Laccase Production by *Pycnoporus* sp. W-9 Using Rose Dregs and Its Application for Phenol Degradation

Xiaohui Liang,[a,b] Dongliang Hua,[a,b*] Yuxiao Zhao,[a,b] Hongyu Si,[a,b*] and Bing Wang[c]

Rose dregs were used for laccase production by the strain *Pycnoporus* sp. W-9 under liquid fermentation, and the obtained laccase was used for phenol degradation. The conditions for laccase production were optimized by Box-Behnken design, and the phenol degradation conditions using the crude laccase were optimized by central composite design. The optimal conditions for laccase production by the strain W-9 were as follows: rose dregs (40 g/L), KCl (0.144 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (0.699 g/L), NaCl (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), $CuSO_4 \cdot 5H_2O$ (0.306 g/L), Tween 80 (0.2 g/L), and $CaCO_3$ (1 g/L), at a pH of 6.0, incubated at 30 °C and 200 rpm for 7 d. The corresponding laccase yield reached 17.4 U/mL, which was approximately 1.8 times the original production. The optimal conditions for phenol removal by the crude laccase were as follows: laccase (12.2 U/mL), 0.1 mol/L citric acid-disodium hydrogen phosphate buffer (pH 3.5), phenol (100 μg/mL), and Triton X-100 (284 μg/mL), incubated at 45 °C for 2 h. The corresponding phenol degradation rate reached 86.6%. These results should be useful for utilization of rose dregs and bioremediation of soil and wastewater.

Keywords: Laccase; Rose dregs; Fermentation; Phenol degradation; Optimization; Pycnoporus sp.

Contact information: a: Energy Institute, Qilu University of Technology (Shandong Academy of Sciences), Shandong Provincial Key Laboratory of Biomass Gasification Technology, Jinan 250014, China; b: School of Energy and Power Engineering, Qilu University of Technology (Shandong Academy of Sciences) Jinan 250014, China; c: College of Chemical Engineering, Qinghai University, Xining 810016, China;
** Corresponding authors: Dongliang hua, huadl@sderi.cn; Hongyu si, sihy@sderi.cn*

INTRODUCTION

Laccase (EC 1.10.3.2) is a class of copper-containing enzymes that can catalyze the oxidation of many substrates with oxygen. The oxidizable substrates include polyphenols, methoxyphenols, aminophenols, and aromatic amines; the final product is water (Rangelov and Nicell 2018). Laccase is widely distributed in plants, microorganisms, and animals, and most laccase has been isolated from fungi such as ascomycetes, deuteromycetes, and basidiomycetes (Du *et al*. 2018; Xu *et al*. 2019). Phenol is a common pollutant from the wastewaters of coal mines, plastics, oil refining, pulp mills, distilleries, pharmaceuticals, leather making, herbicides, and pesticides (Sivasubramanian and Namasivayam 2015; Cordova Villegas *et al*. 2016). It causes systemic diseases in humans and is one of the 129 water pollutants under priority control by the U.S. Environmental Protection Agency (Huang *et al*. 2015). However, the amount of phenol-containing wastewater is increasing with increasing industrial productivity, causing harm to water and soil (Naidu *et al*. 2016; Dong *et al*. 2019). Degradation of phenol by laccase from microorganisms is considered one of the cheapest and safer approaches for remediation of phenol-containing soil and wastewater (Chandra *et al*.

2011).

