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BIOLOGICAL SCIENCES

Effect of tween 40 and ethanol on the secretion, structure and antioxidant activities of exopolysaccharides from *Inonotus rickii*

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Abstract: In this study, the effects of Tween 40 and ethanol supplementation on the secretion, structure and antioxidant activities of exopolysaccharide (EPS) from *Inonotus rickii* were investigated. It was observed that Tween 40 and ethanol displayed a stimulatory effect on EPS secretion. The EPSs obtained by the addition of Tween 40 (EPS-T), ethanol (EPS-E) and control (EPS-C) were purified by Sepharose CL-6B gel chromatography and molecular weights of EPS-T, EPS-E and EPS-C were estimated to be 22.1, 30.0, and 40.5 kDa, respectively. Monosaccharide composition analysis indicated that EPS-T, EPS-E and EPS-C were mainly composed of mannose and glucose. Furthermore, EPS-E exhibited better OH· and DPPH scavenging activities than those of EPS-C and EPS-T, which might be associated with its molecular characterization.

Key words: antioxidant activities, exopolysaccharides, *Inonotus rickii*, molecular mass.

INTRODUCTION

Inonotus rickii is an inedible basidiomycete belonging to the genus *Inonotus*, which causes canker and decay in several ornamental trees (Ramos et al. 2008). Several sesquiterpenoids with moderate anti-tumor activities have been isolated from the fruiting bodies of *I. rickii* (Chen et al. 2014). However, the bioactive polysaccharides from *I. rickii* remain undeveloped. Only a few polysaccharides from the other species such as *I. obliquus* and *I. hispidus* are known, which possess a wide range of pharmacologic properties including antioxidant, anti-diabetes, and anti-tumor activities (Lee et al. 2014, 2012, Wang et al. 2011).

It is well-known that the submerged culture is a suitable method to produce EPS, which has the advantages of space saving, short period, and realization of industrial production. The stimulatory agents, including surfactants and organic solvents, are reported to modify cell membrane composition and thus increasing its permeabilization, or directly affect the synthesis of enzymes involved in the cell growth and biosynthesis of intracellular products (Kang et al. 2013, Xu et al. 2015). On the other hand, they are inexpensive and can be removed by simple method. Hence, the surfactants (Tween 40, Tween 60, and Tween 80) and organic solvents (toluene, chloroform, ethanol, and dimethyl sulfoxide), are used widely and effectively for cell permeabilization (Reese & Maguire 1969, Stasinopoulos et al. 1990).

In this study, the effects of several surfactants and organic solvents were used to enhance EPS secretion in the submerged culture of *I. rickii*. Tween 40 and ethanol were selected out of seven tested stimulatory agents.

After isolation and purification, the molecular weights and chemical characterizations of the EPSs obtained by the addition of Tween 40 (EPS-T), ethanol (EPS-E) and control (EPS-C, *i.e.* without addition) were determined. Finally, their antioxidant activities were also evaluated and compared.

MATERIALS AND METHODS

Microorganism and growth conditions

I. rickii was obtained by tissue isolation from naturally occurring fruiting body collected from Wanda Mountains of Heilongjiang Province. Sample was cut into 0.5 × 0.5 cm^2 and surface-sterilized through 10 % ethanol for 3 min, 3 % sodium hypochlorite for 1 min, 10 % ethanol for 3 min and sterile distilled water, respectively. After surface sterilization, the samples were dried with sterile tissue papers and placed onto potato dextrose agar (PDA) supplemented with 0.5 g/L penicillin G and streptomycin. The plate was incubated at 26 °C for 3 weeks. Then, fungi were isolated into pure culture by hyphal tip isolation technique on potato dextrose agar (PDA) plates without antibiotics according to the procedures of Supaphon et al. (2018) with some modifications. Finally, the fungi was identified by Prof. Xinsheng He (Southwest University of Science and Technology, Mianyang, China). This strain was maintained on PDA slant at the Henan Province Microbiological Culture Collection Center (HPMCC No. 545856a). The strain was transferred into the seed medium (30 g/L glucose, 3 g/L yeast extract) by punching out a disc (5 mm) from the plate culture. The seed culture was grown in a 250 mL flask containing 50 mL of the medium at 26 °C on a rotary shaker incubator (210 rpm) for 4 days.

