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Efficient production of succinic acid from duckweed (*Landoltia punctata*) hydrolysate by *Actinobacillus succinogenes* GXAS137

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ABSTRACT

A novel process of enzyme pretreatment and semi-simultaneous saccharification and fermentation (SSSF) was developed in this work to improve succinic acid (SA) productivity from duckweed (*Landoltia punctata*) and achieve low viscosity. Viscosity (83.86%) was reduced by the pretreatment with combined enzymes at 50 °C for 2 h to a greater extent than that by single enzyme (26.19–71.75%). SSSF was an optimal combination with 65.31 g/L of SA content, which was remarkably higher than those obtained through conventional separate hydrolysis and fermentation (62.12 g/L) and simultaneous saccharification and fermentation (52.41 g/L). The combined approach was effective for SA production. Approximately 75.46 g/L of SA content with a yield of 82.87% and a productivity of 1.35 g/L/h was obtained after 56 h in a 2 L bioreactor. Further studies will focus on increasing the working scale of the proposed method.

1. Introduction

Succinic acid (SA) is an important C4-building chemical platform for many applications, such as food products and completely biodegradable polyester-polybutylene succinate (Pleissner and Venus, 2016). Therefore, SA was included in the top 10 list of platform chemicals that can be produced from biomass by the US Department of Energy in 2004 and 2010 (Bozell and Petersen, 2010; Werpy et al., 2004). Commercial SA is mainly produced through petrochemical processes that use petroleum-derived compounds (Almqvist et al., 2016), but petrochemical processes are unsustainable because of decreased oil reserve, increased cost and environmental pollution (Ferone et al., 2017). SA is alternatively manufactured using bio-based feedstock through microbial fermentation, which has been widely explored because of CO_2 consumption, thereby reducing greenhouse gases (Olajuyin et al., 2016).

Fermentative SA production has been accomplished by different microorganisms, such as wild-type strains, including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens* and *Basfia succiniciproducens* (Olajuyin et al., 2016), and genetically engineered strains, such as *Escherichia coli* (Olajuyin et al., 2016), *Corynebacterium glutamicum* (Xu et al., 2016) and *Saccharomyces cerevisiae* (Ito et al., 2014). Although these organisms have achieved a competitive performance, *A. succinogenes* is promising because of its ability to form SA naturally at appreciable yields and productivities from a broad range of carbon sources (Gunnarsson et al., 2014).

SA is generated by *A. succinogenes* from pure sugars, such as glucose (Herselman et al., 2017), cellobiose (Jiang et al., 2013), xylose (Bradfield and Nicol, 2016), and from inexpensive and renewable substrates, such as starch, bakery waste, cane molasses, glycerol and agricultural residues, including stalk and straw (Barisik et al., 2016). Although bio-based SA production has been successful to some extent, its high costs limit its applications compared with conventional petrochemical routes (Fu et al., 2014). For example, feedstock cost is a key issue in SA production. To address this problem, researchers investigated lignocellulose-rich materials, such as plant residues, because they are inexpensive, abundant and renewable. However, many technical problems, such as pretreatment processes, are complex and expensive (Szymanowska-Powałowska et al., 2014). Therefore, utilisation of cheap nonfood starchy materials can be an effective approach to reduce SA production cost.

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Duckweed (*Landoltia punctata*) provides many advantages. For example, it is able to grow on and in water bodies, easily harvested, conveniently saccharified and uncompetitive with most grains and vegetables for arable land; it also contains high starch content and low lignin content (Gusain and Suthar, 2017). Therefore, this organism has received increasing attention as a promising inexpensive and sustainable source of nonfood biomass for the production of low-value biofuels and high-value chemicals, such as ethanol, butanol, biogas and hydrogen (Tonon et al., 2017; Xu and Deshusses, 2015). To obtain sugars that can be fermented to SA, García proposed that polysaccharides in duckweed should be hydrolysed with acids or enzymes (García et al., 2014). Dilute acid hydrolysis is an effective pretreatment method to obtain high sugar and SA yields (Shen et al., 2016), but this method has several disadvantages, such as production of inhibitory products and corrosion of equipment. Enzymatic hydrolysis is a specific approach that occurs under mild working conditions and does not form side products. However, nonstarch polysaccharides in duckweed create a highly viscous solution, which causes difficulty in mixing; as a consequence, additional power consumption is required for SA production. Thus, decreasing duckweed paste viscosity is a prerequisite to obtain high SA yields.

The configuration of enzymatic hydrolysis and fermentation can contribute to production efficiency and cost reduction. This configuration can be described as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and semi-simultaneous saccharification and fermentation (SSSF). Each process provides advantages and disadvantages. In SHF, enzymes and microbes can be operated under optimum conditions, thereby obtaining the maximum yield in each stage. However, this process produces a high glucose concentration and poses a high contamination risk (Chng et al., 2016). In SSF, hydrolysis and fermentation occur simultaneously and maintain low sugar levels, consequently alleviating substrate inhibition and decreasing the contamination risk during fermentation; nevertheless, optimal temperatures and pH for enzyme activities and microbial growth are mismatched (Zhou et al., 2016). SSSF is a good alternative that involves a short presaccharification period with a faster hydrolytic rate before the main SSF process. With the combined advantages of SSF and SHF, SSSF produces higher yields than SSF and SHF do (Perez-Pimienta et al., 2017). SSSF also reduces the viscosity of slurry at high substrate loadings (Carrillo-Nieves et al., 2017). Despite these advantages, the application of SSSF in SA production has yet to be evaluated.

