Short Communication

Pretreatment of spent mushroom substrate for enhancing the conversion of fermentable sugar

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HIGHLIGHTS

- Analysis of composition of spent mushroom substrate before and after pretreatment.
- A one-step method was established to convert lignocellulose of SMS to sugars.
- SMS hydrolysates were used for cultivating *Bacillus thuringiensis*.
- RP-HPLC with pre-column derivatization method was used to analyze SMS hydrolysates.

ARTICLE INFO

ABSTRACT

To develop a cost-effective biopesticide, spent mushroom substrate (SMS) extract was studied as a potential carbon source for cultivating *Bacillus thuringiensis* (Bt). Several pretreatments were compared to determine the optimal method for degrading cellulose to produce reducing sugars, including dilute sulfuric acid (0.5–2.0% v/v, 50–121 °C, 1 h), sodium hydroxide (0.5–2% w/v, 50–121 °C, 1 h), calcium hydroxide (0.2–4% w/v, 50–121 °C, 1 h), and hot water (50–121 °C, 1 h). Pretreatment was followed by standard enzymatic hydrolysis and fermentation. Results showed that the highest cellulose degradation was obtained using 2% dilute sulfuric acid pretreatment at 121 °C for 1 h, resulting in a high yield of reducing sugar (284.24 g/kg SMS). Sporulation was also highest using the same pretreatment. Use of SMS is not only an alternative way to commercialize Bt-based biopesticide, but also a potential solution for the environmental pollution associated with accumulation of the spent substrate of the mushroom industry.

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1. Introduction

Biopesticides derived from *Bacillus thuringiensis* (Bt) are the most commonly used biological agents for controlling insect pests. Application of this insecticide is minor because of the high cost of the insecticide, accounting for only 2% of the insecticidal market (Bravo et al., 2011). Recently, a variety of agro-industrial refuses have successfully replaced the previous expensive synthetic media for Bt production (Ozcan et al., 2010; Yezza et al., 2006). These studies indicate that Bt could grow well in various carbon and nitrogen sources. Spent mushroom substrate (SMS) is a remnant of the production of mushrooms. In 2010, approximately 50 million tons of SMS were produced by mushroom production in China

(Zhang et al., 2012). Due to the problem of economic waste and environmental pollution, the mushroom industry has paid much attention to the treatment or recycling of the spent substrate. SMS, as a lignocellulosic substance, could be a potential source of reducing sugars to produce biofuels (Lee et al., 2008) and cultivate microorganisms (Qiao et al., 2011). To date, however, the use of SMS in the production of Bt has not been reported. Containing a wealth of nutrients, such as carbon, nitrogen and metal ions, SMS is a potential raw material for Bt production. It may not only greatly reduce the cost of Bt production, but also solve the problem of excess SMS in terms of economy and environment. Aspects of the method, however, such as extracting nutritional ingredients of lignocellulosic substances, producing sugar from cellulose, and polysaccharide and protein from hyphae, have not been studied. Qiao et al., (2011) used SMS for cultivation of *Lactococcus lactis* after dilute sulfuric acid and enzyme treatment. Alkali (NaOH/Ca(OH)$_2$) has also been reported as a pretreatment for lignocellulosic substances (Nlewem and Thrash, 2010). In this study, four

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pretreatments were evaluated to optimize the conversion of SMS to sugar, the total yield of reducing sugars, and the spore count of Bt.

2. Methods

2.1. Chemicals and media

SMS was generously provided by Mycological Research Center of Fujian Agriculture and Forestry University. The mushroom substrate used for cultivating velvet shank, *Flammulina velutipes* (Curt. ex Fr.) Singer is composed of cotton seed hulls, wheat bran, sawdust, light calcium carbonate and lime. After air drying, the SMS was milled with a laboratory grinder to pass through a 40 mesh screen (425 μm) and then stored in air-tight plastic bags at 4 °C for further use. 1-Phenyl-3-methyl-5-pyrazolone (PMP) used for pre-column derivatization was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, PR China). HPLC-grade acetonitrile and methyl alcohol were purchased from Merck Company (Shanghai, PR China). *B. thuringiensis* subsp. *israelensis* (Bti) LLP29 was isolated from leaves of *Magnolia denudata* in previous studies (Zhang et al., 2010, 2011). The Bti was subcultured and streaked on Luria–Bertani (LB) agar plates, incubated for 48 h at 30 °C and then preserved at 4 °C for future use.

2.2. SMS composition analysis

The moisture content, ash content, the amount of hot water extracts and benzene–ethanol extracts of the untreated SMS were all determined according to National Standard of the People's Republic of China as follows: GB/T 2677.2-93, 2677.3-93, 2677.4-93 and 2677.6-94. The yield of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The content of cellulose, hemicellulose, acid soluble lignin (ASL) and acid insoluble lignin (AIL) were determined by the National Renewable Energy Laboratory (NREL) Analytical Procedure: Determination of Structural Carbohydrates and Lignin in Biomass (Sluiter et al., 2008). The HPLC method of the NREL analytical procedure using a Biorad Aminex HPX-87H column was replaced by a reversed phase-HPLC (RP-HPLC) method, using Agilent Zorbax SB-C18 column, with pre-column derivatization with PMP (Dai et al., 2010). All the composition percentages of raw and pretreated solid samples are calculated on a dry-weight basis. All experiments were conducted in triplicate. The data were given as means ± SD, and were analyzed by analysis of variance (ANOVA) using Microsoft Excel 2010 for windows.

