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Low Cytotoxic D-mannitol Isolated from the Industrial Wastewater of *Agaricus bisporus*

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Abstract *Agaricus bisporus* is an edible mushroom, and mainly made in canned food for storage and trading. However, large amount of industrial wastewater containing various components is generated during the production of canned mushroom. This study describes the method for purification of D-mannitol and evaluation of its bioactivity from the industrial wastewater of *A. bisporus*. Its structure was confirmed by GC, IR, UV, HPLC, HPLC-MS, DSC analysis. The content of D-mannitol in the concentrated industrial wastewater (≥45°Be') of *A. bisporus* was determined to more than 32.0%. Comparing with the D-mannitol standard, this purified MN6 had a higher purity and lower cytotoxicity to L929 fibroblast evaluated by MTS assay. These results revealed that the *A. bisporus* industrial wastewater is a good resource for obtanning natural D-mannitol with high quality.

Keywords: *D-mannitol, Agaricus bisporus, wastewater, lower cytotoxicit*

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1. Introduction

A. bisporus (Lange) Sing commonly known as mushroom, is the most widely cultivated and one of the most economically important mushroom in the world, which accounts for about 25% of the world's total output of edible fungus [1]. Because of its short storage period, the mushroom is mainly made in canned products for international trade. In the production process, the fresh mushrooms are precooked firstly, in which the weight is reduced by 35% ~ 40%, and a large quality of industrial wastewater—the liquid of mushroom from the precooking process (LMPP) is generated. In China, about 80% of mushroom is precooked and then made in canned food. It leads to about 30% of the component of fresh mushroom become the industrial wastewater containing plenty of nutrients. The discharge of the LMPP into the surrounding areas definitely will cause environmental pollution. Therefore, development method of utilizing the LMPP is important for the ecological protection and the comprehensive utilization of agricultural resources.

Due to the chemical diversity of the LMPP showed in the preliminary chromatography screening, it was concentrated and purified for compounds. The polysaccharides with hepatoprotective effect against CCl_4-induced hepatic injury in mice [2] and D-mannitol were obtained. In this paper, the isolation of D-mannitol from *A. bisporus* industrial wastewater, the structural elucidation, and its cytotoxicity to L929 fibroblast are reported.

2. Materials and Methods

2.1. Chemicals

Inositol, erythritol, sugar alcohol, xylitol were purchased from Sigma; Indium was purchased from TA, USA; D-mannitol purchased from Amresco was used as standard; CellTiter 96 AQ single solution cell proliferation test kit was purchased from Promega, USA.

2.2. Isolation and Purification of D-mannitol

The LMPP concentrated from industrial wastewater (Brix = 65%) of white button mushroom was provided by Fujian Keren Biological Technology Co. Ltd. It was concentrated to ≥45°Be', and crude crystal was generated by cooling crystallization. It was decolored and subjected for purification by ion exchange chromatography. Finally one component was obtained and referred to as MN6.

2.3. Gas Chromatography Analysis

GC using an instrument (GC3800, Varian, USA) equipped with an WCOT fused silica capillary column (30 m × 0.25 mm × 0.25 μm) and a flame-ionization detector (FID) according to the following temperature program: the oven temperature was initially set at 250°C and maintained for 1 min, then increased to 220°C at the rate of 20 °C/min and held at 220°C for 10 min. The heater temperatures of the injector and detector were 250°C and

280°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 15 mL/min.

The Hexa-*O*-acetyl-myo-inositol was used as an internal standard. The sample solution was prepared by mixing 20 mg MN6, 10 mg hydroxylamine hydrochloride, 5 mL internal standard solution, 4 mL pyridine. The standard solution was prepared by mixing 20 mg erythritol, 20 mg xylitol and 20 mg D-mannitol, 5 mL internal standard solution, 4 mL pyridine. Both solutions were inoculated in shakers at 300 rpm in 90°C for 30 min. After being cooled to 25°C, 1 mL acetic anhydride was added for acetylation reaction in 90°C for 30 min. Then both solutions were centrifuged at 12000 rpm for 5 min to obtain supernatants. They were diluted with pyridine (*v*:*v* 1:10) and subjected for GC analysis, respectively.

