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Efficient production of succinic acid from herbal extraction residue hydrolysate

Caixia Wang[a,1], Xinyao Su[a,d,1], Wei Sun[a], Sijing Zhou[c], Junyu Zheng[a], Mengting Zhang[a,e], Mengchu Sun[d], Jianping Xue[d], Xia Liu[e], Jianmin Xing[b], Shilin Chen[a,*]

[a] *Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, No. 16, Nanxiaojie, Dongzhimennei, Beijing 100700, PR China*
[b] *National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, PR China*
[c] *Beijing Radiation Center, Beijing 100015, PR China*
[d] *School of Life Science, Huai Bei Normal University, Huaibei 23500, PR China*
[e] *School of Chemistry, Chemical Engineering and Life Science, Wuhan University of Technology, Wuhan 430070, PR China*

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ABSTRACT

In this study, six different herbal-extraction residues were evaluated for succinic acid production in terms of chemical composition before and after dilute acid pretreatment (DAP) and sugar release performance. Chemical composition showed that pretreated residues of *Glycyrrhiza uralensis Fisch* (GUR) and *Morus alba* L. (MAR) had the highest cellulose content, 50% and 52%, respectively. Higher concentrations of free sugars (71.6 g/L total sugar) and higher hydrolysis yield (92%) were both obtained under 40 FPU/g DM at 10% solid loading for GUR. Using scanning electron microscopy (SEM), GUR was found to show a less compact structure due to process of extraction. Specifically, the fibers in pretreated GUR were coarse and disordered compared with that of GUR indicated by SEM. Finally, 65 g/L succinic acid was produced with a higher yield of 0.89 g/g total sugar or 0.49 g/g GUR. Our results illustrate the potential of GUR for succinic acid production.

1. Introduction

Succinic acid (1, 4-butanedioic acid) is an important chemical building-block used in synthesis of high value-added derivatives used in food and pharmaceutical products, solvents, biodegradable polymers, surfactants, and detergents (YanZhu et al., 2013; Zeikus et al., 1999). The wide range of usage for succinic acid has lead to much investment in its biotechnological production, including bacterial strain engineering, fermentation control, separation and purification optimization, aiming to replace the conventional petrochemical-based succinic acid production method (Morales et al., 2016; Wang et al., 2012). The biotechnological production of succinic acid, although comparable to petro-based production, still has a higher cost (Pinazo et al., 2015). To reduce the cost, agricultural biomass and other renewable material are used for succinic acid production via strain fermentation by hydrolyzing into monosaccharides. Recent research showed that cost savings can reach up to 34.38% just by replacing glucose with corn stalk hydrolysates as the carbon source (Wang et al., 2014b). However, the natural recalcitrant structure of biomass hindered its utilization which required pretreatment to remove lignin and/or hemicelluloses, reduce cellulose crystallinity, increase biomass porosity and thus release fermentable sugars from their polysaccharides (Akhtar et al., 2014). Besides agricultural biomass, many other renewable materials such as algal biomass have also been explored in succinic acid or biofuel production either due to their unique chemical constituents or to their abundance (John et al., 2011).

Increased demand for Chinese herbal medicines (CHMs) is due to their potential benefits in disease prevention and treatment. The functional ingredients in medicinal plants are extracted using water or organic solvents from medicinal plants. The process produces large amounts of herbal extraction residues (HERs) as solid waste with high moisture content (Morikawa et al., 2014; Xi et al., 2015), which easily decay and breakdown in the environment after disposal. It is estimated that over 1.5 million tons of HERs are produced in China every year without any efficient treatment (Liu and Cheng, 2009). The lack of efficient utilization of HERs has become a social problem due to the environmental impacts of pollution, and thus restricting the sustainable development of the Chinese herbal medicine industry. For example, *Glycyrrhiza uralensis* (GU) or Chinese licorice constitutes over $42.1 million USD (Seki et al., 2008) in trade as it is used worldwide both as a

* Corresponding author at: No. 16 Nanxiaojie, Dongzhimennei Ave, Beijing 100700, PR China.
E-mail address: slchen@icmm.ac.cn (S. Chen).
[1] These authors contributed equally to this work.

