Effects of Tween 80 on the liquid fermentation of *Lentinus edodes*

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Received: 26 July 2017 / Revised: 13 February 2018 / Accepted: 19 February 2018 / Published online: 27 February 2018
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Abstract This paper explored the effects of Tween 80 on the biomass, intracellular polysaccharide (IPS) content, fermentation parameters, the pellets size of mycelium, and the antioxidant activity of IPS in *Lentinus edodes* liquid fermentation. With adding to Tween 80, the outputs of biomass and IPS increased during the *L. edodes* fermentation, respectively, while the reducing sugar content was decreased, as well as, the time courses of pH value were different. It was also shown that the addition of Tween 80 could protect the intact of pellets from breaking down. The effects of Tween 80 on the main structure of IPS were no obvious, and the IPS were revealed similar infrared spectrum, as was indicated by the infrared spectrum analysis. Improvements in the scavenging capacity of DPPH radicals of IPS were observed in Tween 80 treated group compared with the control group. Tween 80 exerts impacts on the liquid fermentation of *L. edodes*.

Keywords *Lentinus edodes* · Intracellular polysaccharide · Tween 80 · Pellet size · Antioxidant activity

Introduction

Lentinus edodes, commonly known as *xianggu*, is a world-renowned medicinal and edible fungus [1]. It contains complex constituents and has multiple active ingredients, such as Lentinan. Lentinan is a high molecular weight glucan with multiple biological properties, including anti-tumor, antioxidant, anti-viral, immunomodulatory, and immune-stimulatory activities, which are widely applied in the medicine and food fields [2]. Submerged culture of *Lentinus edodes* produces potential advantages of higher mycelia production in a tight space and shorter time with fewer opportunities of contamination [3]. During fermentation process, the composition of medium, pH value, culture temperature, etc. exert great effects on the quality and yield of *L. edodes* mycelia [4]. Surfactants, as amphiphilic compounds, have the characteristics of solubility and emulsification as well as the abilities to decrease surface and interfacial tensions [5]. Surfactants have been widely deployed in the regulation of microbial fermentation, such as alcohol fermentation, alkaline lipase production, and L-tryptophan production [6–8].

Tween 80 as food additives has been approved by the US Food and Drug Administration for widely uses in many food products [9]. Tween 80 was found to promote the absorption of nutrients from fermentation medium and accelerate the growth of mycelia. Also, Tween 80 can extend the growth cycle of fungus because it maintains the intact structure of the mycelia and alleviates the destructive power brought by the oscillatory force during shaking [10]. Last, Tween 80 was added to fermentation medium for increasing the yield of useful fungal metabolites, acting as an efficient permeabilizer to improving bioactivator, and serving as an elicitor to promote physiological activator biosynthesis, such as mycelial growth and

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exopolysaccharide yield of *Pleurotus tuber-regium* [11], and the production of ginsenoside [12]. A large number of document reports, both domestic and abroad, have examined the effect of Tween 80 on fermentation outputs, such as *Colletotrichum*, *Aspergillus ficuum*, and *Escherichia* sp., and biohydrogen production [13–16].

This study aimed to investigate the effect of Tween 80 the biomass, the yield of intracellular polysaccharide, kinetics of fermentation, the pellets size of mycelium, and the antioxidant activity of IPS in liquid fermentation of *L. edodes* strain No. 7. In this study, the effect of Tween 80 on the fermentation of *L. edodes* was investigated. The influence of various concentrations of Tween 80 on the biomass and the production of intracellular polysaccharide (IPS) were investigated. The effects of Tween 80 on the kinetics of *L. edodes* fermentation and the size of mycelia pellets were also studied. The discrepancy of IPS with and without the addition of Tween 80 was observed by Infrared spectrometer. The antioxidant capacity of IPS was also measured. Our research would present provide the basic parameters for enhancing the yield of IPS by using Tween 80, and provide the technical guidance for the industrial *L. edodes* fermentation.

Materials and methods

Chemicals

Glucose, yeast extract, peptone, agar, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, magnesium sulfate (MgSO_4), dibasic potassium phosphate (K_2HPO_4), monobasic potassium phosphate (KH_2PO_4), Tween 80, vitamin B_1 were purchased from National drug group chemical reagents Co., Ltd. (Shanghai, China).

Microorganism and medium

Lentinus edodes strain No. 7 was provided by Hubei Yuguo Gu Ye Co., Ltd. (Wuhan, China) and was conserved on a plate of complete yeast extract medium (CYM) at 4 °C.