Rosa rugosa is a member of Rosaceae and has been used as a traditional herb and food in China. The flowers of *Rosa rugos*a cv. 'Plena' have been used for production of cakes, scented tea, sauce, wine, essential oil, and other items. Rose dregs (only composed of petals and calyx) are the waste from rose essential oil production, and the production is about 10000 t per year in China. As the main waste of the rose processing industry, rose dregs can be utilized to reduce environmental pollution. There are many useful ingredients in rose dregs, including polyphenols, protein, sugar, and cellulose. The protein and cellulose could be used for foods or feed production; polyphenols could be used for medicine or health food development, such as products for antioxidant and diabetes treating (Liu *et al.* 2018). Polyphenols have been reported to be plentiful in rose dregs (Chen *et al*. 2015; Huang *et al*. 2017). In a former investigation, it was found that total polyphenols composed approximately 6% to 8% of the dry mass of rose dregs from *Rosa rugosa* cv. 'Plena', and they could be used as the inducer for laccase production. In this study, the rose dregs of *Rosa rugosa* cv. 'Plena' were first used for laccase production with the strain *Pycnoporus* sp. W-9. The fermentation conditions were first screened by a Plackett-Burman design, and the significant factors were further optimized by response surface methodology. The crude laccase was used for phenol degradation, and the removal rate was evaluated and optimized. The results should be useful for bioremediation of soil and wastewater.

EXPERIMENTAL

Microorganism

The strain *Pycnoporus* sp. W-9 was isolated from rose dregs and maintained on a potato dextrose agar (PDA) slant at 4 °C. It was transferred to PDA plates incubated at 30 °C for 3 d and then stored at 4 °C. Subculturing was performed every 3 months.

Culture Media and Cultivation

The rose dregs were dried at 80 ^{o}C, then smashed and screened; the powder ($<$ 0.2 mm) was collected and used for laccase production. The basal medium for laccase production by the strain *Pycnoporus* sp. W-9 was composed of rose dregs (40 g/L), KCl (0.15 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (0.5 g/L), NaCl (0.2 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), $CuSO_4{\cdot}5H_2O$ (0.2 g/L), Tween 80 (0.2 g/L), and $CaCO_3$ (1 g/L). The pH value was adjusted to 6.0. The seed medium was composed of glucose (20 g/L), peptone (5 g/L), yeast extract (1 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (0.5 g/L), and $FeSO_4{\cdot}7H_2O$ (0.01 g/L) at a pH of 6.0. The mediums used for laccase fermentation were sterilized at 121^{o}C for 20 min by a sterilizer. The mycelium on the 6-d-old PDA plate was transferred to 50-mL / 250-mL Erlenmeyer flasks and incubated at 30 °C for 48 h. Then, 2 mL of seed culture was added to 250-mL Erlenmeyer flasks containing 50 mL of basal medium and incubated at 30 °C and 200 rpm for 7 d. The sample volumes of 2 mL were taken from the culture media, followed by measurement of laccase activity of the culture broth after centrifugation (10,000 *g* for 10 min at 4 °C), and the supernatant was used for the laccase activity assay.

Laccase Activity Determination

Laccase activity was determined using 2,6-dimethoxyphenol (DMP) as the

substrate (Litthauer *et al*. 2007). The reaction mixture (3 mL) consisted of 0.01 mol/L DMP (0.5 mL) and 0.1 mol/L citric acid-disodium hydrogen phosphate buffer (pH 3.5) (2.4 mL), followed by incubation at 45 °C for 5 min, and 0.1 mL of culture supernatant was added. Oxidation of DMP was monitored by an increase in absorbance at 470 nm (ε_{470}=49,600L/(mol·cm). One unit of laccase activity (U) was defined as the amount of enzyme required to oxidize 1 μmol of DMP per min.

Degradation of Phenol

The reaction mixture consisted of crude laccase with different activities (the activities varying with the experimental design), 0.1 mol/L citric acid-disodium hydrogen phosphate buffer (pH 3.5) (1000 mL), phenol (100 mg), and Triton X-100 (200 μg / mL) incubated at 45 °C for 2 h. Then, the phenol concentration was assayed for the degradation estimation. Samples for phenol concentration determination were centrifuged at 10,000 *g* for 10 min, and the supernatant was passed through a 0.22-μm membrane filter and analyzed by high-performance liquid chromatography (HPLC) as described by Wang *et al*. (2019): H-class C18 column (250 × 4.6 mm; WATERS, American); 40/30 (v/v) methanol in water as mobile phase, flow rate 1 mL / min; detected at 270 nm; column temperature 30 ^{o}C; sample volume 20 mL.