Flask culture and the treatment of surfactant and organic solvent

The flask culture experiments were performed in a 500 mL Erlenmeyer flask containing 150 mL of the culture medium (lactose 60 g, yeast extract 4 g, distill water 1,000 mL, pH 5.0) at 26 °C for 12 days after inoculating with 4 % (by volume) of the seed culture. To find a suitable surfactant and organic solvents for EPS secretion in *I. rickii*, various surfactant and organic solvents were supplied at a concentration of 0.2 % (by volume) after 5 days of cultivation and were continued to culture up to 10 days according to our preliminary experiments. All the determinations were done in triplicate to ensure the reproducibility.

Mycelial dry mass and EPS determination

The mycelial pellets and culture broth were separated by filtration. The mycelial dry mass was measured after washing and drying the pellets several times. Crude EPS was obtained after procedure of concentration, ethanol precipitation, and centrifugation. The total sugar content was determined by the phenol sulfuric acid method using glucose as the standard (He et al. 2012).

Purification and molecular characterization of EPS

The crude EPS was purified by deproteinization, dialysis and gel filtration chromatography on Sepharose CL-6B. After hydrolyzation, reduction, and acetylation, the alditol acetates of refined EPS were analyzed by GC-MS. The infrared spectrum of the refined EPS was measured through a Bruker Tensor 27 FT-IR spectrometer (Bruker Optics, Germany) using KBr disc technique. The molecular mass of EPS in solution was determined by size-exclusion chromatography using a Sepharose CL-6B column. The separation was carried out at 22 °C at a flow rate of 0.6 mL/min. The calibration curve of *K*av (partition

coefficient) versus log molecular mass was prepared using the following equation.

$$Kav = (Ve-Vo)/(Vt-Vo) \quad (1)$$

Where, *V*e is the elution volume, *V*o is the column void volume and *V*t is the total column bed volume.

Thermogravimetric analysis of the polysaccharide was conducted in a TAQ5000IR TGA apparatus using 15 mg EPS of the test material. TGA (Thermal Gravimetric Analyzer) curve plot TGA signal (converted to percent weight change on the Y-axis) against the reference material temperature (on the X-axis).

Antioxidant assays

The scavenging activities of hydroxyl and DPPH radicals were evaluated for the antioxidant activity of the samples according to our previous report (Zheng et al. 2014).

Statistical analysis

The results were reported as mean ± S.D. One-way analysis of variance (ANOVA) was carried out for the compassion of mean values. $p < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Effect of surfactants and organic solvents on EPS secretion

Among the surfactants and organic solvents tested, Tween 40 and ethanol were not favorable for the mycelial growth (Table I). However, the concentration of EPS was improved by 70.6 % or 40.4 % when Tween 40 in the surfactants or ethanol in the organic solvents was employed, respectively. The similar results were observed by Hsieh et al. (2008) also found that Tween 80 exhibited a remarkable promoting effect on EPS production by *Grifola frondosa*, while Tween 20 addition was shown to have serious inhibition on cell growth. The results indicated that the surfactants and organic solvents might modify the composition of the mycelial cell membrane, thus change the permeability of mycelia cell in *I. rickii* and enhance the EPS secretion (Liu & Wu 2012, Zhang et al. 2011). Hence, the EPS with the higher molecular mass could be secreted when surfactants and organic solvents are added to the culture medium. It was of interest to find that Tween 60, chloroform and DMSO had positive effects on the mycelial biomass, which suggested that they could promote nutrient uptake at a certain concentration and enhance the biomass accumulation.

