126.6 s	
4	26.4 t	3.39 dd (12.0, 6.0)	65.3 d	4.46 br s
		2.89 m		
5	42.8 t	3.71 dd (12.0, 6.0)	50.8 t	3.26 dd (13.6, 1.2)
		3.54 dd (12.0, 6.0)		3.05 dd (13.6, 2.4)
6a	54.5 d	4.37 dd (14.0, 4.8)	54.5 d	3.72 dd (14.0, 4.8)
7	33.7 t	3.22 dd (14.0, 4.8)	35.3 t	2.80 dd (14.0, 4.8)
		3.00 m		2.68 d (14.0)
7a	123.0 s		123.3 s	
8	116.1 d	6.77 s	116.1 d	6.69 s
9	148.0 s		147.6 s	
10	148.4 s		148.0 s	
11	112.0 d	7.68 s	112.2 d	7.62 s
11a	126.5 s		128.6 s	
OCH_2O	102.7 t	6.15 d (1.2)	102.4 t	6.00 d (1.2)
		6.00 d (1.2)		5.86 d (1.2)
OCH_3	56.5 q	3.87 s	56.6 q	3.85 s

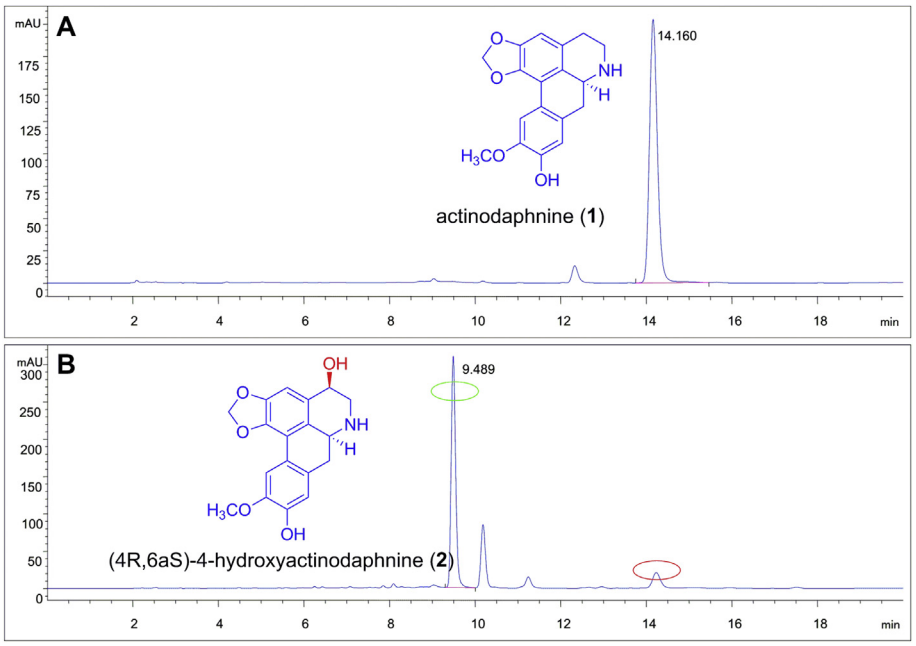

FIG. 2. HPLC chromatograms of UFIA (A) and CFIA (B).

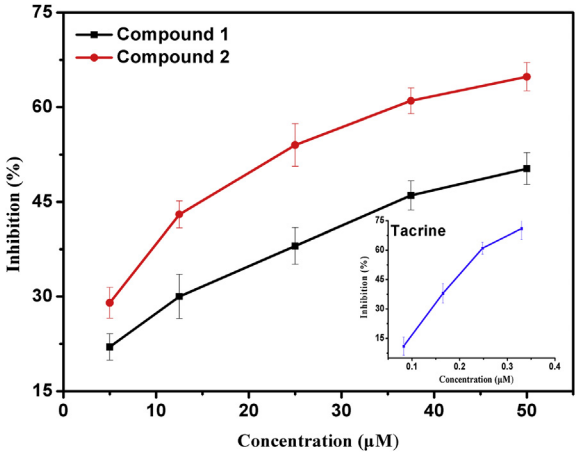

FIG. 3. AChE inhibitory activities of compounds **1** and **2** at different concentrations.

actinodaphnine (**1**) in *I. aromatica* after fermentation at different temperatures and times were determined. As shown in Fig. 4, 30-day fermentation converted actinodaphnine (**1**) into (4*R*,6a*S*)-4-hydroxyactinodaphnine (**2**) to obtain a maximum conversion rate. Fermentation of traditional Chinese medicines is frequently carried out at room temperature in industrial production. Hence, room temperature (ranging from 10°C to 25°C every day), 28°C, and 37°C were selected for determining the effect of temperature. Fig. 4 shows that fermentation at 37°C did not yield transformation. Fermentation at 28°C was more effective than that of room temperature during 5–25 days of fermentation time. However, 30-day fermentation at room temperature exhibited a similar conversion to that at 28°C. Therefore, the fermentation of *I. aromatica* with *C. rogersoniana* at ambient temperature for 30 days is sufficient to improve AChE inhibitory activity.