2.4. FTIR and UV Spectroscopy Analysis

FT-IR from 500 to 4000 cm^{-1} was used for the determination of the functional groups present in MN6 and D-mannitol standard on a Nicolet Fourier transform infrared spectrometer (NICOLET iS10, Thermo). 20 mg MN6 and standard were mixed with KBr (*w*:*w* 1:50-100), respectively, and then tableted for infrared spectrum detection.

The ultraviolet spectra of MN6 and D-mannitol standard were dissolved in ddH_2O and determined with an ultraviolet spectrophotometer (UV-3200pc, Mapada) in the range of 190–340 nm.

2.5. HPLC - MS Chromatographic Analysis

The purity and molecular weight of MN6 were determined by a HPLC-MS system (HPLC: Ultimate 3000, Thermo; Mass spectrometer: Agilent 6300). The HPLC system was fitted with Carbomix Ca-NP5 column (5 μm, 8% cross-linking. 7.8 mm × 300 mm) and a RI detector. Column and detector temperatures were maintained at 35°C. 20 μL of 0.8% (*w*/*w*) MN6 or D-mannitol standard dissolved in ultrapure water were injected into the column, eluted with ultrapure water at a flow rate of 0.3 ml/min.

Ion source: electrospray ionization, negative ion mode (ESI⁻). Capillary voltage: 3.0 kV. Ion source temperature: 120°C. Desolvation gas: N_2. Desolvation temperature: 350°C. Desolvation gas flow rate: 750 L/h. Collision gas: high purity Ar. Scanning mode: multiple reaction monitoring (MRM).

2.6. Differential Scanning Calorimetry Analysis

Differential scanning calorimeter was used to determine the purity of MN6. The temperature was calibrated with Indium, and the baseline was calibrated at 0.7°C/min before determination. 2.5000g MN6 was determined according to the following program: the heating-rate was 0.7°C /min, the gas flow rate of N_2 was 40 mL/min, and the initial equilibrium temperature and the termination temperature were 25.0°C and 190.0°C, respectively.

2.7. Content analysis of the D-mannitol in Concentrated LMPP

The content of D-mannitol in the concentrated LMPP and the purity of MN6 were determined according to colorimetric analysis [3].

2.8. The Cytotoxicity Test

Mouse fibroblast cell L929 (ATCC, Shanghai cell bank of Chinese academy of sciences) was used for evaluation of the cytotoxicity of MN6 by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. The cells line was plated in the wells of 96-well plates (Falcon, USA) in 1640 cell culture medium containing 10% FBS (Gibco, USA). The cells were treated in sextuplicate with 1%, 3%, 5%, 10%, 15% of D-mannitol standard or MN6 for 72 h in 5% CO_2 incubator at 37°C. Then 20 μL MTS was added in each well and the plate was incubated for 4 h. The absorbance of each well was determined by a microplate reader (Infinite 200 pro, Tecan) with a 570 nm wavelength. Growth inhibition rates were calculated. The relative cell proliferation rate (RGR) was calculated according to the following formula.

$$RGR = A / A_0 \times 100\%$$

Annotation: A — optical density of D- mannitol group. A_0 — optical density of the control group.

3. Results

3.1. Morphology and Qualitative Analysis of Crystal MN6

The crystal of MN6 was rod and colorless (Figure 1A), similar to that of the D-mannitol standard, (Figure 1B), and it had a sweetness taste. Therefore, the characterization analysis of alditol compound was further carried out. 0.5 mL 10% (*w*/*v*) $FeCl_3$ and 0.5 mL 4.3% (*w*/*v*) NaOH were added into 1 mL saturated aqueous solution of MN6. The reaction generated a brown yellow precipitate which was stable under oscillating condition. And it was dissolved immediately by adding excess NaOH solution [4]. This result was similar to that of the qualitative identification of D-mannitol from the National Pharmacopoeia, China. Hence, we determined the crystal might be as D-mannitol.

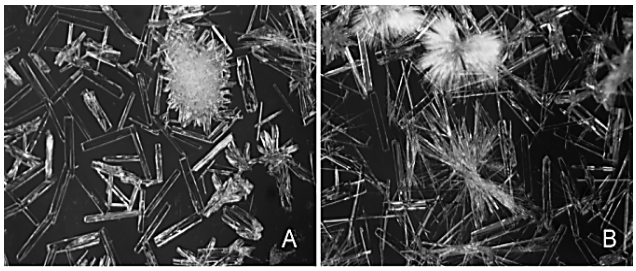

Figure 1. The crystals under stereomicroscope SMZ18 (× 40), A: Crystal of MN6, B: Crystal of standard of D-mannitol

3.2. GC Analysis of MN6

The GC analysis showed symmetrical peaks for the D-mannitol and the internal standard at 7.4 min and 8.1 min in both samples of MN6 (Figure 2, upper graph) and the standard (Figure 2, lower graph). And MN6 was pure with no other alditols, like erythritol (Ery, 2.4 min) and Xylitol (Xyl, 4.4 min) *et. al.*, which are commonly existed in fungi extracts.

