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Production of polymalic acid and malic acid from xylose and corncob hydrolysate by a novel *Aureobasidium pullulans* YJ 6–11 strain

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ABSTRACT

Polymalic acid (PMA) is a water-soluble biopolymer with many attractive properties for pharmaceutical applications. Its monomer, L-malic acid, is a potential C4 platform chemical, which is widely used in the food industry. In this study, a new strain with a high PMA yield from xylose was isolated and identified as *Aureobasidium pullulans* based on the morphological and phylogenetic analyzes. This strain produced PMA from xylose at a comparable or higher rate than glucose, and PMA produced from xylose had a similar molecular weight to that produced from glucose. A high final PMA titer of 80.4 g/L (91.2 g/L of malic acid after hydrolysis) was achieved using fed-batch fermentation with xylose in a 5-L fermentor at a PMA productivity of 0.52 g/L h. Corncob hydrolysate could be better utilized producing 28.6 g/L of PMA (or 32.4 g/L malic acid) in batch fermentation. PMA was further characterized for its chemical structure. This strain can be used to produce PMA and malic acid from xylose and lignocellulosic biomass hydrolysates.

1. Introduction

Polymalic acid (PMA) is a water-soluble biopolymer with many attractive properties, including biocompatibility, degradability, and water solubility. Its monomer, L-malic acid, is extensively used in the food and pharmaceutical industries, which is also an important C4 platform chemical. PMA has been increasingly used as a drug carrier in the past few years. It can be used to make various compression-molded pellets, films, microparticles, and nanoparticles for drug delivery [1,2].

PMA can be produced via chemical synthesis or microbial fermentation. However, it is associated with some disadvantages such as low molecular weight product and complex reaction routes [3,4]. Recently, the development of microbial fermentation for producing PMA has attracted significant attention. To date, bioproduction of PMA has been mostly studied using *Physarum polycephalum* and *Aureobasidium pullulans*. *A. pullulans* isolated from different environments is more capable of achieving a high titer of PMA than *P. polycephalum* [5,6]. In our previous study, a high-yield PMA-producing strain, *A. pullulans* ZX10, was isolated, and a novel process was developed through acid hydrolysis of PMA for the production of L-malic acid from glucose [7].

Various *Aureobasidium* strains have been reported to produce PMA from a variety of carbon sources, including glucose, fructose, sucrose, and maltose [5,6,8]. In another study, a low-cost feedstock, raw sweet potato, was used for PMA fermentation. The maximum PMA production and productivity of 57.5 g/L and 0.34 g/L h, respectively, were achieved; however, the yield of PMA was relatively low (~0.2 g/g) [9]. Therefore, the high cost of PMA has seriously limited its commercial production and wide applications in many industrial areas. In the past two decades, remarkable advances in pentose-specific bioconversion have led to the production of biofuels and chemicals such as ethanol, butanol, fumaric acid, and xylitol [10–13]. The use of xylose, a major intermediate derived from lignocellulosic biomass, has attracted much attention to enable the sustainable recycling of lignocellulosic biomass and circumvent excessive nonfood use of glucose. However, few studies have been conducted to produce PMA and malic acid from xylose or lignocellulosic biomass hydrolysate. In this study, a novel isolate,

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A. pullulans YJ 6–11, was successfully screened from fresh plant samples, which could produce PMA from xylose with a high yield without the formation of dark pigments. Interestingly, compared with glucose, this strain had a better potential to produce PMA when xylose was used as the carbon source. The purpose of this study is to evaluate the use of xylose and corncob hydrolysate for the production of PMA and malic acid. These results will be of utmost importance for the development of a novel process with lignocellulosic biomass and the green production of PMA and malic acid chemicals.

2. Materials and methods

2.1. Isolation and identification of A. pullulans YJ 6–11

The strain was isolated from fresh plants or peach tree samples collected at several locations around Jinyun Mountain in Beibei, Chongqing, China, using the reported method with modifications [14]. With xylose as the sole carbon source, the viable yeast-like colonies were enriched in the plate and were transferred to a potato dextrose agar (PDA) medium. The selected strains were then screened by shake-flask fermentation to evaluate their PMA production levels. The strain that produced maximum PMA was maintained on the PDA slant and characterized by sequencing its deoxyribonucleic acid (DNA) internal transcribed spacer (ITS), including ITS region 1, 5.8 S DNA, and ITS region 2, using the primers ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAG-3′). The similarities of the resulted sequences were searched in GenBank via Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/). The ITS sequences were automatically aligned using Clustal X 2.0 [15], and the phylogenetic tree was constructed by the neighbor-joining method using MEGA 4.0 [16].

2.2. Preparation of corncob hydrolysate

Acid hydrolysis of corncob was performed by adding 1.0% H_2SO_4 (v/v) at a solid-to-liquid (corncob to diluted acid) ratio of 1:10 (w/v), and the mixture was autoclaved at 121 °C for 40 min. Then, 10,000 U/g each of xylanase and cellulase from Aladdin, China, were further added to the mixture at a base of 0.01-g/g corncob, respectively, and the enzyme was hydrolyzed at 50 °C for 48 h. The hydrolysate was centrifuged at 4000 × *g* for 15 min to remove the insolubles, and the residue supernatant was concentrated in vacuum at 70 °C to prepare certain sugar concentrations.

