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Production of polyhydroxyalkanoates by *Burkholderia cepacia* ATCC 17759 using a detoxified sugar maple hemicellulosic hydrolysate

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Abstract Sugar maple hemicellulosic hydrolysate containing 71.9 g/l of xylose was used as an inexpensive feedstock to produce polyhydroxyalkanoates (PHAs) by *Burkholderia cepacia* ATCC 17759. Several inhibitory compounds present in wood hydrolysate were analyzed for effects on cell growth and PHA production with strong inhibition observed at concentrations of 1 g/l furfural, 2 g/l vanillin, 7 g/l levulinic acid, and 1 M acetic acid. Gradual catabolism of lower concentrations of these inhibitors was observed in this study. To increase the fermentability of wood hydrolysate, several detoxification methods were tested. Overliming combined with low-temperature sterilization resulted in the highest removal of total inhibitory phenolics (65%). A fed-batch fermentation exhibited maximum PHA production after 96 h (8.72 g PHA/L broth and 51.4% of dry cell weight). Compositional analysis by NMR and physical–chemical characterization showed that PHA produced from wood hydrolysate was composed of polyhydroxybutyrate (PHB) with a molecular mass (M_N) of 450.8 kDa, a melting temperature (T_m) of 174.4°C, a glass transition temperature (T_g) of 7.31°C, and a decomposition temperature (T_{decomp}) of 268.6°C.

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Introduction

Polyhydroxyalkanoates (PHAs) are a class of biodegradable polyesters synthesized by a variety of microorganisms as intracellular carbon and energy reserve materials under certain limiting nutritional conditions while in the presence of excess carbon [11, 19, 35]. PHAs exhibit similar physical–chemical properties to petroleum-derived thermoplastics but are totally biodegradable to CO_2 and H_2O [35].

Burkholderia cepacia ATCC 17759 is a Gram-negative bacterium that can utilize a wide variety of carbon sources to produce short-chain-length (SCL) PHAs including the homopolymers polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) as well as copolymers of hydroxybutyrate and hydroxyvalerate (PHB-co-PHV) under specific feeding strategies [11]. Although SCL-PHAs have been widely studied and produced commercially, the high production cost still hinders the widespread application of these materials. Since the cost of the carbon source accounts for over 50% of the total cost of PHA production, inexpensive feedstocks for PHA production could significantly decrease the total expenditure for this process [5].

Wood hydrolysate is a potentially inexpensive and renewable feedstock that can be produced through enzymatic or dilute acid hydrolysis of cellulose or hemicellulose to fermentable sugars, such as glucose, galactose, xylose, and mannose. Wood hydrolysate has already been utilized for the production of ethanol and xylitol [1].

However, during the process to produce fermentable sugars, other by-products are released during the treatment of

hemicellulose and lignin under conditions of high temperature and low pH [31, 32]. Besides acetic acid, which is generated with the release of sugars, furfural and 5-hydroxymethyl furfural (HMF) are formed from the degradation of sugars. Subsequent degradation of furfural and HMF leads to the formation of formic acid and levulinic acid. Additionally, phenolic compounds are directly generated from the degradation of lignin. These compounds are also exceedingly toxic to microorganisms during subsequent fermentation processes. In order to increase the fermentability of wood hydrolysate, a number of detoxification methods were tested to remove potential inhibitors. Overliming [21, 33], activated charcoal [3], membrane filtration [18], ion exchange resins [27], and biological treatments [29] are among the most frequently used treatments. Wood charcoal and low-temperature sterilization have also been employed in some studies [16, 24, 37].

In this study, sugar maple hemicellulosic hydrolysate was utilized as the carbon source for PHA production. The inhibitory effects of selected inhibitors from wood hydrolysate were evaluated for effects on cell growth, PHA production, and physical–chemical properties of PHAs. Subsequently, membrane-purified wood hydrolysate, detoxified to remove phenolics, was used to produce PHAs by fermentation. PHA samples were taken and further analyzed for molecular mass, thermal properties, and NMR spectra.

