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A simple method for the simultaneous decoloration and deproteinization of crude levan extract from *Paenibacillus polymyxa* EJS-3 by macroporous resin

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ABSTRACT

A simple method for the simultaneous decoloration and deproteinization of crude levan extract from the endophytic bacterium *Paenibacillus polymyxa* EJS-3 was developed through static and dynamic adsorption tests of macroporous resins. S-8 resin demonstrated the highest decoloration and deproteinization ratios among various resins tested. Under optimized static adsorption conditions (pH 6.0, 35 °C and adsorption time of 70 min), the ratios of decoloration, deproteinization and polysaccharide recovery for S-8 resin were 76.8%, 78.9% and 69.0%, respectively. Under optimized dynamic adsorption condition (flow rate of 2 BV/h, 160 ml of 2.5 mg/ml crude levan extract), higher ratios of decoloration, deproteinization and polysaccharide recovery for S-8 resin (84.6%, 91.7% and 81.3%, respectively) were observed. The method developed will provide a potential approach for large-scale production of levan from *P. polymyxa* EJS-3.
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1. Introduction

Levan, a β-2,6-linked fructose polymer with β-2,1-linked side chains, is an important bioactive polysaccharide with a wide variety of applications. It can be used in medicine as hypo-cholesterol, antitumor and immunostimulating agents, and in food as emulsifier, stabilizer, thickener and encapsulating materials (Bae et al., 2008; Bekers et al., 2005; Oliveira et al., 2007). Levan is primary produced by microbial fermentation from sucrose-based media (Bae et al., 2008; Yoon et al., 2004). The conventional method used to extract levan from the fermentation broth is performed by centrifugation to remove bacterial cells, followed by precipitation of the supernatant with ethanol repeatedly (Bae et al., 2008; Calazans et al., 2000; Poli et al., 2009; Yoo et al., 2004). Notably, many impurities (especially pigments and proteins) are co-precipitated with levan by ethanol, making the subsequent purification process very difficult. Till now, several methods including H_2O_2 and Sevag reagent (Staub, 1965) have been used for the decoloration and deproteinization of crude polysaccharides (Yang and Zhang, 2009); however, these chemical methods probably cause partial hydrolysis of polysaccharides, resulting in variable bioactivities. In addition, the chemical reagents used are environmentally disadvantageous and may cause adverse impacts on the human body when the polysaccharides are added to food and medicine (Wang et al., 2007). Therefore, it is necessary to develop novel methods for the decoloration and deproteinization of crude levan extract.

Macroporous resins are durable polar, non-polar or slightly hydrophilic polymers with high adsorption capacity (Fu et al., 2006). They can selectively adsorb the targeted constituents from aqueous as well as non-aqueous system through electrostatic force, hydrogen bonding interaction, complexation and size sieving action (Gao et al., 2007). Therefore, macroporous resins have been widely used in the separation of targeted component from other impurities in crude biological samples (Crini, 2006; Fu et al., 2005; Hatano et al., 2009; Jia and Lu, 2008; Silva et al., 2007; Wan et al., 2008; Ye et al., 2009; Zhang et al., 2008); however, there is little information available on employing macroporous resins for the decoloration or deproteinization of crude polysaccharide extracts (Li et al., 2006; Wang et al., 2005). Compared to the chemical methods, the application of macroporous resins as the decoloration or deproteinization reagents for crude polysaccharide extracts should have some advantages, such as low operating costs, less solvent consumption and easy regeneration (Gao et al., 2007). Moreover, macroporous resins can simultaneously remove pigments and proteins from crude polysaccharide extracts without destroying the structures and bioactivities of polysaccharides.

Recently, we have found that the endophytic bacterium *Paenibacillus polymyxa* EJS-3, isolated from the root tissue of *Stemona japonica* (Blume) Miquel, produced high levels of levan (a yield of 35.26 g/L). In addition, we have demonstrated that both crude and purified levans showed strong superoxide and hydroxyl radical scavenging activities *in vitro* (Liu et al., 2009, 2010). Due to the fact that the crude levan extract contains a lot of pigments and proteins, the aim of this study was to develop a simple method for the simultaneous decoloration and deproteinization of crude levan

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extract by macroporous resin. Firstly, various macroporous resins were investigated to select one with the highest decoloration and deproteinization efficiencies. Then, both static and dynamic adsorption experiments were carried out to investigate the effects of different parameters (pH, temperature, adsorption time, flow rate, and sample concentration) on decoloration and deproteinization efficiencies of the selected resin. Finally, the levan solutions before and after adsorption by the resin were measured by UV–vis spectroscopy, digital camera and HPLC to validate the decoloration and deproteinization efficiencies. To the best of our knowledge, this is the first report on the simultaneous decoloration and deproteinization of crude levan extract by macroporous resin.

