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Efficient and repeated production of succinic acid by turning sugarcane bagasse into sugar and support

Pengcheng Chen, Shengtao Tao, Pu Zheng *

Jiangnan University, The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Wuxi 214122, China

HIGHLIGHTS

- Application of agricultural waste for bioconversion into high valued chemical.
- Optimization of multi-enzyme cocktail to obtain reducing sugars.
- Diminishing the restriction of $MgCO_3$ in pH modulating.
- Using sugarcane bagasse residue as cell adsorption support to realize reusability.

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ABSTRACT

Here we reported an endeavor in making full use of sugarcane bagasse for biological production of succinic acid. Through NaOH pre-treatment and multi-enzyme hydrolysis, a reducing sugar solution mainly composed of glucose and xylose was obtained from the sugarcane bagasse. By optimizing portions of cellulase, xylanase, β-glucanase and pectinase in the multi-enzyme "cocktail", the hydrolysis percentage of the total cellulose in pre-treated sugarcane bagasse can be as high as 88.5%. *A. succinogenes* CCTCC M2012036 was used for converting reducing sugars into succinic acid in a 3-L bioreactor with a sugar-fed strategy to prevent cell growth limitation. Importantly, cells were found to be adaptive on the sugarcane bagasse residue, offering possibilities of repeated batch fermentation and replacement for $MgCO_3$ with soluble $NaHCO_3$ in pH modulation. Three cycles of fermentation without activity loss were realized with the average succinic acid yield and productivity to be 80.5% and 1.65 $g{\cdot}L^{-1}{\cdot}h^{-1}$.

1. Introduction

Agricultural waste is organic waste produced in the course of agricultural activities. Owing to the rapid growth of global population and economy, the amount of agricultural waste is increasing every year, particularly in Asian countries, which results into a big question of how to deal with it. In China, maize, wheat, rice, and sugarcane are the four agricultural crops with maximum production as well as cultivation area, and are accounting for the majority of lignocellulosic agricultural waste. The traditional methods for disposing these wastes include landfilling, incineration and composting. Unfortunately, the capacity of landfills is limited and the method of landfilling is against the idea of green development (Othman et al., 2013). Incineration is favorable in terms of energy recovery, but can potentially cause air pollution (Ngoc and Schnitzer, 2009). Composting has a relatively low environmental impact, but the biological compost is more expensive than chemical fertilizers and thus suffers from a limited market (Gajalakshmi and Abbasi, 2008). In a word, it is urgent to develop new ways of handling agricultural waste economically and efficiently.

Recent studies have reported the utilization of lignocellulosic agricultural waste as a potential resource for bioconversion into high valued chemicals (Avci et al., 2013; Saini et al., 2015). Compared with chemical conversion which is usually associated with petroleum-based processes and strict reaction conditions, the bioconversion method is safer, more environmentally friendly and easier to scale up. By turning lignocellulosic agricultural waste into fermentable sugars, valuable platform chemicals such as lactic acid (Yadav et al., 2011), citric acid (Liu et al., 2015), succinic acid (Carvalho et al., 2014; van Heerden and Nicol, 2013), and biopolymers such as polyhydroxyalkanoates (Xu et al., 2010) and polyhydroxybutyrates (Patel et al., 2015) can be produced by microorganisms. Among these chemicals, succinic acid, a

* Corresponding author.
E-mail address: zhengpu@jiangnan.edu.cn (P. Zheng).

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dicarboxylic acid which is used as a precursor for many chemicals, is at the forefront of biotechnological research. Up to now, microorganisms like *A. succinogenes* (Maharaj et al., 2014), *Mannheimia succiniciproducens* (Lee et al., 2002), and metabolic engineered strains of *Escherichia coli* (Chen et al., 2014) and *Saccharomyces cerevisiae* (Okino et al., 2005) have been developed for producing this acid. *A. succinogenes* is able to produce a relatively large amount of succinic acid from a broad range of reducing sugars including arabinose, fructose, glucose, lactose, xylose, and sucrose under CO_2 (Beauprez et al., 2010; Xi et al., 2013). Thus, it is estimated that the combination of agricultural waste utilization and greenhouse gas consumption can create a green and viable pathway for balancing the initial high capital of biorefinery.

Notably, sugars are natural intermediates in the biological conversion of lignocellulosic agricultural waste, but access to sugars is often hindered by the recalcitrance of plant cell walls (Jiang et al., 2016). Straw, sugarcane bagasse, cotton stalk and wheat bran are all rich in lignocellulose and contain cellulose, hemicellulose, lignin and extractives. Cellulose forms a skeleton that is surrounded by hemicellulose (Ingram and Doran, 1995). Lignin acts as matrix and encrusting materials, which cannot be converted into reducing sugars (Sun and Cheng, 2002). Enzymatic hydrolysis of cellulose and hemicellulose is generally considered to be a sustainable approach of obtaining reducing sugars for *A. succinogenes*, and cellulase is the most frequently used enzyme. Although the compositions and activities of cellulases differ from their sources, at least three groups of enzymes are included: endoglucanase which attacks regions of low crystallinity in the cellulose fiber to create free chain-ends, exoglucanase which removes cellobiose from the free chain-ends to degrade the molecule further, and β-glucosidase which converts cellobiose into glucose (Coughlan and Ljungdahl, 1988). Obviously, the composition of cellulase can be optimized, or it can even be synergized with other enzymes to improve the saccharification effect.