Experimental Design and Statistical Analysis

Design Expert (version 8.0, Stat-Ease, Inc., Minneapolis, MN, USA) was used for the Box-Behnken design and central composite design and regression analysis and ANOVA in this study.

Characteristics of the Rose Dregs

In this study, the total sugars and cellulose were determined by anthrone-sulfuric acid colorimetry. Total phenols and total protein were determined by the Folin-Ciocalteu method. Ash content was determined by combustion method, and C/N ratio was determined by the potassium dichromate and Kjeldahl methods (Zhou *et al.* 2010; Lan *et al.* 2013; Chen *et al.* 2015; Liu *et al.* 2019), respectively.

RESULTS AND DISCUSSION

Characteristics of the Rose Dregs

The composition of the rose dreg used in this study is listed in Table 1. The total phenols were 7.9%, which was enough for laccase induction. The total sugar content was 21.9%, cellulose was 12.6%, and total protein was 16.2%. Thus, the nutrients of the rose dregs were suitable for the growth of most microorganisms.

Table 1. Characteristics of the Rose Dregs

Total Sugars (%)	Cellulose (%)	Total Phenols (%)	Total Protein (%)	Ash (%)	C/N Ratio
21.9	12.6	7.9	16.2	13.6	6.1

Optimization of Laccase Production

In the experiments, three factors (KCl (*A*), K_2HPO_4 (*B*), and $CuSO_4 \cdot 5H_2O$ (*C*)) were selected and further optimized by Box-Behnken design (Table 2). From the

experimental design and the results of the Box-Behnken design, the final estimated response model equation was obtained (Eq. 1),

$$Y = 16.1 + 2.44A - 0.23B + 1.24C + 0.28AB - 1.25AC - 0.38BC - 1.35A^2 - 1.43B^2 - 1.15C^2 \quad (1)$$

where Y is the response factor (laccase production, U/mL), and A, B, and C represent the three independent factors, KCl (g/L), K_2HPO_4 (g/L), and $CuSO_4 \cdot 5H_2O$ (g/L), respectively.

The ANOVA results are shown in Table 3. In the table, the fit of the model was checked by the coefficient of determination (R^2), which was calculated as 0.96, indicating that approximately 96% of the variability in the response could be explained by this model. The statistical significance of the model equation was evaluated by the F-test for ANOVA. The p-value was also very low ($p < 0.01$), indicating the significance of the model. The lack-of-fit was insignificant at 5% level. The linear and quadratic items were significant. Overall, the statistical results showed a good fit between the model and the experimental data.

Table 2. Box-Behnken Design and Results for Laccase Production

Run No.	KCl (*A*) (g/L)	K_2HPO_4 (*B*) (g/L)	$CuSO_4 \cdot 5H_2O$ (*C*) (g/L)	Laccase Yield (*Y*) (U/mL)
1	1(0.15)	1(0.9)	0(0.3)	15.9
2	1(0.15)	0(0.7)	-1(0.2)	16.6
3	1(0.15)	-1(0.5)	0(0.3)	15.8
4	-1(0.05)	1(0.9)	0(0.3)	10.3
5	1(0.15)	0(0.7)	1(0.4)	15.3
6	0(0.1)	-1(0.5)	-1(0.2)	11.5
7	-1(0.05)	0(0.7)	-1(0.2)	9.4
8	-1 (0.05)	-1(0.5)	0(0.3)	11.3
9	0(0.1)	1(0.9)	-1(0.2)	11.8
10	0(0.1)	-1(0.5)	1(0.4)	16.0
11	-1(0.05)	0(0.7)	1(0.4)	13.1
12	0(0.1)	1(0.9)	1(0.4)	14.8
13	0(0.1)	0(0.7)	0(0.3)	16.1
14	0(0.1)	0(0.7)	0(0.3)	16.1
15	0(0.1)	0(0.7)	0(0.3)	16.1

The data in parentheses are the coded factor levels.