2. Methods

2.1. Preparation of crude levan extract

Levan was produced by using *P. polymyxa* EJS-3 according to our reported method (Liu et al., 2010). Briefly, the fermentation broth was properly diluted and centrifuged at 10,500*g* for 15 min to remove bacterial cells after incubation on a rotary shaker incubator at 24 °C for 60 h. The resultant supernatant was mixed with four volumes of anhydrous ethanol, stirred vigorously and kept overnight at 4 °C. The precipitate from the ethanol dispersion was collected by centrifugation at 10,500*g* for 15 min, dissolved in distilled water and dialyzed against distilled water to afford the crude levan extract for further use.

2.2. Adsorbents

Macroporous resins including D3520, D4020, AB-8, D101, NAK-II and S-8 were provided by Chemical plant of Nankai University (Tianjing, China) and their physical properties are listed in Table 1. These resins were pretreated with 1 M HCl and NaOH solutions sequentially to remove the monomers and porogenic agents trapped inside the pores during the synthesis process, and then dried at 60 °C under vacuum. Prior to adsorption experiments, pre-weighed amounts of resins were soaked in 95% ethanol and washed thoroughly with deionized water.

2.3. Static adsorption tests

Static adsorption tests were performed as follows: pre-weighed amounts of hydrated adsorbent (equal to 1 g dry resin) and 50 ml of 5 mg/ml crude levan solution were added to an air-tight Erlenmeyer flask. The flask was shaken at 150 rpm in a constant temperature (25 °C) water-bath shaker for 12 h. After the resin was separated from the sample solution by filtration, the decoloration, deproteinization and polysaccharide recovery ratios of the resin were measured as described in Section 2.5. The preliminary selection of the resins was evaluated by their decoloration, deproteinization and polysaccharide recovery ratios towards crude levan solution. Then, static adsorption tests were done at different pH values (4, 5, 6, 7, 8 and 9), different temperatures (25, 30, 35 and 40 °C) and adsorption time (60, 120, 180 and 240 min) on the decoloration, deproteinization and polysaccharide recovery ratios of the selected resin were investigated with a shaking of 150 rpm.

2.4. Dynamic adsorption tests

Dynamic adsorption tests were carried out as follows: 180 ml of crude levan solution (5 mg/ml) was loaded continuously at a constant flow rate to a glass column (2.0 × 30 cm) wet-packed with the selected resin. The bed volume (BV) of the resin was 10 ml. The decoloration and deproteinization ratios of the effluents (10 ml/tube collected) were monitored as described in Section 2.5. In this way, the effects of flow rate and sample concentration on decoloration and deproteinization efficiencies of the selected resin were investigated.

2.5. Analytical methods

2.5.1. Determination of decoloration ratio

The decoloration ratio was determined by the method of Wang et al. (2005) with some modifications. Briefly, sample solution was adjusted to pH 7.0 with 1 M HCl and NaOH, and then centrifuged at 5000 rpm for 20 min. Subsequently, the absorbance of the resultant supernatant was measured at 420 nm on a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). The following equation was used to quantify the decoloration ratio of the resin:

$$\text{Decoloration ratio } (\%) = (A_o - A_e)/A_o \times 100 \quad (1)$$

where A_o and A_e were the absorbance of the samples at 420 nm before and after adsorption by resin, respectively.

2.5.2. Determination of protein concentration and deproteinization ratio

The concentration of protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard. The following equation was used to quantify the deproteinization ratio of the resin:

$$\text{Deproteinization ratio } (\%) = (C_o - C_e)/C_o \times 100 \quad (2)$$

where C_o and C_e were the concentrations of protein (μg/ml) in the solutions before and after adsorption by resin, respectively.

2.5.3. Determination of levan concentration and its recovery ratio

The concentration of levan was measured by the phenol–sulfuric acid method using fructose as standard (Dubois et al., 1956). The recovery ratio of levan was calculated by the following equation:

$$\text{Polysaccharide recovery ratio } (\%) = M_e/M_o \times 100 \quad (3)$$

where M_o and M_e were the concentrations of levan (μg/ml) in the solutions before and after adsorption by resin, respectively.

2.5.4. Characterization of levan before and after adsorption by resin

The UV–vis spectra of the levan solutions before and after adsorption by resin were determined by a Shimadzu UV-2450 spectrophotometer. In addition, the levan solutions before and after adsorption by resin were photographed by a Sony T700 digital camera (Sony Corp., Park Ridge, NJ).

Table 1
Physical properties of the macroporous resins used.

Resin	Particle diameter (mm)	Surface area (m^2/g)	Average pore diameter (nm)	Appearance	Polarity
D3520	0.3–1.25	480–520	8.5–9.0	Milk white	Non-polar
D4020	0.3–1.25	540–520	10–10.5	Milk white	Non-polar
D101	0.2–0.6	400–600	10–12	Milk white	Non-polar
AB-8	0.3–1.25	480–520	13–14	Milk white	Weak-polar
NAK-II	0.3–1.25	160–200	14.5–15.5	Reddish brown	Polar
S-8	0.3–1.25	100–120	28–30	Slight yellow	Polar

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