2.3. Pretreatment and enzymatic hydrolysis

As shown in Table 1, SMS samples (5 g per replicate) were pretreated with dilute sulfuric acid, sodium hydroxide solution, calcium hydroxide solution and water of different concentrations. The samples were placed in 50 ml of water or pretreatment liquor (solid to liquid ratio of 1:10) in an autoclave for 1 h at different temperatures. To avoid further reaction, the pretreated samples were cooled promptly and neutralized with HCl or NaOH. The neutralized samples were washed 4 times with distilled water and centrifuged at 10,000 rpm at 4 °C for 3 min. The combined liquor was further diluted to 500 ml total. One portion of the liquid, which was used for determining the yield of sugars by HPLC and the DNS method (Miller, 1959), was sealed in a 50 ml centrifuge tube and stored at −20 °C, and the remaining portion was used for fermentation of Bt. The solid residues were transferred thoroughly to a pre-weighed petri dish, and dried at 40 °C to determine the solid recovery, and then stored at 4 °C for later enzymatic hydrolysis. One gram of pretreated sample (dry basis) was hydrolyzed by 35 FPU/g Celluclast 1.5 L and 61.5 CBU/g Novozym 188 as described by Xu et al., (2010). The supernatant was used to determine the yield of reducing sugars by the DNS method (Miller, 1959).

Table 1
Sugar production data, remaining mass data and spore count data for SMS pretreatment and enzymatic hydrolysis.

Test No.	Pretreatment method	Parameters			Yield of reducing sugars after pretreatment (g/ kg raw SMS)[a]	Yield of reducing sugars after pretreatment (g/ kg raw SMS)[b]	Yield of reducing sugars after enzymatic hydrolysis (g/kg raw SMS)	Remaining mass of SMS after pretreatment (g/ kg raw SMS)	Spore count (10^6 CFU/ ml)
		Temperature (°C)	Concentration (%)	Pressure (MPa)					
1	Dilute	121	0.5	0.5	134 ± 7.1	119 ± 1.9	66 ± 2.3	677 ± 37.8	5.24 ± 0.21
2	sulfuric	121	0.75	0.5	190 ± 2.7	185 ± 3.3	96 ± 4.7	606 ± 34.8	5.38 ± 0.42
3	solution	121	1	0.5	277 ± 4.8	236 ± 3.1	80 ± 1.5	602 ± 15.1	6.58 ± 0.46
4		121	2	0.5	315 ± 8.8	284 ± 2.1	93 ± 3.5	555 ± 34.7	6.06 ± 0.28
5		50	0.75	0.1	14 ± 0.5	11 ± 0.1	81 ± 0.8	739 ± 56.0	5.04 ± 0.35
6		100	0.75	0.1	43 ± 0.4	36 ± 0.3	136 ± 9.0	734 ± 35.8	5.14 ± 0.26
7	Sodium	121	0.5	0.5	9 ± 0.5	ND[c]	122 ± 3.1	638 ± 47.1	0.46 ± 0.03
8	hydroxide	121	0.75	0.5	13 ± 1.1	ND	200 ± 6.5	562 ± 13.1	0.34 ± 0.02
9	solution	121	1	0.5	18 ± 0.8	ND	251 ± 16.9	521 ± 43.0	0.40 ± 0.03
10		121	2	0.5	26 ± 2.0	ND	122 ± 7.9	438 ± 30.8	0.54 ± 0.03
11		50	0.75	0.1	12 ± 0.1	ND	137 ± 2.9	780 ± 5.2	2.40 ± 0.53
12		100	0.75	0.1	5 ± 0.3	ND	202 ± 6.7	763 ± 44.2	0.78 ± 0.02
13	Calcium	121	0.2	0.5	13 ± 1.1	ND	75 ± 1.6	795 ± 10.7	3.26 ± 0.13
14	hydroxide	121	1	0.5	4 ± 0.2	ND	224 ± 8.5	696 ± 24.5	3.38 ± 0.19
15	solution	121	2	0.5	8 ± 0.8	ND	264 ± 10.1	676 ± 24.1	2.02 ± 0.10
16		121	4	0.5	14 ± 1.1	ND	267 ± 3.6	678 ± 12.4	4.74 ± 0.32
17		50	1	0.1	12 ± 1.0	ND	155 ± 7.5	796 ± 14.5	3.42 ± 0.24
18		100	1	0.1	9 ± 0.3	ND	218 ± 9.4	755 ± 12.7	2.36 ± 0.13
19	Water	121	0	0.5	15 ± 1.0	11 ± 0.8	159 ± 9.7	769 ± 25.0	4.68 ± 0.23
20		50	0	0.1	18 ± 0.9	17 ± 0.1	99 ± 2.1	808 ± 37.6	2.76 ± 0.16
21		100	0	0.1	12 ± 0.6	9 ± 0.1	83 ± 1.9	823 ± 22.0	2.36 ± 0.13

[a] Determined by DNS method.
[b] Determined by HPLC analysis method.
[c] ND: Not detectable.