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sweetening agent and medicinal herb. High consumption of GU has emphasized the need for appropriate *Glycyrrhiza uralensis* residue (GUR) utilization method.

Current methods exist to reuse HERs; specifically, re-extraction of other ingredients, cultivation of edible and medicinal fungi, utilization as organic fertilizer, implantation in waste water treatment, and biogas production (Cheng and Liu, 2010; Zhao and Zhou, 2016). It is notable that the main components of HERs are cellucose, hemicellucose and lignin, which make them attractive as a renewable source of succinic acid production. Unlike agricultural biomass, HERs are produced through the extraction of medicinal plants under higher temperatures (60–80 °C). It is likely that the extraction process destroys the recalcitrant structure of HERs, avoiding the need of further severe treatments, giving it an advantage as biomass for succinic acid production. Despite this, a significant gap about using HERs in the literature exists concerning chemical composition analysis, enzymatic hydrolysis performance evaluation, and succinic acid production. Thus, intense research is required in this area for efficient utilization of HERs to produce succinic acid.

Based on the above background, HERs were explored to produce succinic acid to achieve the recycle of HERs and meanwhile alleviate their environmental pollution. Unlike agricultural biomass, there is very little published research about this issue. In order to do this, six representative HERs were selected and tested. Chemical composition of these representative HERs was measured. Enzymatic hydrolysis was conducted to assess their performance in free sugar release. The enzymatic hydrolysis process was optimized for pretreated GUR. Batch and fed-batch succinic acid fermentation was conducted using hydrolysates of pretreated GUR. Based on the authors' knowledge, HERs have been explored systematically for the first time for succinic acid production, providing possible solution of HERs usage.

2. Materials and methods

2.1. Bacterial strains, media, and cultivation

E. coli MG-PYC (pTrchisA-pyc, △ldhA) was used for succinic acid fermentation in this study. In this strain, lactate dehydrogenase A (ldhA) has been deleted to decrease the production of lactic acid and heterologous pyruvate carboxylase (pTrchisA-pyc) is overexpressed for increased succinic acid production.

For bacteria cultivation, a Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter of water) was used but for the licorice extraction medium the ingredients were dissolved in 1 L licorice extraction liquid instead of water. The fermentation medium was composed of 10 g yeast extract, 20 g $MgSO_4$, 1.37 g K_2HPO_4, 1.53 g KH_2PO_4, 1.5 g NaCl, 0.05 g $MnCl_2$, 0.38 g $CaCl_2$, and 5 g $MgCO_3$ per liter, and corresponding enzymatic hydrolysates. The pH of fermentation media was set at 6.8 by10 M NaOH. All media were sterilized at 115 °C for 30 min, and glucose or enzymatic hydrolysates were sterilized separately. A 250 mL flask with 100 mL of fresh fermentation medium was used for succinic acid production, and the size of the inoculum was 5%. Reagents used in the study were of analytical grade and were from Oxoid (UK) or from Sinopharm Chemical Reagent Beijing Co., Ltd. (China) unless otherwise indicated.

Batch fermentation in flask was conducted using the following protocols. After 12 h aerobic cultivation in LB medium at 37 °C, high-density stock culture (optical density (OD_{600}) of 12) was obtained, and was then subsequently centrifuged at 5000 rpm for 10 min. The pellet was resuspended in water and added to each fermentation flask to an OD of 4 to start anaerobic fermentation. 23.8 mg/L IPTG (Isopropyl-D-thio-galactoside) was added to the medium to induce expression of pyruvate carboxylase in the aerobic phase at OD = 0.6. Succinic acid fermentation using enzymatic hydrolysate media was conducted for 60 h.