In this work, sugarcane bagasse, one of the most common lignocellulosic agricultural waste in China, was used as the raw material for biological production of succinic acid by *A. succinogenes* CCTCC M2012036. Chemical pre-treatment of sugarcane bagasse to remove lignin was followed by enzymatic hydrolysis in generating reducing sugars. A multi-enzyme "cocktail" contained cellulase, xylanase, β-glucanase and pectinase was optimized in the saccharification process. Importantly, during succinic acid production, $MgCO_3$ is reported to be the most satisfactory neutralization reagent used for pH control and providing CO_2, because it prevents cell flocculation and prolongs the stationary phase (Dorado et al., 2009; Du et al., 2008; Liu et al., 2008). However, $MgCO_3$ is expensive and hardly soluble in water, which brings difficulty in large-scaled industrial operations, especially in the sterilization process. This work pioneered in the attempt in replacing $MgCO_3$ with soluable $NaHCO_3$. Moreover, the residue of sugarcane bagasse after saccharification was used as support for cell adsorption and recovery. This work is hoped to pave the way for an efficient, economically viable and large-scale biological production of succinic acid.

2. Methods and methods

2.1. Materials

Sugarcane bagasse was kindly supplied by Professor X.R. Liao's group in School of Biotechnology. It was grinded, sieved through a 425-μm screen and dried at 65 °C. Cellulase (ZSS-2100, 163.5 filter paper unit (FPU)/g) was purchased from Zesheng Bioengineering Technology Co., Ltd (Shandong, China). Xylanase (SNbase-XP, 1044 U/g) and β-glucanase (SNbase-BP, 5000 U/g) was bought from Sinoenzymes Biotechnology Co., Ltd (Shanghai, China). Pectinase (64007033, 158 U/g) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Yeast extract and corn steep powder were obtained from Angel Yeast Co., Ltd (Hubei, China) and Henghui Starch Sugar Co., Ltd (Jiangsu, China), respectively. All other chemicals were of analytical grade and used without further purification.

2.2. Pre-treatment and enzymatic hydrolysis of sugarcane bagasse

Sugarcane bagasse was separately pre-treated by acid and alkali. For the acid pre-treatment, sugarcane bagasse was immersed in H_2SO_4 solution with a liquid to solid ratio of 15:1 (v/w). The mixture was kept in an autoclave at 121 °C for 2 h. The alkaline pre-treatment of sugarcane bagasse was conducted with NaOH solution at 121 °C for 2 h. The liquid to solid ratio was also 15:1 (v/w). The concentrations of H_2SO_4 and NaOH solutions were listed in Table 1. After filtration and thorough washing processes, the pre-treated sugarcane bagasse (PSB) was dried at 65 °C.

The compositions of sugarcane bagasse before and after pre-treatment were determined by the method announced by National Renewable Energy Laboratory (NREL, 2008). Briefly, 0.5 g of sugarcane bagasse was hydrolyzed by 3.0 mL of concentrated H_2SO_4 solution (concentration: 72 wt.%, solvent: deionized water) at 30 °C for 60 min. Then deionized water was added into the mixture to dilute the H_2SO_4 solution to a 4.0 wt.% concentration. The obtained mixture was kept at 121 °C for 45 min. In these two acid hydrolysis processes, cellulose and hemicellulose can be converted into monosaccharides. After that, the mixture was filtered. pH value of the filtrate was adjusted to 2.0 by a 8.0 wt.% NaOH solution. The amount of cellulose in sugarcane bagasse was calculated by glucose and the hemicellulose amount was determined by xylose and arabinose. The residue was thoroughly rinsed by deionized water and dried at 105 °C and then burned at 550 °C. The amount of lignin was calculated from the mass balance between the dried residue and the ash left after burning.

In the enzymatic hydrolysis, PSB was respectively immersed in multi-enzyme solutions with different enzyme proportions at a liquid to solid ratio of 15:1 (v/w). pH values of these enzyme solutions were tailored to 5.0 by 1.0 M NaOH or HCl solutions. Then the mixture was shaken in a 50 °C water bath at 120 rpm for 30 h and at every 4 h time interval, 0.2 mL of hydrolysate was withdrawn to determine the contents of sugars produced. An orthogonal design $L_9(3^4)$ was applied to analyze the following factors: cellulase concentration (*A*), xylanase concentration (*B*), β-glucanase concentration (*C*) and pectinase concentration (*D*). Three levels of each factor were chosen for these experiments (Table 2). Totally nine tests were carried out and the order of the tests was randomized in order to prevent the error and bias.

Table 1
Pre-treatment of sugarcane bagasse under different acidic and alkaline conditions.

	Concentration (M)	Cellulose retention (%)	Hemicellulose retention (%)	Lignin removal (%)
NaOH	0.10	82.2 ± 0.12	88.5 ± 0.12	95.0 ± 0.15
	0.25	97.9 ± 0.06	87.3 ± 0.08	93.3 ± 0.10
	0.50	85.3 ± 0.13	55.5 ± 0.13	94.0 ± 0.12
	0.75	92.0 ± 0.15	39.5 ± 0.23	90.3 ± 0.15
	1.00	96.8 ± 0.12	26.8 ± 0.24	94.0 ± 0.08
H_2SO_4	0.10	81.0 ± 0.17	62.3 ± 0.18	65.9 ± 0.14
	0.25	72.4 ± 0.19	39.5 ± 0.17	66.6 ± 0.13
	0.50	75.2 ± 0.23	32.6 ± 0.10	41.6 ± 0.25
	0.75	81.1 ± 0.07	29.3 ± 0.12	45.5 ± 0.18
	1.00	56.4 ± 0.29	22.6 ± 0.10	42.3 ± 0.20