Complete yeast extract medium (g/L): glucose 20, peptone 2, yeast extract 2, MgSO_4 0.5, K_2HPO_4 1, KH_2PO_4 0.46, agar 18.

The liquid seed medium (g/L): corn flour 15, yeast extract 20, glucose 10, KH_2PO_4 0.3, MgSO_4 0.15, vitamin B_1 0.02.

The liquid fermentation medium was the same as the liquid seed medium.

Preparation of liquid seed

Lentinus edodes strain was cultured on the CYM culture dish for 5 days at 25 °C. Then, 4 blocks of agar discs (67.2 mm diameter) punched from the CYM culture dish were inoculated into the 250 mL flask containing 100 mL of the liquid medium and subsequently incubated at 25 °C for 7 days with shaking at 150 rpm.

Effect of different Tween 80 concentrations on the biomass and polysaccharide of *L. edodes*

The liquid fermentation experiments were performed in 250 mL flasks contained 100 mL of the liquid fermentation medium and 0–1.2% Tween 80 with 10% (by volume) of the seed culture. The *L. edodes* was cultured at 25 °C for 10 days with shaking conditions at 150 rpm and the dry biomass and the content of intracellular crude polysaccharide were subsequently measured.

Influence of Tween 80 on the kinetics of *L. edodes* liquid fermentation

The liquid fermentation experiments carried out in 250 mL flasks containing 100 mL of the liquid fermentation medium and 0 or 1% Tween 80 with 10% (by volume) of the seed culture. All flasks were cultured at 25 °C with shaking condition at 150 rpm. The dry biomass, intracellular crude polysaccharide content, pH value, and the content of reducing sugar were measured every 3 days from the flasks. Each test was repeated triple at the same time.

Analysis method of fermentation

The fermentation broth was centrifuged at 3170g for 15 min to obtain mycelium pellets and the supernatant.

Mycelium pellets were washed with distilled water for 3 times, and then dried at 60 °C until the dry mycelium weigh (DMW) was constant. The dry mycelium pellets were shattered and sieved, and added 15 volumes of distilled water, extracted at 90 °C for 2 h. Then, the filter residue was extracted for 2 more times. The extracted filtrate was gathered and condensed, then, precipitated by adding 3 volumes of ethanol and kept at 4 °C for 12 h. The precipitates were isolated by centrifugation, and washed 3 times by adding 75% ethanol, then dried at 60 °C. The crude IPS was obtained. The IPS production and DMW were expressed as grams per 100 mL.

The centrifuged supernatant was used for the measurements of reducing sugar and pH. Reducing sugar content of the solution was determined by the 3,5-dinitrosalicylic acid method (DNS) using D-glucose as standard at 540 nm. The

pH value was detected with digital pH meter (PHS-3C, China).

Determination of mycelium pellet size

Thirty mycelium pellets were randomly taken from shake flask and lined up in single file, and measured overall length, and then calculated mean value. Repeat the above operation at least three times so as to obtain the average pellet size.

Purification of intracellular polysaccharides

The crude IPS was re-dissolved with distilled water, and then proteins were removed by adding sewage reagent (chloroform: *n*-butanol = 4:1 [v/v]). Then, 1% of the activated carbon was added to decolorize the supernatant. After all, the solutions were dialyzed in distilled water for 24 h by 3.5 kD dialysis bag. The dialyzate were then condensed, precipitated, and dried to obtain the purified IPS.

Infrared spectrum

Infrared analysis of the purified IPS was carried out using the Fourier transform infrared (FTIR) spectrometer L1600301 (Perkin Elmer, USA). 2 mg of IPS was mixed with KBr, pressed into pellets and then scanned in the range of 4000–400 cm^{-1} .

DPPH radical scavenging assay

The DPPH radical scavenging activity assay was conducted according to the method of Wang et al. [17] with some modifications. 2 mL of the purified IPS with different concentrations (200–1000 $\mu\text{g/mL}$) was mixed with 2 mL of DPPH (0.2 mmol/L) and 2 mL of absolute ethanol in a tube. The mixtures were reacted for 30 min in dark and then the absorbance of the mixtures was measured at 517 nm. The DPPH radical scavenging activity of each sample was calculated by the following equation:

$$\text{Scavenging activity (\%)} = (1 - (A_1 - A_2)/A_0) \times 100\%$$

where A_1 is the absorbance in the existence of the sample solution and DPPH; A_2 is the absorbance in the existence of the absolute ethanol and the sample solution; and A_0 is the absorbance in the existence of the absolute ethanol and DPPH.