Table I. Effect of surfactants and organic solvents on mycelial growth and exopolysaccharide (EPS) production by *Inonotus rickii* in shake flask cultures.

	mycelia (g/L)	EPS (g/L)
Control	2.83±0.02	1.09±0.09
Surfactant		
Tween 40	2.64±0.03	1.86±0.09
Tween 60	4.03±0.07	0.91±0.10
Tween 80	3.97±0.08	1.09±0.02
Organic solvents		
Chloroform	4.67±0.01	1.22±0.03
Ethanol	1.83±0.01	1.53±0.04
DMSO	4.67±0.02	1.01±0.02
Acetone	4.43±0.01	1.28±0.07

Fermentations were carried out for 10 days at 26 °C. The surfactants and organic solvents were added to the basal medium at a volume fraction of 0.2 % after 5 days of cultivation. Values are mean±S.D. of triplicate determinations.

Comparison of molecular and structural properties of EPS

EPS groups designated as EPS-T, EPS-E and EPS-C were obtained from the EPSs cultivated by the addition of Tween 40, ethanol and control, respectively (Fig. 1a). IR spectra for the three EPS groups were depicted in Fig. 1b. All samples have similar characteristic absorption peaks of polysaccharides, and it showed a broad stretching intense characteristic peak

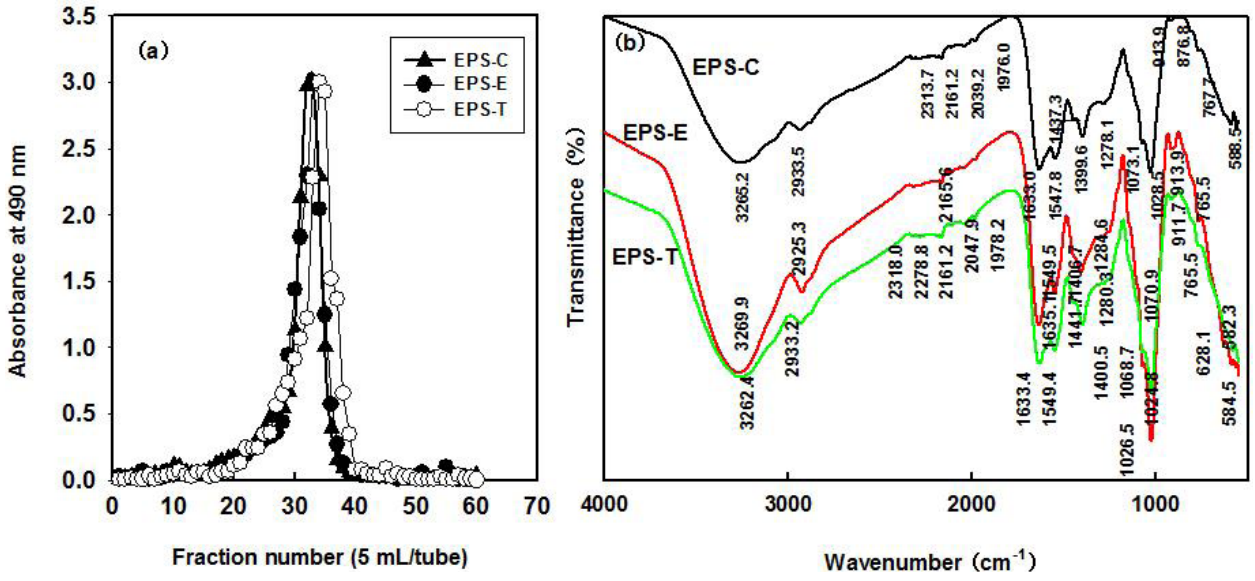

Figure 1. Elution profile (a) in Sepharose CL-6B chromatography and the FT-IR spectra (b) of the EPS produced by submerged culture of *I. rickii* by treatment of Tween 40 (EPS-T) and ethanol (EPS-E), respectively. EPS-C means without addition. Elutes were analyzed by measuring the absorbance at 480 nm for carbohydtate.

typical of -OH groups at 3265 cm^{-1} as well as a weak C–H absorption band at 2930 cm^{-1}. The characteristic band at 1634 cm^{-1} could be correlated to the stretching vibration of the C=O of the polysaccharide. Bands at 1025-1029 cm^{-1} suggested the presences of C-O type of linkages. The absorption peak at 890 cm^{-1} proved that all the samples are β-configuration (Wang et al. 2011). The results suggested that the function of Tween 40 and ethanol does not really affect the function groups of the EPS.