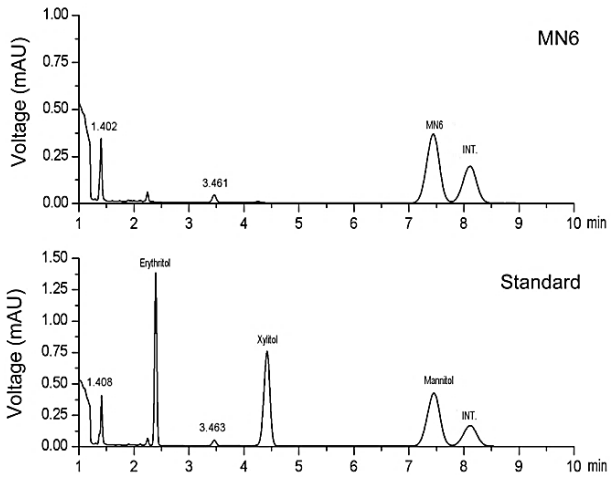

Figure 2. Gas chromatogram analysis of MN6 and standard

3.3. FT-IR and UV Assay

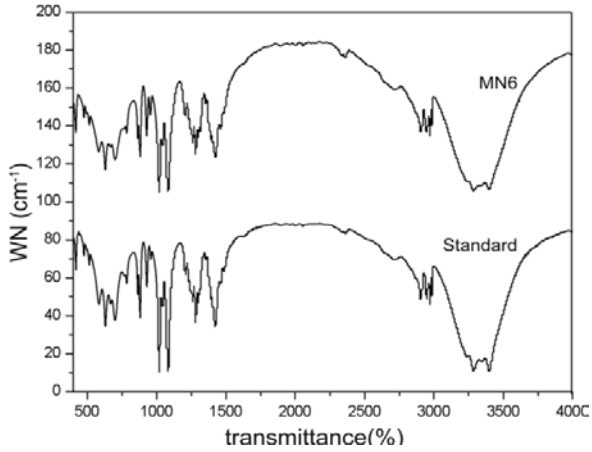

Figure 3. IR spectrum analysis of D-mannitol standard and MN6

The FT-IR analysis ranging from 4000 cm^{-1} to 500^{-1} showed that MN6 had a highly similar carbohydrate pattern with the standard D-mannitol (Figure 3). The strong broad bands near 3300 cm^{-1} (MN6: 3397.86 cm^{-1}, 3285.65 cm^{-1}, Standard: 3397.86 cm^{-1}, 3285.65 cm^{-1}) were contributed to the characteristic of OH stretching in hydrogen bonds. The weak bonds near 2950 cm^{-1} (MN6: ν_{max}/cm^{-1} 2968.60, 2946.81 and 2910.95; Standard: ν_{max}/cm^{-1} 2968.60, 2946.81 and 2910.95) were the characteristic of C-H stretching and bending vibrations. The bands near 1100 cm^{-1} (MN6: ν_{max}/cm^{-1} 1415.37, 1280.87, 1082.31 and 1018.26; standard: ν_{max}/cm^{-1} 1415.37, 1280.87, 1079.11 and 1015.06) were belonged to the C-O and C-C stretching. The peaks at 1000-667 cm^{-1} (MN6: ν_{max}/cm^{-1} 928.59, 880.55 and 701.21; standard: ν_{max}/cm^{-1} 928.59, 880.55 and 698.01) were contributed to the C-C connections. The small difference in this IR spectrum should be due to the preparation of samples [5].

The UV spectra analysis showed MN6 had identical absorption peak λ_{max} (ddH_2O)/nm 193.4 with standard D-mannitol, providing the reference wavelength for HPLC analysis [6].

