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Chromatographic separation of phenyllactic acid from crude broth using cryogels with dual functional groups

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ABSTRACT

Phenyllactic acid (PLA) is an important organic acid with wide antimicrobial activities against gram-positive and gram-negative bacteria and some fungi. This interesting compound can be synthesized by the microbial fermentation or the bioconversion using phenylpyruvic acid (PPA) as the key substrate and microorganisms as the whole-cell biocatalysts. However, the isolation of high-purity PLA with a high recovery from the crude fermentation or conversion broth is a challenging task. In this work, the separation of PLA from the crude conversion broth prepared by employing *Lactobacillus buchneri* cells as the whole-cell catalysts was achieved by the chromatography using the poly(hydroxyethyl methacrylate) (pHEMA)-based cryogel with a combination of anion-exchange and hydrophobic benzyl groups. The static adsorption behaviors of PLA under different salt concentrations and the adsorption capacities of PLA on the cryogel were measured experimentally. The chromatographic performance of PLA from the crude conversion broth was compared with that from the clarified broth. The results showed that the pHEMA-based cryogel has a high capacity of PLA, i.e., 14.64 mg mL^{-1} cryogel, and the adsorption of PLA was influenced by the salt concentration. By using deionized water as running buffer, PLA with a high purity of 97.6% was obtained with one step elution using 0.3 M NaCl as the elution solution with the recovery at the range of 80.2–90.8% from crude feedstock without any pretreatment at various flow velocities. These values were close to those obtained for the clarified broth, i.e., the purity of 98.4% and the recovery of 92.3% under the same chromatography conditions at 1 cm min^{-1}. The cryogel was then applied to separate PLA from clarified feedstock, high purity (>96.7%) and recovery (>91.4%) of PLA were found with 20 cycles, which verified the selectivity and robustness of prepared pHEMA-VBTAC cryogel. Therefore, the chromatography using pHEMA-based cryogel with the dual functional groups is an effective approach for the isolation of PLA directly from the crude bioconversion broth and thus could be interesting in the separation and production of high-purity PLA in industry.

1. Introduction

Compared with the traditional chemical synthesis, the biosynthesis of chemicals via fermentation or biotransformation has received an increasing attention due to its sustainable and friendly advantages. The biosynthesis of organic acids is one of such examples and these important bio-chemicals are of great importance in food, cosmetic, pharmaceutical, material and chemical industries [1–4]. Phenyllactic acid (PLA) is an important organic acid, which can be produced via microbial fermentation or whole-cell biotransformation with the strains of lactic acid strains [5–14], propionibacteria [15,16], phototrophic bacteria [17], *Geotrichum candidum* [18] and *Wickerhamia fluorescens* [19]. This interesting organic acid can be used as the food preservative, feed additive, pharmaceutical agent, and building block chemical for new polymers of poly(phenyllactic acid)s [6,11,12,19–22], and therefore, has potential applications in chemical, pharmaceutical, biotechnological and food industries.

The fermentation production of PLA with different microorganisms has been intensively investigated by researchers in the past years, as reviewed recently by Mu et al. [11] and Ni et al. [23]. However, most wild strains of lactic acid bacteria in the traditional culture medium like DeMan-Rogosa-Sharpe (MRS) broth, have produced PLA at a very low concentration, i.e., less than 2.5 g/L [7–10,18,24] due to the inhibitory effects in the metabolic pathways regarding the transamination process of phenylalanine. Recently, recombinant *E. coli* strains expressing the *Wickerhamia fluorescens* gene encoding a phenylpyruvate reductase has been developed

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to synthesize PLA using renewable cellulose, sorghum bagasse or sweet sorghum as the feedstock [21,25–27]. The concentration of PLA obtained was ten times more than those observed in the fermentation processes with wild strains. By using phenylpyruvic acid (PPA) as the key substrate, the production of PLA was improved further by either the fermentation or the whole-cell conversion with wild or recombinant strains [10,14,28–30]. The final concentration of PLA reported was in the range from 5.6 to 100 mg mL^{-1}, which could be very interesting in the potential industrial production of this important organic acid.

Although much efforts have been done to produce PLA by microbial fermentation or whole-cell bioconversion using various strains, the direct and effective separation of PLA from those crude fermentation broths or biotransformation feedstocks was rarely investigated due to the complex of these suspension mixtures, i.e., the product always together with microbial cells, substrates and by-products [9,31]. Several methods, such as chromatography with capillary electrophoresis, preparative high performance liquid chromatography (HPLC), extraction and ion-exchange adsorption, have been suggested for the separation of PLA [24,32–37]. Zhou et al. [32] developed a capillary electrophoresis method for the separation of PLA enantiomers with a resolution factor of 8.08 by using a derivate of β-cyclodextrin as the chiral selector. Ström et al. [33] developed a preparative HPLC method using C18 columns for the separation, purification and characterization of D,L-PLA and enantiomers from biological samples. Both of these methods are suitable for the separation of PLA at analysis scale. The extraction was achieved by using organic solvents as the extractant for the separation of PLA and this method had advantages like the high performance and possible to be scaled up, but suffered some disadvantages like the complex procedure and large amounts of solvents and wastes. Thinh [34] has proposed the ion-exchange adsorption using macroporous resins for the isolation of PLA from fermented solution. The capacity of the resin for PLA was 0.153 mg mg^{-1} with a recovery of 87.3%. However, several pretreating steps like the filtration and decolorizing with active carbon at 80 °C were required for the feedstock before the adsorption and the adsorption and separation were also time-costly.