The molecular mass of EPS-T, EPS-E and EPS-C were estimated to be about 22.1, 30.0, and 40.5 kDa, respectively (Fig. 2). The EPS-E had moderate molecular mass than other two EPSs. The EPSs were hydrolyzed, acetylated and analyzed by GC-MS; and the monosaccharide composition of EPSs obtained was shown in Table II. Results indicated that mannose and glucose were the major monosaccharide from all EPSs though the ratio of them has been changed. Similarly, Xu et al. (2015) also observed that the EPS from *I. obliquus* under different culture conditions had the large amount of mannose and glucose in

Table II. Carbohydrate composition of the purified *Inonotus rickii* exopolysaccharides (EPS) produced with Tween 80 or acetone.

Carbohydrate/ %		EPS	
	EPS-C	EPS-T	EPS-E
Xylose	0.14± 0.05	0.40±0.00	0.22±0.04
Mannose	63.96±2.17	60.73±2.01	65.08±1.02
Galactose	0.51±0.07	1.09±0.09	0.28±0.07
Glucose	35.07±1.83	37.49±2.08	34.42±1.11
Gluconic acid	0.32±0.02	0.29±0.00	0.00±0.00

the chemical compositions. It is interesting to note that EPS-E had highest mannose content and no gluconic acid content. Though it was still in doubt, this result indicated that Tween 40 and acetone could alter the internal metabolism at a certain level by affecting the enzymatic syntheses (De et al. 2004).

Thermal gravimetric analysis of EPS

Some polysaccharides are extensively used in the development coated solid dosage form for specific drug delivery. On the other hand, the chemical modification of polysaccharides

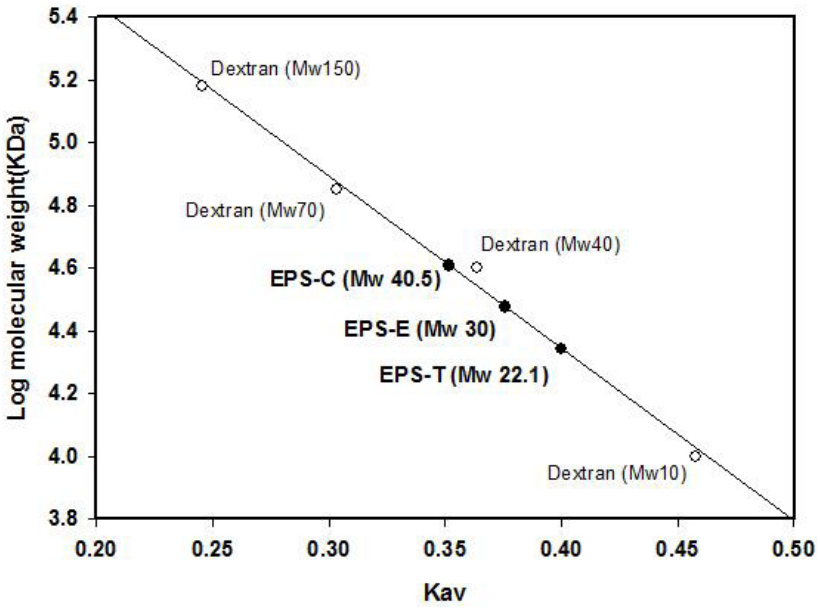

Figure 2. Molecular mass determination of the purified EPSs produced by submerged culture of *I. rickii* by treatment of Tween 40 (EPS-T) and ethanol (EPS-E), respectively, in Sepharose CL-6B chromatography. Elutes were analyzed by measuring the absorbance at 480 nm for carbohydrate. EPS-C means without addition. Three groups of EPS were denoted as closed circles.