3.4. HPLC - MS Analysis of MN6

The HPLC analysis (Figure 4) showed that 20 μL 0.8% (*w*/*w*) of MN6 and standard had the same symmetrical peaks at 10.0 min with the peak area of 1.4×10^4 mAUs. This result indicated that MN6 was a homogenous sample with no other peak excepting a solvent peak at 3.9 min. MN6 was further confirmed to be D-mannitol by analysis of the mass spectrum data (MN6: *m*/*z* 181.0 $[M-H]^-$; standard: *m*/*z* 181.1 $[M-H]^-$) .

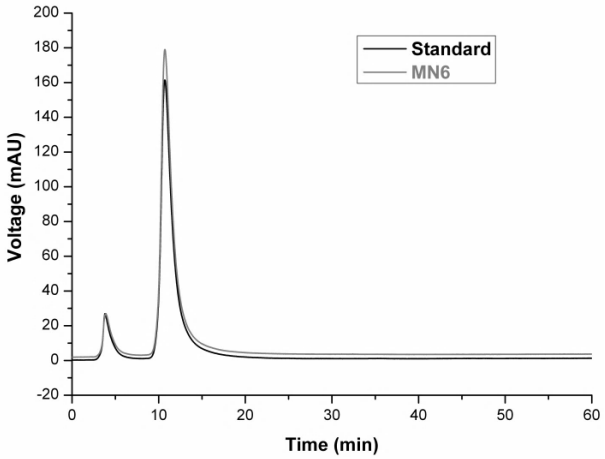

Figure 4. HPLC chromatogram of D-mannitol standard and MN6

3.5. DSC Analysis

The DSC analysis indicated MN6 was pure with mp 168°C, which was in the melting range of D-mannitol standard at 165-168°C (Figure 5). This result further suggested MN6 isolated from LMPP was D-mannitol.

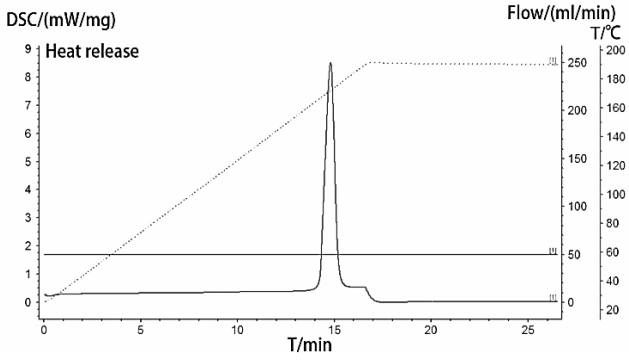

Figure 5. DSC analysis of MN6

3.6. Content Analysis of D-mannitol in Concentrated LMPP

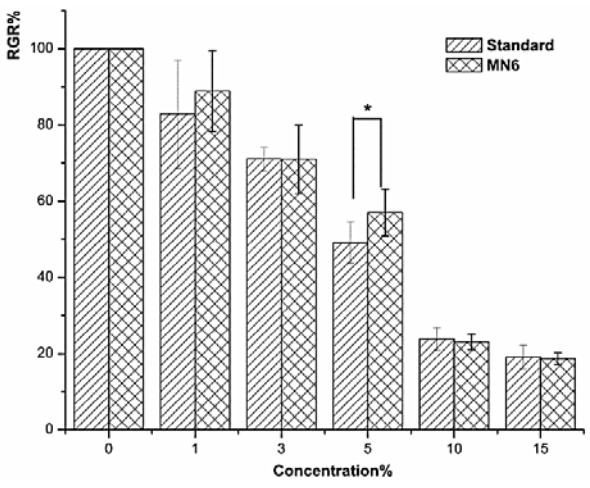

Figure 6. RGR of L929 cells in different concentration of D-mannitol

As MN6 had been identified to be D-mannitol, we tried to determine its content in concentrated LMPP. A regression equation (y=0.0102 x + 0.031, R^2=0.997, y: absorbance value; x: content of D-mannitol, %) of standard was established. According to this equation, the content of D- mannitol in concentrated LMPP and the purity of MN6 were calculated. They are 32.16% and 101.16%, respectively. This result indicated that concentrated LMPP is rich of D-mannitol, and suggesting that this method affords D-mannitol with high purity.