Materials and methods

Microorganism and media

Burkholderia cepacia ATCC 17759 was cultivated in a carbohydrate mineral salts medium initially reported by Bertrand et al. [2]. However, the $(NH_4)_2SO_4$ was reduced to 1.5 g/l to introduce nitrogen limitation for PHA production [11].

Preparation of sugar maple hemicellulosic hydrolysate

The hemicellulosic hydrolysate of sugar maple was provided by the Department of Paper and Bioprocess Engineering (SUNY-ESF) and produced using dilute sulfuric acid as described by Amidon et al. [1] and Hu et al. [9]. Briefly, wood extracts, which were obtained by processing sugar maple chips at 160°C for 120 min, were then hydrolyzed with 2% sulfuric acid at 95°C for 20 min. After concentration, neutralization, and centrifugation to remove precipitates, a product containing 160.7 g/l xylose, 0.7 g/l furfural, 0.1 g/l HMF, 0.66 g/l total phenolics and 0.78 M acetic acid was obtained (Table 1). Subsequently, a two-stage filtration was performed to separate sugars from acetic acid, furfural, and HMF using two membranes with molecular weight cut-offs of 200 and 130 Da, respectively [18]. The final product contained 71.9 g/l xylose, 0.05 M acetic acid, 0.007 g/l HMF, 0.60 g/l total phenolics and no detectable furfural (Table 1).

Table 1 Concentrations of xylose, furfural, HMF, acetate, and total phenolics in wood hydrolysate before and after membrane filtration

	Before membrane treatment	After membrane treatment
Xylose	160.7 g/l	71.9 g/l
Furfural	0.69 g/l	N.D.
HMF	0.12 g/l	0.007 g/l
Acetate	0.78 M	0.05 M
Total phenolics (g/l GAE)	0.66 g/l	0.60 g/l

GAE gallic acid equivalents; *N.D.* not detected

Characterization of wood hydrolysate by gas chromatography-mass spectrometry (GC-MS)

To prepare samples for GC–MS, 2 ml of wood hydrolysate sample was mixed with 4 ml of methanol. The resulting precipitates were subsequently removed by a 0.45-μm syringe filter and the filtrate was concentrated under argon/nitrogen to 2 ml. The concentrate was then extracted with 4 ml of ethyl acetate three times. Extracts were then collected and concentrated under nitrogen to 1 ml. Derivatization was performed by incubating 1 ml of concentrated extract with 300 μl of 1:1 NO-Bis(trimethylsilyl)trifluoro acetamide (BSTFA) and anhydrous pyridine (Sigma-Aldrich Co., St. Louis, MO) for 30 min at 70°C.

Characterization of wood hydrolysate was achieved using a Thermo Electron Focus gas chromatograph connected to a Thermo Electron Polaris Q mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Gas chromatography was performed with an RTX-5MS column (30 m × 0.25 mm I.D. and 0.5-mm film thickness, Restek Co., Bellefonte, PA) under the following conditions: helium 39 ml/min; injector temperature 260°C; transfer-line temperature 280°C; energy of 70 eV; oven temperature 110°C for 4 min followed by increasing temperature from 110–260°C at a ramp rate of 4°C/min, and finally holding temperature at 260°C for 25 min. Splitless injection was used to introduce 1 μl of sample. Pure reference standards for frequently reported sugars and lignin derivatives such as vanillin, vanillic acid, syringic acid, syringaldehyde, gallic acid, *p*-coumaric acid, cinnamic acid, HMF, and furfural were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Fermentation

Shake-flask experiments were performed using 500-ml baffled flasks with 100 ml of nitrogen limiting mineral salt

medium at an initial pH of 7. Seed cultures were grown in 3% xylose (w/v) at 30°C and 150 rpm for 48 h and stored at 4°C before use. Samples were taken for analyses of sugars, organic acids, potential inhibitors, and PHA content. Microbial biomass was harvested after fermentation by centrifugation at 5,000 rpm for the extraction of PHAs and subsequent characterization.