always need heat to high temperature (Mehta et al. 2011). Hence, the decomposition behavior of the polysaccharide is of great importance to ascertain the thermochemical stability of the material in various applications, such as drug discovery. Thermogravimetric analysis involves the determination of mass changes with temperature variation, being a highly useful technique to analyze samples that either gain or lose mass upon heating. Experimental results for the TGA analysis of purified EPS fractions have been included in Fig. 3. According to the TGA curve of each fraction, the degradation temperature of EPS-T, EPS-E and EPS-C was determined to be 124, 93, and 120 °C, respectively. These findings suggested that the stability of the EPS fractions was compromised at temperatures above the observed degradation temperature. Furthermore, a significant mass loss was recorded in each fraction at temperatures around 275 °C, and gradually decreased to leave a final residue of 31.3 %, 27.4 % and 29.2 % of the original EPS mass for EPS-T, EPS-E and EPS-C, respectively. In any case, TGA confirmed that the three fractions generally possessed a high thermal stability. EPS-T and EPS-C have a similar thermal stability and degradation behavior, possibly due to their similar carbohydrate structures. This result suggests that the degradation behavior of EPS is highly related to the structure of EPS.

Antioxidant activities

In vitro antioxidant activities of three EPS groups of *I. rickii* were evaluated using hydroxyl and DPPH radical scavenging assays. The DPPH scavenging rates of three EPSs depicted in Fig. 4a proved that the hydroxyl scavenging activities of EPS-E were superior to those of EPS-T and EPS-C, reaching a maximum of 65.5 % at 10 mg/mL. Similarly, it has been reported that addition of Tween 80 into the culture medium could improve the antioxidant capacity (Trolox-Equivalent Antioxidant Capacity) of EPS of *Phellinus* sp. (Ma et al. 2013). The EPS-E showed the strongest DPPH radical scavenging capacities, though all three samples exhibited no notable differences at higher concentration of 10 mg/mL (Fig. 4b). The stronger antioxidant activities of EPS-E than that of EPS-C and EPS-T may be attributed to their difference of carbohydrate composition

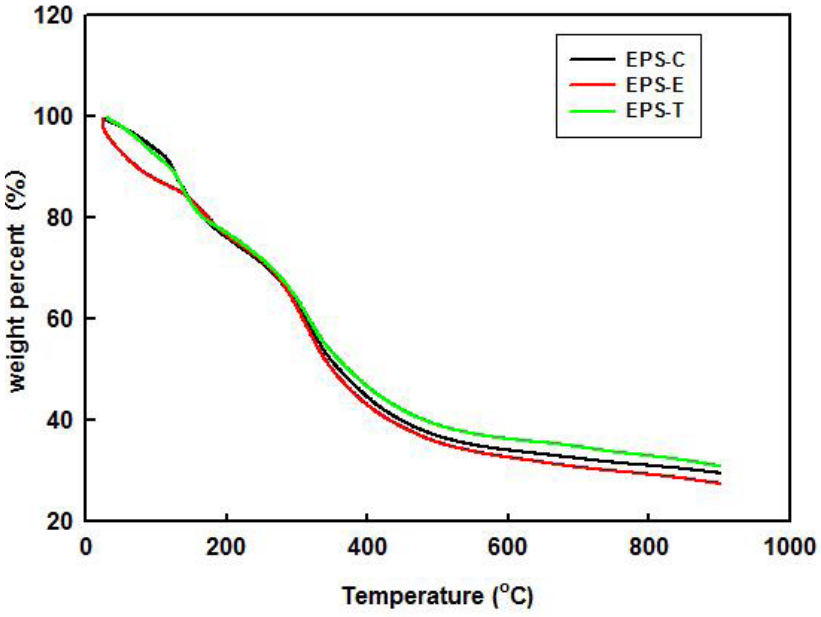

Figure 3. TGA thermogram of the purified EPSs produced by submerged culture of *I. rickii* by treatment of Tween 40 (EPS-T) and ethanol (EPS-E), respectively. EPS-C means without addition.