The solid–liquid rate is another factor for solid-state fermentation. Different solid (*I. aromatica*, g)-liquid (water, mL) rates of 1:5, 1:3, 1:2, 1:1, and 1:0.5 were selected to obtain an optimal rate. As shown in Fig. 5, *I. aromatica* containing 100–200% (v/w) water was suitable for fermentation. Therefore, *I. aromatica* immersed in

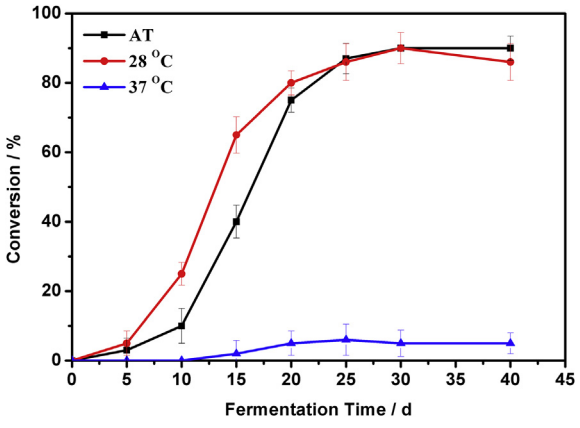

FIG. 4. The conversion rates after fermentation at different times and temperatures.

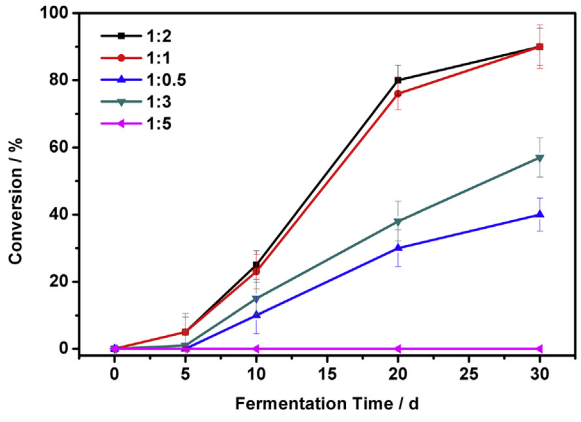

FIG. 5. The conversion rates after fermentation of UFIA at different solid (*I. aromatica*, g)-liquid (water, mL) rates.

100−200% water and fermented with *C. rogersoniana* at ambient temperature for 30 days is conducive to the biotransformation of actinodaphnine (**1**), which improves AChE inhibitory activity of *I. aromatica*.

In conclusion, fermentation of *I. aromatica* with *C. rogersoniana* significantly improved AChE inhibitory activity. The biotransformation of actinodaphnine (**1**) into (4*R*,6a*S*)-4-hydroxyactinodaphnine (**2**) played an important role in the improvement of AChE inhibitory activity of *I. aromatica*. Moreover, it was determined that *I. aromatica* immersed in 100−200% water and fermented with *C. rogersoniana* at ambient temperature for 30 days was optimal for facilitating this biotransformation. The present study provides a novel approach for improving the AChE inhibitory effect of *I. aromatica* and suggests that CFIA may be used as an alternative AChE inhibitor.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.04.012.