Results and discussion

Effects of different concentration Tween 80 on *L. edodes* fermentation

The effects of different concentrations of nonionic surfactant Tween 80 on the DMW and crude IPS production during *L. edodes* fermentation were shown in Fig. 1. The addition of Tween 80 to the culture medium increased the yield of the biomass and polysaccharide in a concentration depend manner. It was consistent with the result of Taoka et al. [18] that the addition of Tween 80 into medium could increase the biomass and the yield of metabolites. Studied have indicated that Tween 80 can reduce the surface tension of the contact surface between the fungal bacteria and medium, and increase the membrane permeability of certain micro-organisms [19], then further promote nutrient uptake by the cells, thus raise the output [20]. When the concentration of Tween 80 increased to 0.8%, further increase did not result in significant increases in DMW and LPS production.

Effects of Tween 80 on the kinetics of liquid fermentation of *L. edodes*

The fermentation of *L. edodes* with or without 1% Tween 80 is shown in Fig. 2 shows. In the fermentation process, the biomass and IPS production of *L. edodes* increased, and reducing sugars and pH decreased with time. The biomass growth rate was faster in medium with 1.0% Tween 80 than in the media without Tween 80 (control) during the 15 days' culture [Fig. 2(A)]. At 12th day, the DMW reached 1.6254 g/100 mL, increased by 22.19% compared with the control. Correspondingly, the

Fig. 1 Effects of different concentrations of Tween 80 on the DMW and crude IPS production during *L. edodes* fermentation. Values are given as mean $\pm$ standard deviation ($n = 3$). Bars denoted with different letters are significantly different ($P < 0.05$)

Fig. 2 Comparison of *L. edodes* liquid fermentation with or without 1% Tween 80. (A) The biomass (DMW), (B) IPS, (C) pH and (D) the content of reducing sugar. Values are given as mean $\pm$ standard deviation (n = 3)

IPS biosynthesis rate was much higher than that of the control [Fig. 2(B)]. The result that Tween 80 could enhance metabolites production was similar to the findings of Reddy et al. [21], which studied the effect of additives on pullulanase production using *Clostridium thermosulfurogenes* SV2. When separately adding Triton X-100, CHAPS, Tween 80, and sodium taurocholate into medium, the production of pullulanase exhibited 114, 146, 47 and 28% more than the control (no surfactants adding), respectively. The maximal IPS production with the addition of 1% Tween 80 was 0.2572 g/100 mL at 12th day, which showed an increase of 20.85% when compared to the control. Furthermore, the reducing sugar of fermentation broth dramatically reduced throughout the fermentation process, and decreasing rate was higher in the 1% Tween 80 treated group [Fig. 2(D)]. At the end of the process, the content of reducing sugar in medium with 1% Tween 80 (0.07 g/100 mL) was lower than the the control group (0.163 g/100 mL). Tween 80 can increase mycelia

cell membrane permeability and the uptake efficiency of nutrients from the fermentation medium [11, 22]. Therefore, these results suggested that Tween 80 was an effective enhancer of DMW and IPS biosynthesis.

The pH value of the ferment liquid is one of the most vital environment factors for the *L. edodes* mycelia growth and biosynthesis of Lentinan in submerged fermentation. The time courses of pH value with and without the addition of Tween 80 were investigated [Fig. 2(C)]. There was little change in the pH value during the prior fermentation period, and then it decreased rapidly and finally slightly increased. The pH value of fermentation liquor with the addition of Tween 80 decreased faster than the control during the middle and late fermentation. It was reported that the pH value depends on the Oxygen supply and substrate consumption [23]. Adding Tween 80 could enhance the consumption of substances and improved the utilization of culture medium. Then, at the end of the fermentation, the pH value of the group without the addition

of Tween 80 was reduced to the minimum value (3.41) at the 12th day and then slightly increased to 3.52. At the same time, the minimum pH value in the media with the addition of 1% Tween 80 was 3.6 and then slightly increased to 3.81. Adding Tween 80 could avoid the pH value dropping to very low and alleviate the influence of pH. Tween 80 is a hydrophilic surfactant, with a certain role of the cell membrane rupture, which is conducive to the release of intracellular material. It is generally known that the intracellular components would be released in the late fermentation stage, which acts as buffering role to maintain the pH of the fermentation broth [24]. Results showed that Tween 80 was able to protect the integrity of the mycelium, hence enhance the yield of mycelia biomass and Lentinan.