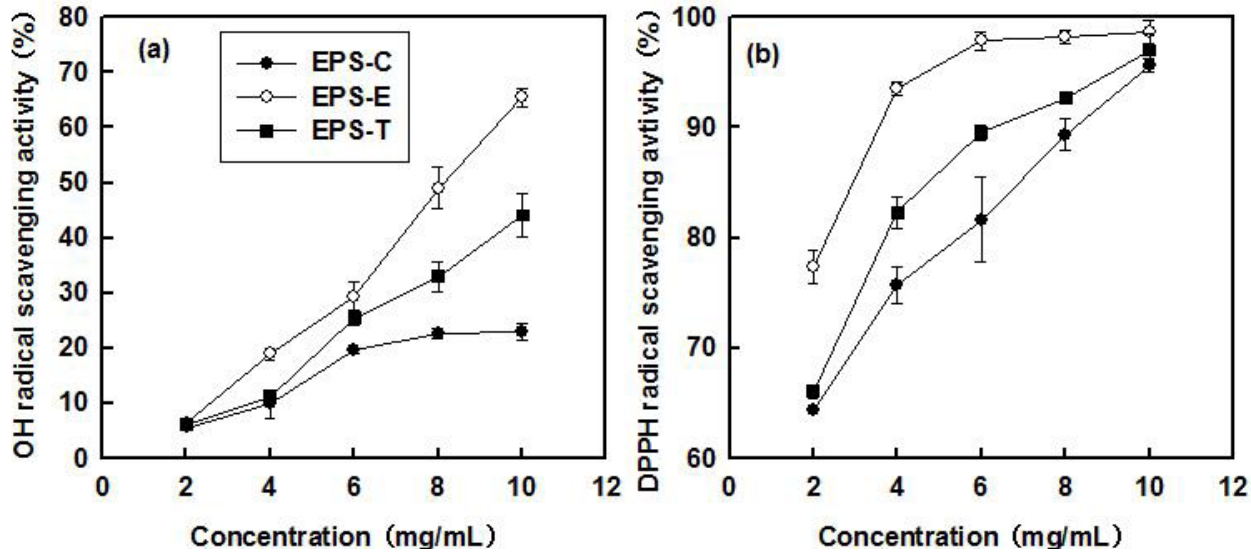

Figure 4. Bioactivity of EPS produced by submerged culture of *I. rickii* by treatment of Tween 80 (EPS-T) and ethanol (EPS-E), respectively. The results represent mean ± S.D. (n = 3). OH• (a) and DPPH (b) radical scavenging activity of EPS. EPS-C means without addition.

and molecular mass (Wang et al. 2010, Yang & Zhang 2009). The results were in agreement with the report of Mu et al. (2012), who showed that the polysaccharide extracts of *I. obliquus* had strong antioxidant activity.

CONCLUSIONS

We investigated the effects of surfactant and organic solvents on secretion, characterization and bioactivity of EPS of *I. rickii*. The results demonstrated that Tween 40 and ethanol could

enhance EPS secretion but interfere the mycelia growth. The molecular characterizations and bioactivities of EPS-T, EPS-E and EPS-C were compared. EPS-E showed potent hydroxyl and DPPH radical scavenging activities, which might be related to its molecular characterization. The optimization of EPS biosynthesis in the fungal cells and improvement of other biological activities by different concentration of Tween 40 and ethanol need further investigation.

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REFERENCES

CHEN HP, DONG WB, FENG T, YIN X, LI ZH, DONG ZJ, LI Y & LIU JK. 2014. Four new sesquiterpenoids from fruiting bodies of the fungus *Inonotus rickii*. J Asian Nat Prod Res 16: 581-586.

DE LA, GARCIA B & BARBA RAP. 2004. *Periplasmic penicillin* G acylase activity in recombinant Escherichia coli cells permeabilized with organic solvents. Process Biochem 39: 301-305.

HE PX, GENG LJ, WANG Z, MAO DB WANG JZ & XU CP. 2012. Fermentation optimization, characterization and bioactivity of exopolysaccharides from *Funalia trogii*. Carbohydr Polym 89: 17-23.