Table 3. ANOVA Results of the Regression Model

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p > F
Model	83.90	9	9.32	14.07	0.005
Error	3.31	5	0.66		
Corrected Total	87.21	14			

$R^2 = 0.96$; $R^2_{adj} = 0.89$; coefficient of variation (CV) = 5.81%

The response surface curves (Fig. 1) were plotted to explain the theoretical combination of KCl (*A*), K_2HPO_4 (*B*), and $CuSO_4 \cdot 5H_2O$ (*C*) *vs*. laccase yield (*Y*). The optimal values of *A*, *B*, and *C* were 0.87, -0.0032, and 0.064, respectively. The corresponding optimum concentrations were (rose dregs 40 g/L, KCl 0.144 g/L, KH_2PO_4

1 g/L, K_2HPO_4 0.699 g/L, NaCl 0.2 g/L, $MgSO_4 \cdot 7H_2O$ (0.3 g/L), $CuSO_4 \cdot 5H_2O$ 0.306 g/L, Tween 80 0.2 g/L, and $CaCO_3$ 1 g/L, pH 6.0, incubated at 30 °C, 200 rpm for 7 d). The predicted maximum laccase production was 17.2 U/mL. To confirm the optimized laccase production conditions, three additional experiments were performed, and the mean value of laccase activity was 17.4 U/mL broth, which agreed well with the predicted yield.

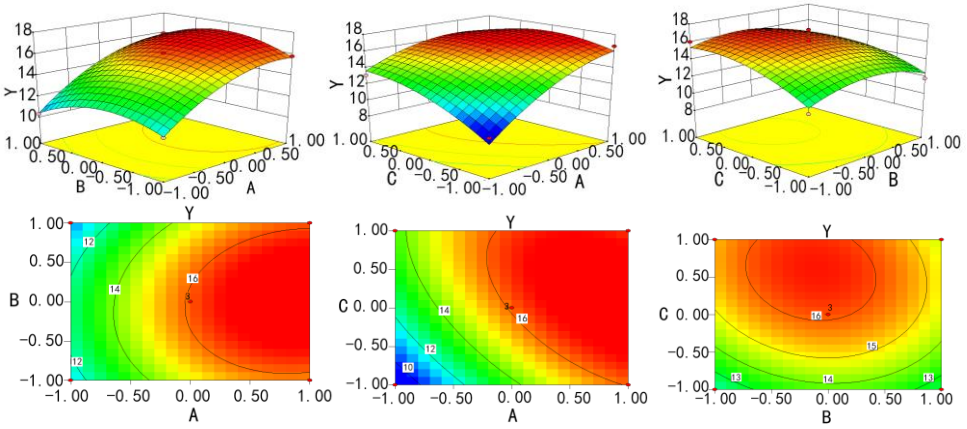

Fig. 1. Response surfaces and contours of laccase production by the strain *Pycnoporus* sp. W-9

Table 4. Central Composite Design of Phenol Degradation by Laccase

Run No.	Laccase activity (*D*) (U/mL)	Triton X-100 (*E*) (μg/mL)	Degradation Rate (*Z*) (%)
1	-1(5)	-1(100)	67.4
2	1(15)	-1(100)	70.8
3	-1(5)	1(300)	75.3
4	1(15)	1(300)	88.7
5	0(10)	-1.414(58.6)	63.4
6	0(10)	1.414(341.4)	82.1
7	-1.414(7.9)	0(200)	69.7
8	1.414(17.1)	0(200)	72.6
9	0(10)	0(200)	83.6
10	0(10)	0(200)	83.6

Optimization of Phenol Degradation

Laccase activity (*D*) and Triton X-100 (*E*) concentration were optimized by central composite design (Table 4). The ANOVA results are shown in Table 5. The coefficient of determination (R^2) of the model was 0.93, indicating that approximately 93% of the variability in the response could be explained by this model. The statistical significance of the model equation was evaluated by the F-test for ANOVA. The p-value was low ($p < 0.05$), indicating the significance of the model. Overall, the statistical results showed a good fit between the model and the experimental data.