The chromatography using cryogels has been suggested as an interesting separation method for the direct isolation of biomolecules from crude feedstocks in recent years [38–48]. Cryogels are a novel class of sponge-like adsorbents prepared via cryo-polymerization under freezing conditions. These adsorbents have well-interconnected supermacropores with sizes from several to hundreds microns, which permit the free passage of microbial cells. Cryogels also have high permeability and efficient convective mass transfer within the pores, and thus permit the rapid adsorption and chromatographic separation of target biomolecules such as adenosine triphosphate (ATP), cytidine triphosphate (CTP), plasmid DNA, *Glycyrrhiza* polysaccharides, serum albumin, antibody proteins and lactoperoxidase from complex crude biological suspensions or fermentation feedstocks [49–56]. Recently, we have prepared novel dual functional cryogels with a combination of functional ligands by anion-exchange amine and accessorial hydrophobic benzyl groups, i.e., benzyl-quaternary amine, by grafting the monomer chains of (vinylbenzyl)trimethylammonium chloride (VBTAC) onto a polyacrylamide (pAAm)-based matrices and the composite poly (hydroxyethyl methacrylate) (pHEMA)-based cryogel with embedding macroporous cellulose beads [54,55]. These interesting cryogels have remarkable chromatography adsorption and separation properties for the isolation of bioactive proteins of immunoglobulin G (IgG) antibody with a purity of 98.2% and albumin with a purity of 96.8% from rabbit blood serum, and IgG with the purity of 83.2% and albumin with the purity of 98% from human blood serum, respectively.

In this work, we provide a direct chromatographic method for rapid separation of high-purity PLA from crude whole-cell biotransformation broth of a *Lactobacillus buchneri* strain by using pHEMA-based cryogels with dual functional groups via grafting of VBTAC. The equilibrium adsorption of PLA on the cryogels under various salt concentrations and buffer pH values was investigated. The chromatographic and elution behaviors of PLA from the crude and clarified broth were also investigated at various conditions of salt stepwise elution and the flow velocities. The purities and recoveries of the obtained PLA were analyzed and the separation results were compared.

2. Experimental

2.1. Materials

L-PLA (98%), PPA (98%), 2-Hydroxyethyl methacrylate (HEMA, 97%), poly(ethylene glycol) diacrylate (PEGDA, 99%, M_n ~250 g/mol), VBTAC (99%) and *N,N,N,N′*–tetramethylethylenediamine (TEMED, 99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium persulfate (APS, 98%) and other chemicals were analytical grade from local resources. *L. buchneri* GBS3 strain isolated in our group and deposited at the China Center for Type Culture Collection (CCTCC NO: M 201615), was used as the whole-cell biocatalysts in the bioconversion.

2.2. Preparation and characterization of the cryogel

The pHEMA cryogel grafted with functional ligand of VBTAC, i.e., pHEMA-VBTAC cryogel, was prepared according to the method reported in our previous work [54,55]. Typically, 4.3 mL reactive solution containing HEMA (11.6%, w/w) and PEGDA (3.5%, w/w) initiated by TEMED and APS (both with the mass ratio of 0.8% to the total mass of HEMA and PEGDA) was poured into a glass column with inner diameter of 10 mm. Free radical cryopolymerization was conducted under freezing condition to get the cryogel. The obtained cryogel was grafted with 1 M VBTAC solution using 0.056 M $K_5[Cu(HIO_6)_2]$ as the initiator [54,55].

The properties of the pHEMA-VBTAC cryogel including the permeability, porosity, height of theoretical plate (HETP), residence time distributions (RTDs) and axial dispersion coefficient (D_{ax}) were measured as those methods used in references [53,55,57]. The pore morphology of the cryogel was investigated by scanning electron microscope (SEM, S-4700, Hitachi, Japan). The samples were cut from the middle of the column, directly dehydrated using an ethanol-water solution (10-30-50-70-80-90-99.5%), dried at critical point, coated with gold and then observed by SEM.

2.3. Equilibrium adsorption of PLA

The static adsorption behaviors of PLA under various NaCl concentrations were measured by re-cycling of a given amount of PLA solution to the cryogel at 25 °C. In each run, 25 mL PLA solutions with a known concentration of 1, 5, 10, 15 or 20 mg mL^{-1} in a NaCl solution of a given concentration (0, 0.01, 0.05, 0.1, 0.2, or 0.3 M), were recycled through the cryogel column at a constant flow velocity of 1 cm min^{-1} to ensure the adsorption. The PLA concentration in the outlet effluent was monitored by the on-line UV detector at 220 nm. After the equilibrium (about 3–12 h for the concentration from 20 to 1 mg mL^{-1}) was reached, the cryogel column was washed with deionized water to remove the unbound PLA and then eluted with 1 M NaCl. The concentration of PLA in the elution effluent was measured by high performance liquid chromatography (HPLC) and the amount of PLA bound by the cryogel was determined by the concentration, the volume of the elution solution and the volume of the cryogel bed. The adsorption isotherm of