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References

1. **Flora of China Editorial Committee:** Flora Reipublicae Popularis Sinicae, vol. 30. Science Press, Beijing (2004).
2. **Chen, J.-J., Lee, T.-H., Kuo, W.-L., Sung, P.-J., Chen, I.-S., Shu, C.-W., Cheng, M.-J., Wang, T.-C., Lim, Y.-P., and Xu, R.:** (+)-(6a*R*,7*R*)-7-Hydroxy-*N*-butyrylcaaverine, a new aporphine alkaloid from the roots of *Illigera luzonensis* with cytotoxic activity, Chem. Nat. Compd., **51**, 739−742 (2015).
3. **Chen, K.-S., Wu, Y.-C., Teng, C.-M., Ko, F.-N., and Wu, T.-S.:** Bioactive alkaloids from *Illigera luzonensis*, J. Nat. Prod., **60**, 645−647 (1997).
4. **Ross, S. A., Minard, R. D., Shamma, M., Fagbule, M. O., Olatunji, G., and Gbile, Z.:** Thaliporphinemethine: a new phenanthrene alkaloid from *Illigera pentaphylla*, J. Nat. Prod., **48**, 835−836 (1985).
5. **Liu, S. L.:** Studies on the alkaloids of *Illigera luzonensis* (Presl.) Merr, J. Chin. Chem. Soc., **24**, 209−211 (1977).
6. **Chen, J.-J., Hung, H.-C., Sung, P.-J., Chen, I.-S., and Kuo, W.-L.:** Aporphine alkaloids and cytotoxic lignans from the roots of *Illigera luzonensis*, Phytochemistry, **72**, 523−532 (2011).
7. **Huang, C.-H., Chan, Y.-Y., Kuo, P.-C., Chen, Y.-F., Chang, R.-J., Chen, I.-S., Wu, S.-J., and Wu, T.-S.:** The constituents of roots and stems of *Illigera luzonensis* and their anti-platelet aggregation effects, Int. J. Mol. Sci., **15**, 13424−13436 (2014).
8. **Yuan, A., Qin, L., and Kang, S.:** Chemical constituents of *Illigera khasiana* C. B. Clarke, Chin. Bull. Bot., **29**, 324−326 (1987).
9. **Xie, L., Li, P., Gong, Z., and Ou, Y.:** Study on chemical consituents of *Illigera aromatica* S. Z. Huang et S. L. Mo, Contemp. Med., **17**, 31−32 (2011).
10. **Huang, S. Z.:** A new species and some medicinal plants of *Illigera* Bl. from Guangxi, Guihaia, **5**, 17−20 (1985).
11. **Dong, J. W., Cai, L., Li, X. J., Wang, J. P., Mei, R. F., and Ding, Z. T.:** Monoterpene esters and aporphine alkaloids from *Illigera aromatica* with inhibitory effects against cholinesterase and NO production in LPS-stimulated RAW264.7 macrophages, Arch. Pharm. Res., **40**, 1394−1402 (2017).
12. **Dong, J. W., Cai, L., Li, X. J., Shu, Y., Wang, J. P., and Ding, Z. T.:** A novel sesquiterpene derivative with a seven-membered B ring from *Illigera aromatica*, Nat. Prod. Res., **32**, 2589−2595 (2018).
13. **Qin, D., Shen, W., Wang, J., Han, M., Chai, F., Duan, X., Yan, X., Guo, J., Gao, T., Zuo, S., and Dong, J.:** Enhanced production of unusual triterpenoids from *Kadsura angustifolia* fermented by a symbiont endophytic fungus, *Penicillium* sp. SWUKD4.1850, Phytochemistry, **158**, 56−66 (2019).
14. **Eom, S. J., Hwang, J. E., Kim, K. T., and Paik, H. D.:** Increased antioxidative and nitric oxide scavenging activity of ginseng marc fermented by *Pediococcus acidilactici* KCCM11614P, Food Sci. Biotechnol., **27**, 185−191 (2018).
15. **Zhou, S. D., Xu, X., Lin, Y. F., Xia, H. Y., Huang, L., and Dong, M. S.:** On-line screening and identification of free radical scavenging compounds in *Angelica dahurica* fermented with *Eurotium cristatum* using an HPLC-PDA-Triple-TOF-MS/MS-ABTS system, Food Chem., **272**, 670−678 (2019).
16. **Zhang, Z. P., Ma, J., He, Y. Y., Lu, J., and Ren, D. F.:** Antioxidant and hypoglycemic effects of *Diospyros lotus* fruit fermented with *Microbacterium flavum* and *Lactobacillus plantarum*, J. Biosci. Bioeng., **125**, 682−687 (2018).
17. **Qi, K., Xia, X.-X., and Zhong, J.-J.:** Enhanced anti-oxidative activity and lignocellulosic ethanol production by biotin addition to medium in *Pichia guilliermondii* fermentation, Bioresour. Technol., **189**, 36−43 (2015).
18. **Li, X.-J., Dong, J.-W., Cai, L., Mei, R.-F., and Ding, Z.-T.:** Improving the acetylcholinesterase inhibitory effect of *Illigera henryi* by solid-state fermentation with *Clonostachys rogersoniana*, J. Biosci. Bioeng., **124**, 493−497 (2017).
19. **Cai, L., Dong, J.-W., Zhao, L.-X., Zhou, H., Xing, Y., Li, Y., Li, Z.-J., Duan, W.-H., Li, X.-J., and Ding, Z.-T.:** An improved water-soluble/stereospecific biotransformation of aporphine alkaloids in *Stephania epigaea* to 4*R*-hydroxyaporphine alkaloids by *Clonostachys rogersoniana*, Process Biochem., **51**, 933−940 (2016).