Fed-batch fermentation of GUR hydrolysate was conducted in a 3 L bioreactor (Bioflo 110, New Brunswick Scientific Co., Inc., USA USA) with 1.0 L fermentation media in which an initial total sugar was 45 g/L. GUR hydrolysate was sterilized together with fermentor at 115 °C for 30 min and other components including nitrogen source and mineral salt were sterilized separately under same condition. These components were mixed after sterilization in the fermentor before fermentation. Seed culture was prepared by inoculating several colonies into a 250 mL flask containing 50mLculture medium, and incubating at 30 °C and 250 rpm for 12 h. The amount of seed inoculums was 5% relative to the fermentation media. Dual-phase fermentation strategy was adopted in the fed-batch fermentation. During the aerobic phase, pH was maintained at 7.0 with 10 M NaOH and 10% H_2SO_4 (v/v), and the temperature was maintained at 37 °C. Oxygen-enriched air was sparged at 4 vvm with an agitation of 300–1000 rpm to maintain the dissolved oxygen (DO) above 20% as measured by an online probe (Mettler-Toledo Process Analytical Instruments, Wilmington, MA). IPTG was added as that in batch fermentation. After cultivation for 16 h, anaerobic phase was started. During the anaerobic phase, CO_2 was sparged into the media and the strain started to produce succinic acid. pH was controlled at 6.7 by 10 M NaOH. During this period, the agitation rate was set at 150 rpm to mix the sparged CO_2. The evaporated concentrated GUR hydrolysate containing 400 g/L total sugar was added intermittently when it was used up at 16, 33, 50, 69 h. Fed batch fermentation was finished after 130 h cultivation and samples were taken at certain time point for analysis.

2.2. Pretreatment and enzymatic hydrolysis of herbal-extraction residues

Six representative herbal-extraction residues were selected according to their specific medical parts. Residues of *Glycyrrhiza uralensis Fisch* (GUR), *Sophora flavescens Ait.* (SFR), *Scrophularia ningpoensis Hemsl* (SNR) were extracted from the roots, while residues of *Morus alba* L. (MAR) and *Cinnamomum cassia Presl* (CCR) were derived from the root cortex and bark cortex respectively. Whole plant residues were extracted for *Equisetum arvense* L. (EAR).

Herbal-extraction residues were provided by Shanxi TianZhi Run Biological Technology Co., Ltd. Herbal-extraction residues were first dried then milled in a vegetation disintegrator and separated by passing through a 40-mesh screen. Before use, the HER raw material was dried at 80 °C to a constant weight.

HERs were pretreated by the dilute-acid method, where 0.1 M HCl solution was used and the loading amount was 10%. After, HERs were further pretreated in the autoclave at 121 °C for 30 min. The pH of the pretreated HER solution was adjusted to 5.0 by 10 M NaOH, which was followed by enzymatic hydrolysis.

Enzymatic hydrolysis was performed with cellulose (Xiasheng Company, Ning Xia Province, China) at pH 5.0 for 24 h for batch hydrolysis and 72 h for fed-batch hydrolysis. To adjust the pH, 10% H_2SO_4 or 10 M NaOH was used. Enzymatic hydrolysis of six HERs utilized 20 filter paper unit (FPU)/g per dry matter (DM), whereas 10, 20, 40, 80 FPU/g per DM were tested for the appropriate enzyme-loading amount of GUR. Celllose FPU was provided by the manufacture. The enzymatic hydrolysis yield (Y) was defined as:

$$Y\,(\%) = \frac{C_{glu}\,(mg/ml) \times V\,(ml) \times 0.9}{Mw\,(mg) \times C_{cellu}\,(\%)} \times 100\%$$

where C_{glu} is the glucose concentration of the enzymatic hydrolysate, V is the total volume of the enzymatic hydrolysate, Mw is the mass of biomass used, and C_{cellu} is the cellulose content of the herbal-extraction residue.

2.3. Analytical methods

Bacterial growth was estimated from the OD_{600mn} of the medium using a spectrophotometer (723 N, Shanghai Precision & Scientific

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