Table 5. ANOVA Results of the Regression Model

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p > F
Model	578.61	5	115.72	10.03	0.022
Error	46.13	4	11.53		
Corrected Total	624.74	9			

R^2 = 0.93; R^2_{adj} = 0.83; CV = 4.48%

From the experimental design and the results of the central composite design, the final estimated response model equation was obtained (Eq. 2),

$$Z = 83.6 + 2.61D + 2.5DE - 5.33D^2 - 4.53E^2 \quad (2)$$

where Z is the degradation rate (%), and D and E represent the two independent factors, laccase activity (U/mL) and Triton X-100 (μg/mL), respectively. The response surface curves (Fig. 2) were plotted, and the optimal values of D and E were 0.44 and 0.84, respectively, while the predicted maximum degradation rate was 86.9%. The corresponding optimum concentrations were laccase 12.2 U/mL, pH 3.5, phenol 100 μg/mL, Triton X-100 284 μg/mL, incubated at 45 °C for 2 h, and the actual maximum degradation rate of the phenol was 86.6%.

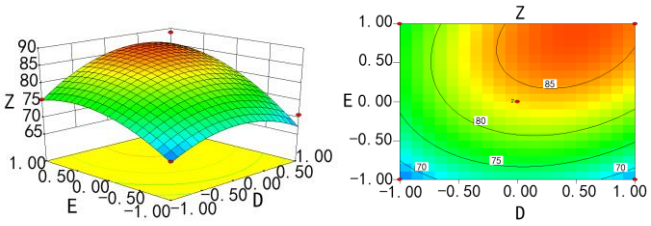

Fig. 2. Response surface and contour of phenol degradation by laccase

In this study, rose dregs were first used for laccase production by the strain *Pycnoporus* sp. W-9, and laccase yield reached 17.4 U/mL, and maximum degradation rate of the phenol was 86.6%, which provides a new way for the resource utilization of rose dregs. However, the laccase yield and the degradation rate of phenol were relatively lower than the reports at present. Zhao *et al.* (2018) expressed a kind of thermotolerant laccase from *Pycnoporus sanguineus* in *Trichoderma reesei* and its application in the degradation of bisphenol A, and it was found that the activity of laccase reached 17.7 IU/mL after 144 h fermentation. The degradation rate of bisphenol A reached 95%. Tian *et al.* (2019) reported the purified laccase of the stain *Pycnoporus* sp. SYBC-L10 could degrade tetracycline or oxytetracycline 100% within 5 min. Furthermore, laccase can also decolorizes anthraquinone, azo, and triphenylmethane dyes, and the crude laccase was effective in the decolorization of azo dye (RB220), with more than 90% of decolorization in 24 h; and anthraquinone dye (RBBR), with 60% decolorization, triphenylmethane dye (MG), with 68% decolorization, both in 72 h (Cardoso *et al.* 2018). In this work, the conditions of laccase produced in a fermenter, and the degradation of other materials such as antibiotics, dyes, pollutants should also be investigated in further study.

CONCLUSIONS

1. Rose dregs were used as the unique carbon and nitrogen sources for laccase production with the strain *Pycnoporus* sp. W-9, and optimal conditions were obtained: the laccase yield reached 17.4 U/mL.
2. The obtained laccase was used for phenol degradation, and the optimal conditions for phenol removal were obtained, and the corresponding phenol degradation rate reached 86.6%.
3. The results of this work will provide a new way for the resource utilization of rose dregs. Laccase produced by rose dregs can be used for pollutants degradation and environmental protection.