HSIEH C, WANG HL, CHEN CC, HSU TH & TSENG MH. 2008. Effect of plant oil and surfactant on the production of mycelial biomass and polysaccharides in submerged culture of *Grifola frondosa*. Biochem Eng J 38: 198-205.

KANG BY, ZHANG XH, WU ZQ, QI HS & WANG ZL. 2013. Solubilization capacity of nonionic surfactant micelles exhibiting strong influence on export of intracellular pigments in *Monascus* fermentation. Microb Biotechnol 6: 540-550.

LEE KR, LEE JS, SONG JE, HA SJ & HONG EK. 2014. Inonotus obliquus-derived polysaccharide inhibits the migration and invasion of human non-small cell lung carcinoma cells via suppression of MMP-2 and MMP-9. Int J Oncol 45: 2533-2540.

LIU YS & WU JY. 2012. Effects of Tween 80 and pH on mycelial pellets and exopolysaccharide production in liquid culture of a medicinal fungus. J Ind Microbiol Biotechnol 39: 623-628.

MA XK, ZHANG H & FAM H. 2013. Influence of rutin, $FeSO_4$, Tween 80, aspartate and complex vitamins on synthesis of fungal exopolysaccharide. Carbohydr Polym 92: 1188-1196.

MEHTA TJ, RAJPUT SS, PATEL MR, PATEL KR, PATEL NM & MOTHILAL M. 2011. Formulation, development and optimization of metronidazole compression coated tablets. Int J Sci Eng Res 3: 94-103.

MU HB, ZHANG AM, ZHANG WX, CUI GT, WANG SC & DUAN JY. 2012. Antioxidative properties of crude polysaccharides from *Inonotus obliquus*. Int J Mol Sci 13: 9194-9206.

RAMOS AP, CAETANO MF & MELO I. 2008. *Inonotus rickii* (Pat.) Reid: an important legnicolous basidiomycete in urban trees. Rev Fac Cienc Agrar 31: 159-167.

REESE ET & MAGUIRE A. 1969. Surfactants as stimulants of enzyme production by microorganisms. Appl Microbiol 17: 242-245.

STASINOPOULOS SJ & SEVIOUR RJ. 1990. Stimulation of exopolysaccharide production in the fungus *Acremonium persicinum* with fatty acids. Biotechnol Bioeng 36: 778-782.

SUPAPHON P, KEAWPIBOON C, PREEDANON S, PHONGPAICHIT S & RUKACHAISIRIKUL V. 2018. Isolation and antimicrobial activities of fungi derived from *Nymphaea lotus* and *Nymphaea stellate*. Mycosci 59: 415-423.

WANG J, ZHANG QB, ZHANG ZS, SONG HF & LI PC. 2010. Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from *Laminaria japonica*. Int J Biol Macromol 46: 6-12.

WANG JG, MA ZC, ZHANG LN, FANG YP, JIANG FT & PHILLIPS GO. 2011. Structure and chain conformation of water-soluble heteropolysaccharides from *Ganoderma lucidum*. Carbohydr Polym 86: 844-851.

WANG ZB, SUN CY, LI DH & MENG YU. 2011. Extraction and immune function of polysaccharide from the fruit body of *Inonotus hispidus*. Sci Technol Food Ind 32: 383-392.

XU X, SHEN M & QUAN L. 2015. Stimulatory agents simultaneously improving the production and antioxidant activity of polyphenols from *Inonotus obliquus* by submerged fermentation. Appl Biochem Biotechnol 176: 1237-1250.

YANG LQ & ZHANG LM. 2009. Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. Carbohydr Polym 76: 349-361.

ZHANG BB & CHEUNG PC. 2011. A mechanistic study of the enhancing effect of Tween 80 on the mycelial growth and exopolysaccharide production by *Pleurotus* tuber-regium. Bioresour Technol 102: 8323-8326.

ZHENG JQ, MAO XJ, GENG LJ, YANG GM & XU CP. 2014. Production optimization, preliminary characterization and bioactivity of exopolysaccharides from *Incutis tamaricis* (Pat.) Fiasson & Niemela. J Taiwan Inst Chem Eng 45: 725-733.

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