This study aimed to improve SA productivity by optimizing the technological parameters of enzyme pretreatment and fermentation configuration from duckweed by *A. succinogenes* GXAS137 strain. Enzymatic components and loading were optimised to reduce viscosity and obtain high SA yields. Uploading strategies for enzyme hydrolysis and SA fermentation by SSF, SHF and SSSF processes were also evaluated, and scale-up studies were performed in a 2 L bioreactor to produce SA.

2. Materials and methods

2.1. Chemicals and materials

Duckweed (*Landoltia punctata*) was collected from a pond in Sichuan province, China and cultivated to accumulate starch. The wet biomass was dried at 60 °C, ground and meshed (300 μm mesh screen) to obtain homogeneous particle sizes. The chemical compositions of duckweed powder were as follows: 7.5% moisture, 92.5% total solids, 39.8% starch, 12.4% protein, 14.5% cellulose, 3.4% lignin and 0.2% reducing sugar based on the dried weight (w/w). More details regarding the feedstock chemical constituents were reported previously (Shen et al., 2016). Analytical grade SA, acetic acid, lactic acid and formic acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercially available Viscozyme L (120 FBG/mL activities, Novozymes, Bagsvard, Denmark), Celluclast 1.5 L (91 PFU/mL activities, Novozyme, Bagsvaerd, Denmark), Pectinex Ultra SP-L (10292 PGU/mL activities, Novozyme, Bagsvaerd, Denmark), a-amylase Liquozyme Supra (120 KNU/g protein activities, Novozymes, Wuxi, China), glucoamylase Suhong GAII (500 AGU/g protein activities, Novozymes, Wuxi, China), neutral protease Neutrase 0.8 L (1.5 AU/g protein activities, Novozymes, Wuxi, China) and pullulanase (5×10^4 U/g protein activities, Sigma P1067) were of technical grade and used to hydrolyse the reducing sugar resources that could be derived from cell wall sugars and starch. Yeast extract (YE) was purchased from OXOID (Cambridge, UK). All of the other chemicals used in this study were obtained from Sinochem (Shanghai, PR China) unless otherwise specified.

2.2. Microorganism and growth conditions

A. succinogenes GXAS137 (CCTCC M 2011399, China Centre for Type Culture Collection, Wuhan, China) isolated from bovine rumen (Shen et al., 2016) was used in the present study. Cells were grown in 100 mL sealed anaerobic bottles containing 50 mL of medium containing the following (in g/L): glucose (20.0), YE (5.0), corn steep liquor powder (CSLP, 5.0), $NaH_2PO_4{\cdot}H_2O$ (8.5), K_2HPO_4 (15.5) and $NaHCO_3$ (2.0). Glucose was autoclaved and aseptically added to the medium. All liquid cultures were incubated in a rotary shaker (100 rpm) at 37 °C.

2.3. Enzyme hydrolysis

2.3.1. Viscosity reduction of duckweed mash by enzymatic pretreatment hydrolysis

Duckweed powder was mixed with tap water in a 500 mL Erlenmeyer flask to achieve a concentration of 160 g/L dry solid in a final volume of 200 mL. To reduce the viscosity of the duckweed mash, different enzymatic pretreatment hydrolyses with Celluclast 1.5L 0.4 PFU/g dry matter, Viscozyme L 0.2 FBG/g dry matter, Pectinex Ultra SP-L 40 PGU/g dry matter and combined enzymes of Viscozyme L 0.2 FBG + Pectinex Ultra SP-L 40 PGU/g dry matter were performed at 50 °C, pH 4.8 and 220 rpm for 4 h under agitation on a water bath shaker (Srichuwong et al., 2009). pH was adjusted using 10 M hydrochloric acid or 10 M potassium hydroxide. Viscosity reduction was calculated using Eq. (1).

$$\text{Viscosity reduction}(\%) = \frac{[\text{Viscosity1}]-[\text{Viscosity2}]}{[\text{Viscosity1}]} \times 100\% \tag{1}$$

where viscosity 1 is the viscosity at the beginning and viscosity 2 is the viscosity after enzymatic pretreatment hydrolysis.

2.3.2. Liquefaction and sacharification

Duckweed mash treated with viscosity-reducing enzyme was then liquefied by α-amylase at a concentration of 0.29 KNU/g dry matter and temperature range between 85 and 90 °C with shaking at 150 rpm for 1 h. The temperature of the liquefied mash was then decreased to 60 °C before saccharification through the addition of 5.90 AGU/g dry matter glucosidase and 31.86 u/g dry matter pullulanase for 8 h under agitation in a water bath shaker. Liquefaction and saccharification were performed according to previously described methods (Shen et al., 2016); thus, process optimization was not investigated in the present work. Hydrolysate samples were analysed in terms of glucose, xylose, arabinose, galactose, cellobiose and fructose contents through HPLC. The yield of enzymatic hydrolysis was calculated using Eq. (2).

$$\text{Hydrolysis yield}(\%) = \frac{[\text{Glu}] + [\text{Xyl}] + [\text{Ara}] + [\text{Gla}] + [\text{Cel}] + [\text{Fru}]}{(1.111)f\,[\text{Biomass}]} \times 100\% \tag{2}$$

where Glu is the glucose concentration (g/L); Xyl is the xylose concentration (g/L); Ara is the arabinose concentration (g/L); Gla is the