3.7. Cytotoxicity Assay

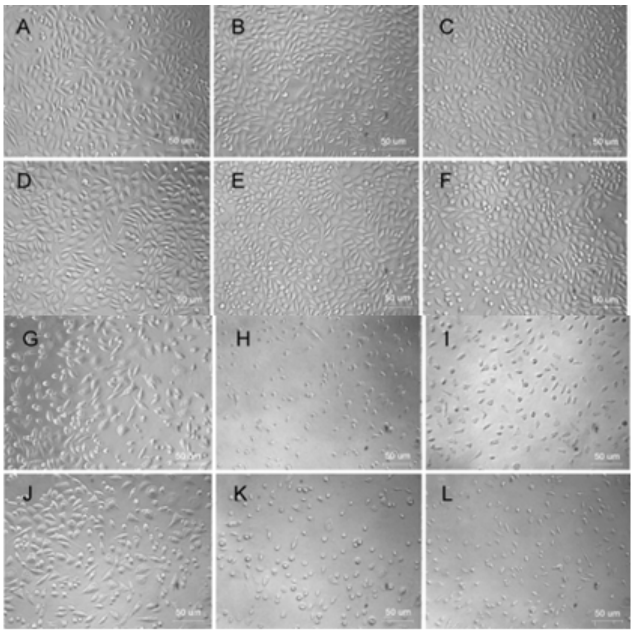

Figure 7. Morphology of L929 cells in different concentrations of D-mannitol (Olympus IX51 inverted microscope biological × 200), Standard (Upper graph): A-0%; B-1%; C-3%; G-5%; H-10%; I-15% MN6 (lower graph): D-0%; E-1%; F-3%; J-5%; K-10%; L-15%

The cytotoxicity of MN6 and standard was evaluated using mouse fibroblast cell L929. The MTS assay result suggested that D-mannitol could reduce the RGR of the cell in a dose dependent manner (Figure 6). The cell morphology was normal in 1%, 3% and 5% of MN6 and standard. But the cellular shrinkage was observed in the concentration of 10% and 15% (Figure 7). However, MN6 had lower cytotoxicity (RGR = 48.3%) than standard (RGR = 56.8%) in the concentration of 5%. This result indicated D-mannitol from *A. bisporus* is biologically safer than that obtaining from chemical synthesis due to the simpler and safer production process.

4. Discussion

D-mannitol, as a sugar alcohol, also known as hexahydroxy hexane, is widely distributed in plants [7], algae, and fungi. Owing to the special physical and chemical properties, it is widely applied in food, medical, pharmaceutical and chemical industry. It plays an important role in growth and development of organisms. It acts as an osmo-regulator to influent the development of the fruiting body of fungi [8,9]. It also could inhibit the ACE (angiotensin converting enzyme) activity and decrease the blood pressure of spontaneously hypertensive rats [10]. It is a good material for diuretic, dehydrating agent, sugar substitutes, and pill vehicle. It can be used to reduce intracranial and intraocular pressure. As D-mannitol improves brain tissue oxygenation, it is applied to cure edema caused by craniocerebral trauma, tumor and hypoxia in brain [11].

Algae was once used to extract D-mannitol, from which homogenous product was obtained without sorbitol. But low extraction rate, high energy consumption and environment pollution leaded it to be replaced by chemical hydrogenation. Currently, D-mannitol is produced by chemical synthesis in industry. The sucrose was firstly hydrolyzed into glucose and fructose by acid for hydrogenation reaction, and the isomer products of sorbitol and D-mannitol (3:1) were obtained. Then the latter was purified from the mixture by crystallization according to their solubility difference [12]. But there are several drawbacks in this method including the need of raw materials with high-purity, high reaction temperature and pressure, removal of the metal residues [13], and the complicated process of separating D-mannitol from its isomer [14]. There are some progress made in producing D-mannitol based on microbial fermentation [15,16,17] and enzymes canalization [12,18,19,20,21,22], but the high cost is the common obstacle for industrial production. Therefore, exploring new natural resource and developing a method for producing D-mannitol with high quality is an important priority.

Although D-mannitol is one of the main carbohydrates in mushrooms, there is few studies on industrial production from fungi. In this study, we developed a method of obtaining D-mannitol from mushroom industrial wastewater with high quality, lower cytotoxicity, without isomer or metal residues. In addition, the high content of D-mannitol in the concentrated LMPP (32.16%) indicated that it is a noble resource for industrial production instead of wastewater discharging directly into the surrounding area, which will definitely lead to the serious pollution.

Acknowledgments

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Conflict of Interests

The authors declare that they have no any conflict of interests.

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