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Estimation of inhibitory effects of hemicellulosic wood hydrolysate inhibitors on PHA production by *Burkholderia cepacia* ATCC 17759 using response surface methodology

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HIGHLIGHTS

- ▶ Using response surface methodology to estimate the inhibitory effects of multiple inhibitors.
- ▶ Utilizing microbial growth and PHA content as response surfaces.
- ▶ Estimating synergistic inhibitory effects among select inhibitors.
- ▶ Monitoring the degradation and metabolism of inhibitors.

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ABSTRACT

Sugar maple hemicellulosic hydrolysate was utilized as a renewable feedstock for polyhydroxyalkanoates production by *Burkholderia cepacia* ATCC 17759. To estimate inhibitory effects of the hydrolysate, response surface methodology was utilized to analyze cell growth and PHA accumulation in the presence of multiple inhibitors. Mixture design was employed to study the correlation between the proportion of phenolics and total inhibition. The resultant models (R^2 as 92.42% and 93.14% for cell growth and PHA production, respectively) indicated syringic acid was the most inhibitory among three phenolics and synergistic inhibition was observed for the combinations of vanillin/syringic acid and vanillic acid/syringic acid. When furfural, levulinic acid, and acetate were also present during the fermentation, central composite design was employed. The regression model using 48 h cell growth as the response surface (R^2 = 87.82%) showed acetate was the most inhibitory. Additionally, strong synergistic effects were observed for the combinations of acetate/phenolics and levulinic acid/furfural.

1. Introduction

Polyhydroxyalkanoates (PHAs) are a class of polyesters synthesized and stored by various bacteria as intracellular carbon sources under metabolic stress or during unbalanced growth conditions (Hazer and Steinbuchel, 2007). Besides being completely biodegradable, PHAs exhibit comparable thermal and material properties to petrochemical-derived plastics and could be considered as potential alternatives to these recalcitrant materials (Madison and Huisman, 1999).

In order to lower the cost of PHA production, inexpensive and renewable feedstocks have been utilized as carbon sources (Choi and Lee, 1999; Du et al., 2012; Waller et al., 2012). As a potentially inexpensive wood-based feedstock, hemicellulosic wood hydrolysate is produced through a dilute acid hydrolysis of wood biomass and contains variable amounts of fermentable sugars composed primarily of xylose, rhamnose, mannose and glucose, as well as inhibitory compounds such as acetic acid, levulinic acid, furan derivatives and phenolic compounds (Amidon et al., 2008; Hu et al., 2010).

Burkholderia cepacia ATCC 17759 is a Gram-negative bacterium capable of producing short-chain-length PHAs including polyhydroxybutyrate (PHB), as well as copolymers of PHB and polyhydroxyvalerate (PHV) when provided with monomer precursors such as levulinic acid, which also inhibits cell growth at high concentrations (Keenan et al., 2004). Meanwhile, *Burkhoderia* strains are widely employed as bioremediation agents due to their ability to degrade toxic compounds such as polyaromatic hydrocarbons, polychlorinated biphenyls, furans and lignin monomers (Goris

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Table 1
Concentrations of vanillin, vanillic acid and syringic acid for a 3-factor mixture design and responses in terms of cell growth represented by OD_{540} and PHA content.

Design point	X_1/vanillin (g/L)	X_2/vanillic acid (g/L)	X_3/syringic acid (g/L)	OD_{540} at 24 h	OD_{540} at 48 h	PHA content (% CDW)
1	1.5	0	0	7.29 ± 0.20	10.14 ± 1.17	37.1 ± 6.9
2	0	1.5	0	8.95 ± 0.13	14.47 ± 0.66	61.0 ± 3.7
3	0	0	1.5	6.50 ± 0.64	5.22 ± 0.22	2.5 ± 1.6
4	0.75	0.75	0	7.14 ± 0.31	12.19 ± 2.28	53.8 ± 10.3
5	0	0.75	0.75	7.74 ± 0.48	7.86 ± 1.72	20.5 ± 7.3
6	0.75	0	0.75	9.50 ± 0.73	12.25 ± 1.97	56.6 ± 11.2
7	1	0.25	0.25	7.84 ± 1.13	12.50 ± 1.43	48.1 ± 11.6
8	0.25	1	0.25	8.63 ± 0.21	12.20 ± 0.47	41.8 ± 12.0
9	0.25	0.25	1	8.60 ± 0.35	6.18 ± 1.41	23.2 ± 7.3
10	0.5	0.5	0.5	7.12 ± 0.30	11.71 ± 1.87	57.2 ± 11.5

et al., 2004; Kim et al., 2003; Mitsui et al., 2010). Therefore, it is conceivable that *B. cepacia* is suitable for PHA production from hemicellulosic hydrolysate.