Fermentation was subsequently scaled up to a 1-l fermentor (Bioflo 410, New Brunswick Scientific, New Brunswick, NJ) to increase PHA yield. The fermentation experiment was initiated with 40% (v/v) detoxified wood hydrolysate and subsequent addition of 100% detoxified wood hydrolysate. The pH was maintained at 7 and the aeration rate was one volume air flow per unit of liquid volume per minute. Dissolved oxygen was also monitored as a parameter for cell growth and cellular activity. Nitrogen was added in the form of $(NH_4)_2SO_4$; subsequently, more undiluted wood hydrolysate was added to enhance the accumulation of intracellular PHA.

Detoxification of wood hydrolysate

Several wood hydrolysate detoxification methods were employed and compared in this study. Overliming was performed as described by Ranatunga et al. [33], whereupon calcium hydroxide was added to adjust the pH of wood hydrolysate to 10 followed by heating to 50°C for 30 min; after filtration through Whatman #1 filter paper, the pH was adjusted back to 7 using 98% sulfuric acid. All precipitates were removed by filtration and samples were stored at 4°C for further analyses.

Activated charcoal (Mallinckrodt Baker Inc., Phillipsburg, NJ), anion exchange resin (AG® 1-X2, chloride form, Bio-Rad Laboratories, Inc., Hercules, CA) and cation exchange resin (Bio-Rex® 70, sodium form, Bio-Rad Laboratories, Inc., Hercules, CA) were mixed with wood hydrolysate in the ratio of 1:20 (w/v) and stirred for 1 h at 60°C. After filtration and neutralization, all samples were stored at 4°C prior to fermentation. Low-temperature sterilization was performed by autoclaving wood hydrolysate at 85°C for 45 min at 15 psi.

Analytical methods

All analyses for carbohydrates and potential inhibitory compounds were performed with a Waters 1525 binary high-performance liquid chromatograph and 717+ autosampler (Waters Co., Milford, MA). Xylose and other sugar components were detected using a refractive index (RI) detector (Waters Co., Milford, MA) and a carbohydrate analysis column (300 mm × 3.9 mm) with a covalently bonded amino packing from Waters Co. (Milford, MA). Acetonitrile and water in a ratio of 80:20 (v/v) were employed as the mobile phase at a flow rate of 1 ml per min and a sample volume of 20 µl was used for all injections. Acetate and levulinic acid were determined using a model 2996 Photodiode Array Detector (Waters Co., Milford, MA) at 210 nm and a Jordi Gel DVB organic acid column (250 mm × 10 mm, Jordi Labs LLC, Bellingham, MA). The mobile phase consisted of 0.2 M of phosphoric acid, methanol, and acetonitrile in a ratio of 90:5:5 (v/v) at a flow rate of 1 ml per min and a sample volume of 20 µl. Furfural, HMF and vanillin were also measured by HPLC with a C18 column (250 mm × 4.6 mm, Grace Davison Discovery Sciences, Deerfield, IL) at 280 nm, using 0.3% acetic acid:methanol in a ratio of 7:3 (v/v) as the mobile phase at a flow rate of 0.5 ml per min.

A spectrophotometer (Cary 300 Bio, Varian, Inc., Palo Alto, CA) was used to estimate growth of *B. cepacia* on xylose by measuring the optical density at 540 nm.

Total phenolics were measured as described by Li et al. [17]. Briefly, the Folin–Ciocalteu reagent (Sigma-Aldrich Co., St. Louis, MO) was diluted tenfold to react with diluted sample extracts and 75 g/l Na_2CO_3 in the ratio of 5:1:4 (v/v). The mixture was then incubated at room temperature for 2 h before measuring the absorbance at 760 nm. Gallic acid (Sigma-Aldrich Co., St. Louis, MO) was used to construct a standard curve and total phenolics were expressed as grams gallic acid equivalents (GAE) per liter wood hydrolysate.