Effects of Tween 80 on the mycelia pellets of *L. edodes*

The mycelia morphology, during submerged cultivations in shake flasks, is influenced by the size inoculums, composition of the culture medium, shear stress, and so on [25]. The time courses of pellets size in the mediums with and without the addition of Tween 80 were shown (Fig. 3). The pellets size increased rapidly from day 0 to day 9 and slowly from day 9 to day 15 in the culture medium with or without Tween 80. At the early stages (from day 0 to day 6), the average pellet diameter with Tween 80 treatment was smaller than that in the control group. The smaller individual pellet size can result in an overall relatively larger surface area and better absorption efficiency of nutrition. Meanwhile, it can provide some protections in the liquid medium for the cells to avoid harmful shear effects [26, 27]. As growth progression, pellets in the

Fig. 3 Effects of Tween 80 on the mycelia *L. edodes*. Values are given as mean $\pm$ standard deviation ($n = 3$)

Tween 80 treated group grew faster and the average pellet diameter was also only a little bit bigger than the control group during the later stages of fermentation (from day 9 to day 15). The results were consistent with the findings of Ma et al. [28]. The addition of Tween 80 to the medium could promote mycelia growth and reduce the presence of dispersed mycelia and fine segments of mycelium. The results indicated that Tween 80 in the culture medium could inhibit the pellets breaking down into smaller size and keep their integrity.

Infrared spectral analysis of polysaccharides production

Infrared spectrum was usually used for the analysis of organic functional groups, especially N–H, O–H, and C=O. Infrared spectra for purified IPS obtained from mycelium in the medium with and without containing 1% Tween 80 were compared in Fig. 4. The comparisons of the characteristic absorption peaks of IPS in the groups with and without the addition of Tween 80 were presented in Table 1. The infrared spectra showed similar features in the Tween 80 and control groups. A broadly-stretched strong peak at 3403.7 cm^{-1} was the characteristic of O–H. A weak band presented at 2928.3 cm^{-1} was attributed to C–H stretching. The relatively strong band at around 1637.1 cm^{-1} demonstrated the existence of C=O. Further characteristic signals emerged at 1404.4 cm^{-1} was due to the bending vibration of C–H. Infrared spectrum analysis showed that the characteristic absorption peaks of pyranose ring were 1153.8 , 1079.4 , and 1023.2 cm^{-1} . A strong

Fig. 4 IR spectra of IPS obtained from *L. edodes* mycelium cultured with or without 1% Tween 80

Table 1 The comparison of FTIR data of purified Lentinan with and without 1% Tween 80

Assignment	Wavenumber (cm ⁻¹)	
	Control	1% Tween 80
O–H stretching	3434.7	3403.7
C–H stretching	2932.8	2928.3
O–C–O stretching	1637.1	1637.1
C–H bending	1404.1	1404.4
Pyranose ring	1157.5, 1079.5, 1025.8	1153.8, 1079.4, 1023.2
β-Type glucosidic bonds	928.8	933.3

absorption in 933.3 cm⁻¹ was the characteristic of the linkage of β-type glycosidic bonds [29].

Effects of Tween 80 on the scavenging activity of DPPH radical of IPS

The DPPH free radical, which is a stable free radical with a maximum absorption peak at 517 nm, can be easily eliminated by antioxidants [30]. The DPPH radical scavenging activity assay has been widely used in the determination of the antioxidant ability of natural compounds, in which the stable diphenylpicrylhydrazine (DPPH-H) was generated in the presence of an antioxidant [31]. Influence of Tween 80 on IPS antioxidant activity (the scavenging activity of DPPH radical) of *L. edodes* was presented in Fig. 5. IPS was found to present DPPH radical scavenging activity in both of the Tween 80 and control groups. Scavenging activity of IPS with adding Tween 80 in medium was significantly increased with the increasing concentration and it exhibited much stronger activity than the control.

It has been reported that addition of Tween 80 into the culture medium could improve the antioxidant capacity (Trolox-Equivalent Antioxidant Capacity) of fungal

extracellular polysaccharides [28]. In our experiments, we found that the use of Tween 80 in the medium could improve the free-radical scavenging capacity of IPS. Tween 80 was a statistically significant effect on the antioxidant activity ($P < 0.05$). Polysaccharides showed many biological activities, which are strongly affected by their structures and chain conformations [32]. The bioactivity of *L. edodes* polysaccharide is closely related to the spiral structure [33]. Tween 80 may have effects on modulating the molecular structure of IPS, and the principle need to further research. These results were really important to the application of Tween 80 in obtaining IPS with high antioxidant activity and yield.

Acknowledgements The authors thank Hubei Yuguo Gu Ye Co., Ltd. for financial and material support.

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Fig. 5 Effects of Tween 80 on IPS antioxidant activity (the scavenging activity of DPPH radical) of *L. edodes*. Values were given as mean $\pm$ standard deviation ($n = 3$)

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