ACKNOWLEDGMENTS

This work was financially supported by the Special Fund for Agro-Scientific Research in the Public Interest (Grant No. 201503135-04), the National Natural Science Foundation of China (No. 51978347), the Key R&D Program of Shandong Province (No. 2019GSF110012 and 2019LYX027, Youth Science and Technology Innovation Team of Shandong Colleges and Universities (2019KJD002), the National Key R&D Program of China (No. 2018YFB1501403).

REFERENCES CITED

Cardoso, B. K., Linde G. A., Colauto, N. B., and Valle, J. S. (2018). "*Panus strigellus* laccase decolorizes anthraquinone, azo, and triphenylmethane dyes," *Biocatalysis and Agricultural Biotechnology* 16, 558-563. DOI: 10.1016/j.bcab.2018.09.026

Chandra, R., Yadav, S., Bharagava, R. N., and Rai, V. (2011). "Phenol degradation by *Paenibacillus thiaminolyticus* and *Bacillus cereus* in axenic and mixed conditions," *World Journal of Microbiology and Biotechnology* 27(12), 2939-2947. DOI: 10.1007/s11274-011-0777-4

Chen, T., Li, J., Chen, J., Song, H., and Yang, C. (2015a). "Anti-hyperplasia effects of *Rosa rugosa* polyphenols in rats with hyperplasia of mammary gland," *Environmental Toxicology and Pharmac*ology 39(2), 990-996. DOI: 10.1016/j.etap.2015.02.014

Chen, L. Y., Cheng, C. W., Liang, J. Y. (2015b). "Effect of esterification condensation on the Folin–Ciocalteu method for the quantitative measurement of total phenols," *Food Chemistry* 170, 10-15. DOI: 10.1016/j.foodchem.2014.08.038

Cordova Villegas, L. G., Mashhadi, N., Chen, M., Mukherjee, D., Taylor, K. E., and Biswas, N. (2016). "A short review of techniques for phenol removal from wastewater," *Current Pollution Reports* 2(3), 157-167. DOI: 10.1007/s40726-016-0035-3

Dong, D., Wang, R., Geng, P., Li, C., and Zhao, Z. (2019). "Enhancing effects of activated carbon supported nano zero-valent iron on anaerobic digestion of phenol-containing organic wastewater," *Journal of Environmental Management* 244, 1-12. DOI: 10.1016/j.jenvman.2019.04.062

Du, W., Sun, C., Wang, J., Wang, B., Yao. Z., Qu, F., Xia, J., Xie, W., Sun, J., and Duan, D. (2018). "Isolation, identification of a laccase-producing fungal strain and enzymatic properties of the laccase," 3 *Biotech* 8, 137-143. DOI: 10.1007/s13205-018-1149-7

Huang, D.-L., Wang, C., Xu, P., Zeng, G.-M., Lu, B.-A., Li, N.-J., Huang, C., Lai, C., Zhao, M.-H., Xu, J.-J., *et al*. (2015). "A coupled photocatalytic-biological process for phenol degradation in the *Phanerochaete chrysosporium*-oxalate-Fe_3O_4 system," *International Biodeterioration & Biodegradation* 97, 115-123. DOI: 10.1016/j.ibiod.2014.11.001

Huang, W., Mao, S., Zhang, L., Lu, B., Zheng, L., Zhou, F., Zhao, Y., and Li, M. (2017). "Phenolic compounds, antioxidant potential and antiproliferative potential of 10 common edible flowers from China assessed using a simulated *in vitro* digestion–dialysis process combined with cellular assays," *Journal of the Science of Food and Agriculture* 97(14), 4760-4769. DOI: 10.1002/jsfa.8345

Litthauer, D., van Vuuren, M. J., van Tonder, A., and Wolfaardt, F. W. (2007). "Purification and kinetics of a thermostable laccase from *Pycnoporus sanguineus* (SCC 108)," *Enzyme and Microbial Technology* 40(4), 563-568. DOI: 10.1016/j.enzmictec.2006.05.011