PHA extraction and content analysis

Wet biomass was obtained by centrifugation of fermentation broth at 5,000 rpm for 10 min followed by resuspension in distilled water and a second centrifugation. Wet biomass was then frozen and lyophilized for 12 h. Dry biomass was mixed with chloroform in a ratio of 1:10 (w/v) and stirred by shaking at 30°C and 100 rpm for 12 h. The extracted biomass was separated by filtration through Whatman #1 filter paper and PHA was then precipitated from the clarified solution with the addition of five volumes of methanol. PHA composition was analyzed by an AOC-20i gas chromatography system (Shimadzu Co., Kyoto, Japan), according to Nomura et al. [28].

Molecular mass determination

Molecular mass of PHAs was determined by gel permeation chromatography (lc-20ad Liquid Chromatograph with SIL-20A auto-sampler and RID-10A refractive index detector, Shimadzu Co., Kyoto, Japan) at 40°C. Chloroform was employed as the mobile phase at a rate of 0.8 ml per min. Polystyrene with low polydispersity was used to construct a standard curve with a range from 682 to 1,670,000 Da [38].

Thermal analysis

Decomposition temperature (T_{decomp}) of the PHA samples was measured by thermogravimetric analysis (TGA 2950, TA Instruments, New Castle, DE) and melting temperature (T_m) and glass transition temperature (T_g) were measured by differential scanning calorimetry (DSC 2920, TA Instruments, New Castle, DE). Ten milligrams of PHA was weighed and heated to 200°C at a ramp rate of 10°C per min and then cooled to −50°C at a rate of 5°C per min. Three cycles were performed whereupon all parameters were analyzed during the second cycle by a Universal Analysis 2000 program [11].

Nuclear magnetic resonance (NMR)

One milligram of solvent-cast film of PHA was dissolved into 1 ml of deuterated chloroform for NMR analysis (Bruker BioSpin AVANCE 600, Bruker Co., Billerica, MA). ^{1}H and ^{13}C NMR spectra were obtained at 300 MHz and data were collected and analyzed using a X-Win-NMR 3.1 program (Bruker Co., Billerica, MA).

Results and discussion

Characterization of hemicellulosic hydrolysate

Hemicellulosic wood hydrolysate was analyzed by HPLC and GC-MS to identify major carbon sources and inhibitory compounds. The chromatographic profile of membrane-treated wood hydrolysate obtained by GC-MS is shown in Fig. 1a. Most of the pertinent peaks eluted within a retention time of 20–30 min (Fig. 1b). Based on standards, the most abundant peaks in the chromatogram were sugars, including xylose, rhamnose, mannose, glucose, and some minor sugar derivatives. Among the sugars, xylose was the most abundant monosaccharide component, and accounted for more than 85% of the total sugar content in the hemicellulosic hydrolysate, followed by mannose, rhamnose, and glucose. These results are consistent with data from HPLC (data not shown) and NMR analyses for sugar contents [9].

Based on HPLC analyses, most of the furfural, HMF, and acetic acid were removed by the two-stage membrane filtration [1]. However, direct fermentation of hydrolysate after filtration still exhibited strong inhibition of cell growth compared to using commercial xylose as the sole carbon source (data not shown). It was hypothesized that the presence of other inhibitors such as lignin derivatives could reduce the fermentability of wood hydrolysate for PHA production. The content of total phenolic compounds was determined to be 0.60 g/l GAE, which represents 91% of the total phenolics in the original wood hydrolysate before membrane filtration (Table 1). It is conceivable that most of the molecular masses of typical lignin derivatives (vanillin, vanillic acid, syringaldehyde, and syringic acid) are between 130 and 200 Da, and therefore would not be efficiently removed by membrane filtration. Further analysis of the GC chromatogram based on comparison with authentic standards also indicated the presence of vanillin, cinnamic acid, syringaldehyde, vanillic acid, syringic acid, and *p*-coumaric acid (Fig. 1b).

The presence of phenolic compounds in wood hydrolysate will vary based on the method of treatment and the ratio of the *p*-hydroxyphenyl, guaiacyl, and syringyl lignins contained in the biomass feedstock [13]. Sugar maple contains syringyl lignin as the most abundant lignin component followed by guaiacyl lignin, which is hydrolyzed to form vanillin and vanillic acid [10]. Similar results to Jőnsson et al. [10] were obtained in this study; syringic acid and syringaldehyde represented relatively higher peaks compared to vanillic acid and vanillin.