However, the presence of several toxic compounds in wood hydrolysate still exhibits significant inhibition of both microbial growth and PHA production. In a previous study, typical inhibitors of hemicellulosic hydrolyate fermentation, such as furfural, hydroxymethylfurfural (HMF), acetic acid, levulinic acid and vanillin, were examined for their inhibitory effects on cell growth and PHA production when they were added as sole inhibitors (Pan et al., 2012). It was observed that 2 g/L vanillin demonstrated significant inhibition of cell growth while 1 g/L only mildly inhibited growth within the first 24 h but was quickly overcome with the accumulation of biomass. Syringic acid and vanillic acid, when utilized as the sole inhibitor, also showed only partial inhibition of microbial growth even at 1 and 1.5 g/L respectively. However, diluted wood hydrolysate (50%), containing only 0.48 g/L total phenolics and very low concentrations of other inhibitors after membrane filtration, exhibited strong inhibitory effects on cell growth during fermentation. Therefore, synergistic inhibitory effects are believed to occur in the presence of multiple inhibitors when *B. cepacia* is grown on wood hydrolysate.

In this study, inhibitory effects of wood hydrolysate inhibitors were estimated using response surface methodology, an effective statistical and mathematical tool to determine the influence of many factors on one or several specific parameters (Lakshman et al., 2004; Triveni et al., 2001). Polynomial mathematical equations were developed for typical inhibitors based on mixture and central composite experimental design.

2. Methods

2.1. Microorganism and medium components

B. cepacia ATCC 17759 was cultivated in a mineral salts medium previously described by Bertrand et al., 1990 and the concentration of ammonium sulfate was established at 1.5 g/L for optimal PHA synthesis (Keenan et al., 2004). Xylose (3%, w/v) was employed as the sole carbon source and the initial pH of the growth medium was adjusted to 7 prior to fermentation.

2.2. Fermentation

Acetic acid, levulinic acid, furfural, vanillin, vanillic acid and syringic acid were purchased from Sigma–Aldrich Co. (St. Louis, MO) and added into 500 mL baffled flasks with 100 mL of nitrogen limiting mineral salts medium containing 3% xylose as the carbon source. Shake-flask experiments based on mixture design and CCD were performed at 150 rpm and 30 °C in a rotary shaker. Seed cultures were grown in the same medium, containing 3% xylose, for 48 h and stored at 4 °C before inoculation. Samples were taken daily or every 12 h for analyzing the consumption of xylose, inhibitors and ammonium sulfate. Microbial biomass was harvested after 48 h and subjected to centrifugation at 5000 rpm and lyophilization for the determination of PHA concentrations and composition by gas chromatography (GC) as described in 2.4 (Nomura et al., 2004; Tappel et al., 2012). A control group was also established without any inhibitor added and subjected to GC analysis.

Table 2
Concentrations of four inhibitors in terms of coded units.

	−2	−1	0	1	2
X_1/acetate (M)	0.048	0.072	0.096	0.120	0.144
X_2/levulinic acid (g/L)	1.0	1.5	2.0	2.5	3.0
X_3/phenolics (g/L)	0.20	0.35	0.50	0.65	0.80
X_4/furfural (g/L)	0.10	0.15	0.20	0.25	0.30

2.3. Experimental design and statistical analysis

Response surface methodology (RSM) was employed in this study for the analysis of inhibitory effects caused by three typical lignin-derived phenolic compounds and for these phenolics plus other inhibitors typically present in hemicellulosic hydrolysate.

The total phenolic content in the wood hydrolysate varied from 0.6–1.5 g/L based on production conditions, pretreatments, detoxification methods and the sterilization method. Syringic acid/syringaldehyde and vanillin/vanillic acid were determined as two major phenolic groups present in sugar maple hemicellulosic hydrolysate by gas chromatography–mass spectrometry (GC–MS) (Pan et al., 2012). The proportion of these two groups, which may affect the total inhibition in the hydrolysate, is determined by the ratio of *p*-hydroxyphenyl, guaiacyl and syringyl lignins contained in the original woody biomass and the method of treatment (Klinke et al., 2004). Therefore, mixture design methodology was applied in this study and vanillin (X_1), vanillic acid (X_2) and syringic acid (X_3) were selected (the water solubility of syringaldehyde is relatively low). In the mixture design, the total amount of phenolics for each experimental run was fixed at 1.5 g/L and the proportions of the three select phenolics were varied (Eq. (1)). An augmented mixture design methodology was selected by establishing more experimental runs involving all inhibitors for the estimation of the complete mixture (Table 1). Initially, a special cubic model (Eq. (2)) was proposed. Microbial growth, represented by optical density, and PHA content were employed as responses. Coefficients $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_2\beta_3$ and $\beta_1\beta_2\beta_3$ represent the potential synergistic or antagonistic effects for different combinations. The significance of these terms was determined by the analysis of variances (ANOVA) and p values (Prob. > F), which are preferred to be less than 0.05.