Lan, Z. P., Qiu, D. M., and Lei, G. D. (2013). "Determination of carbon-nitrogen ratio of citrus peel residue," *Journal of Neijiang Normal University* 28(10), 28-30. DOI: CNKI:SUN:NJSG.0.2013-10-008 (in Chinese)

Liu, K. S. (2019). "Effects of sample size, dry ashing temperature and duration on determination of ash content in algae and other biomass," *Algal Research* 40, 1-5. DOI: 10.1016/j.algal.2019.101486

Liu, L., Yasen, M., Tang, D., Ye, J., Aisa, H. A., and Xin, X. (2018). "Polyphenol-enriched extract of *Rosa rugosa*, thunb regulates lipid metabolism in diabetic rats by activation of ampk pathway," *Biomedicine & Pharmacotherapy* 100, 29-35. DOI: 10.1016/j.biopha.2018.01.143

Naidu, L. D., Saravanan, S., Goel, M., Periasamy, S., and Stroeve, P. (2016). "A novel technique for detoxification of phenol from wastewater: Nanoparticle assisted nano filtration (NANF)," *Journal of Environmental Health Science and Engineering* 14. DOI: 10.1186/s40201-016-0249-8

Rangelov, S., and Nicell, J. A. (2018). "Modelling the transient kinetics of laccase-catalyzed oxidation of four aqueous phenolic substrates at low concentrations," *Biochemical Engineering Journal* 132, 233-243. DOI: 10.1016/j.bej.2018.01.016

Sivasubramanian, S., and Namasivayam, S. K. R. (2015). "Phenol degradation studies using microbial consortium isolated from environmental sources," *Journal of Environmental Chemical Engineering* 3(1), 243-252. DOI:10.1016/j.jece.2014.12.014

Tian, Q. P., Dou, X., Huang, L., Wang, L., Meng D., Zhai, L. X., Shen, Y., You C. P., Guan, Z. B., Liao, X. R. (2020). "Characterization of a robust cold-adapted and thermostable laccase from *Pycnoporus* sp. SYBC-L10 with a strong ability for the degradation of tetracycline and oxytetracycline by laccase-mediated oxidation," *Journal of Hazardous Materials* In press. DOI: 10.1016/j.jhazmat.2019.121084

Wang, X., Wang, Y., Zhang, A., Duo, C., Zhao, C., and Xie, F. (2019). "Isolation of a highly efficient phenol-degrading fungus and the preparation of an effective microbial inoculum for activated sludge and its enhancement for hydrogen production," *International Journal of Hydrogen Energy* 44(30), 16004-16014. DOI: 10.1016/j.ijhydene.2018.10.154

Xu, G., Wang, J., Yin, Q., Fang, W., Xiao, Y., and Fang, Z. M. (2019). "Expression of a thermo- and alkali-philic fungal laccase in *Pichia pastoris* and its application," *Protein Expression and Purification* 154, 16-24. DOI: 10.1016/j.pep.2018.09.015

Zhao, J., Zeng, S. Q., Xia, Y., and Xia, L. M. (2018). "Expression of a thermotolerant laccase from *Pycnoporus sanguineus* in *Trichoderma reesei* and its application in the degradation of bisphenol A," *Journal of Bioscience and Bioengineering* 125(4), 371-376. DOI: 10.1016/j.jbiosc.2017.11.010

Zhou, Z. L., Li, F., and Li, J. W. (2010). "Study on relationship between sugar content and cold–hot nature of 20 kinds of herbs by fisher analysis," *World Science and Technology* 12(4), 558-561. DOI: 10.1016/S1876-3553(11)60020-3.

Article submitted: September 23, 2019; Peer review completed: January 12, 2020;
Revisions accepted: February 7, 2020. Published: February 25, 2020.
DOI: 10.15376/biores.15.2.2621-2629