Besides sugars and phenolic compounds, peaks of furan derivatives were identified in the chromatogram, indicating the presence of furfural and 2-furanyl methanol. Acetic acid was also detected in low amounts by both GC-MS and HPLC.

Based on the library of the analytical program, the remainder of the peaks shown in Fig. 1 are short-chain organic acids with retention times less than 20 min, sugar acids with retention times close to sugars, dimers or oligomers of the derivatization reagent (BSTFA), long-chain unsaturated fatty acids, and steroid derivatives with retention times from 33 to 60 min.

Inhibitory effects of acetate, HMF, furfural, vanillin, and levulinic acid

In order to study the inhibitory effects on cell growth and PHA production, a 48-h fermentation using 3% xylose as the sole carbon source was employed with the addition of each typical inhibitor at certain concentrations. Among them, acetic acid and levulinic acid were proved to affect cell growth by dissociation in the cytosol thereby decreasing intracellular pH, which further leads to intracellular anion accumulation and decreased cellular ATP levels [34]. Furans (especially furfural and HMF) have been reported to affect enzymatic activities and inactivate cell replication, both of which lead to a longer lag-phase for microorganisms [30]. Vanillin was chosen due to its higher water solubility compared to other abundant phenolic compounds, which could lead to the loss of cellular integrity [8]. The concentrations used for each inhibitory compound were established based on the detectable concentrations observed during the preparation of wood hydrolysate.