2.3. Shake-flask fermentation

PDA agar slants were inoculated with cells and cultured at 25 °C for 2 days, and then used for the following seed culture inoculation. The seed culture medium contained 60, 2, 0.1, 0.1, 0.1, 0.5, and 20 g/L of xylose, NH_4NO_3, KH_2PO_4, $MgSO_4$, $ZnSO_4$, KCl, and $CaCO_3$, respectively. The seed culture was grown in a 500-mL shake flask containing 50 mL of liquid medium, and was incubated at 25 °C in a rotary shaker (220 rpm) for 2 days. The fermentation medium contained 90, 2, 0.1, 0.1, 0.1, 0.5, and 30 g/L of xylose, NH_4NO_3, KH_2PO_4, $MgSO_4$, $ZnSO_4$, KCl, and $CaCO_3$, respectively. In order to evaluate the effect of different carbon and nitrogen sources on PMA production, 90 g/L of different carbon sources (e.g., xylose, glucose, fructose, maltose, and sucrose) or 2 g/L of different nitrogen sources (e.g., tryptone, yeast extract, NH_4Cl, $CO(NH_2)_2$, $NaNO_3$, and NH_4NO_3) were taken in 500-mL shake flasks each containing 50 mL of fermentation media. The fermentation cultivation was inoculated with 10% (v/v) of the above-described seed culture broth and kept at 25 °C with stirring at 220 rpm for 4 days.

2.4. Batch and fed-batch fermentations in a 5-L stirred-tank fermentor

Batch and fed-batch fermentation kinetics was studied in a 5-L stirred-tank fermentor (Shanghai Baoxing Co., Ltd., China) containing 3 L of media. The fermentation was inoculated with 300 mL of seed culture grown in a shake flask for 48 h, and operated at 25 °C with agitation and aeration at 400–800 rpm and 1.3 vvm, respectively. During fermentation, the agitation was controled to maintain the dissolved oxygen level >20%. The exhaust gas was monitored using a Hartman PS6000 gas analyzer (PS 6000, Hartman, China) to calculate the carbon dioxide evolution rate (CER) [17]. For fed-batch fermentation, xylose with a concentration of approximately 600 g/L was fed into the fermentor when the residual xylose concentration was <20 g/L. Throughout the fermentation process, broth samples were taken periodically for the analysis of residual xylose or glucose, biomass, and PMA titer. All trials were performed in triplicate.

2.5. Identification and characterization of PMA

The molecular weight of PMA produced from different carbon sources was determined. PMA was precipitated from the fermentation broth by adding three volumes of ethanol, and the precipitate obtained by centrifugation at 8000 × *g* for 20 min was washed twice with ethanol. The number average molecular weight (M_n) and weight average molecular weight (M_w) were determined by gel permeation chromatography (GPC) using an Elite system equipped with a TSK-GEL G3000 PW_{XL} column and refractive index detector (Shodex RI-201H). Polyethylene glycol standards were used for calibration. Sodium phosphate buffer (0.1 M, pH 6.8) was used as the mobile phase, and the flow rate was 1 mL/min.

For characterization of the structure, PMA was separated from the fermentation broth by adsorption with D201 anion-exchange resins (Shanghai Huazhen Sci. & Tech. Co., Ltd., China), and eluted with 0.5 M H_2SO_4 solution. The collected PMA was then freeze-dried for structure analysis. Fourier transform infrared spectroscopy (FTIR) was performed on an IRPrestige-21 FTIR spectrometer with KBr pellets. The ^{13}C nuclear magnetic resonance (NMR) spectra of PMA was tested on a Bruker Avance 400-MHz spectrophotometer (Avance III, Basel, Switzerland).

2.6. Analytical methods

2.6.1. Cell biomass and residual xylose and glucose concentration

The cell density was determined by the dry cell weight (DCW) method. Prior to the measurement, excess $CaCO_3$ was eliminated from the broth by adding 1 M HCl. The cell suspension was centrifuged at 4000 × *g* and then dried overnight at 105 °C [7]. The concentrations of residual xylose and glucose were measured using a high-performance liquid chromatograph (HPLC) equipped with a Hypersil NH_2 analysis column (Elite, China) and refractive index detector (Shodex RI-201H) at 40 °C. The mobile phase with an acetonitrile-to-water ratio of 75:25 (v/v) was used at a flow rate of 1 mL/min.

2.6.2. Assay of PMA production

PMA was analyzed by centrifuging the fermentation broth and then adding 1 mL of resulted supernatant to 1 mL of 2 M H_2SO_4 in an incubator at 85 °C for 8 h. After neutralization of the solution, the hydrolyzed sample was analyzed by HPLC (Agilent 1200, USA) using a Spursil C18-EP organic acid column at 40 °C eluted with 5 mM H_2SO_4 at a rate of 0.6 mL/min to determine its malic acid content [18].