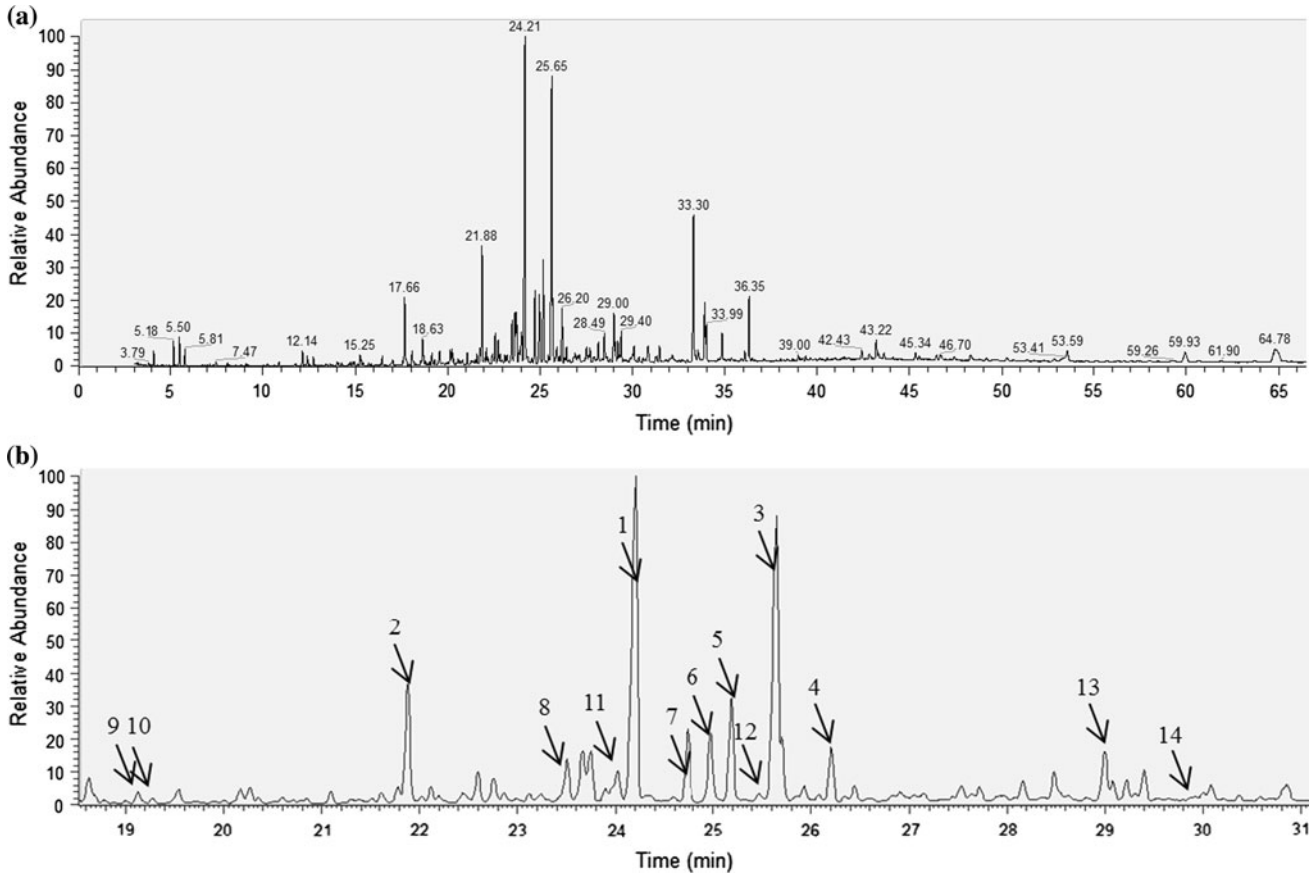

Fig. 1 Chromatogram of membrane-treated hemicellulosic wood hydrolysate (**a** entire chromatogram of wood hydrolysate; **b** expanded view of the region from RT 18.5 to 31.2 min). Sugars and sugar derivatives: xylose (*1*), rhamnose (*2*), mannose (*3*), glucose (*4*), other pentoses or pentose derivatives (*5–8*). Aromatic compounds: vanillin (*9*), cinnamic acid (*10*), syringaldehyde (*11*), vanillic acid (*12*), syringic acid (*13*) and *p*-coumaric acid (*14*). Other potential inhibitors: furfural (RT = 10.87), 2-furanyl methanol (RT = 4.10) and acetic acid (RT = 5.50)

The profiles of cell growth are shown in Fig. 2 and indicate that strong inhibition occurred with 1 g/l furfural, 7 g/l levulinic acid, 1 M acetic acid, and 2 g/l vanillin. Only early inhibition was observed with 0.5 g/l furfural, 5 g/l levulinic acid and 1 g/l vanillin. Additionally, compared to the control, increased cell density after 48 h was observed with low concentrations of levulinic acid (1–5 g/l), acetate (0.1 and 0.5 M) and vanillin (0.1–1 g/l) since these inhibitors, when provided at low concentrations, could be utilized as alternative carbon sources by *B. cepacia*.

Although a major furan derivative present in wood hydrolysate, HMF did not exhibit significant inhibition on either microbial growth or PHA production based on its concentration in wood hydrolysate (Fig. 2; Table 2). However, furfural should be given high priority in any pretreatment process since only 1 g/l completely inhibited microbial growth of *B. cepacia*. Furthermore, synergistic effects among major phenolic compounds, even among phenolics and other residual inhibitors after membrane filtration, are believed to occur in wood hydrolysates since 1 g/l vanillin showed only early inhibition and 2 g/l vanillin significantly inhibited cell growth. However, direct utilization of undiluted wood hydrolysate (containing only 0.96 g/l total phenolics and low amounts of other minor inhibitors after autoclaving) showed complete inhibition during the fermentation. It is conceivable that the presence of other moieties within the hydrolysate exacerbate the effects of vanillin alone.

PHA contents were measured for samples having sufficient biomass after harvest. Except for samples containing levulinic acid, which showed increased PHV content (Table 2) with increasing initial levulinic acid concentrations, the remainder of PHA samples was composed of only PHB. Compared to the control, only 1 g/l vanillin resulted in significantly lower PHA contents. The addition of certain concentrations of levulinic acid and acetate could enhance PHA production by providing alternative carbon sources besides xylose. The remainder of samples showed no significant difference from the control regarding PHA synthesis.

Burkholderia strains have been widely recognized as capable of utilizing many toxic compounds, including

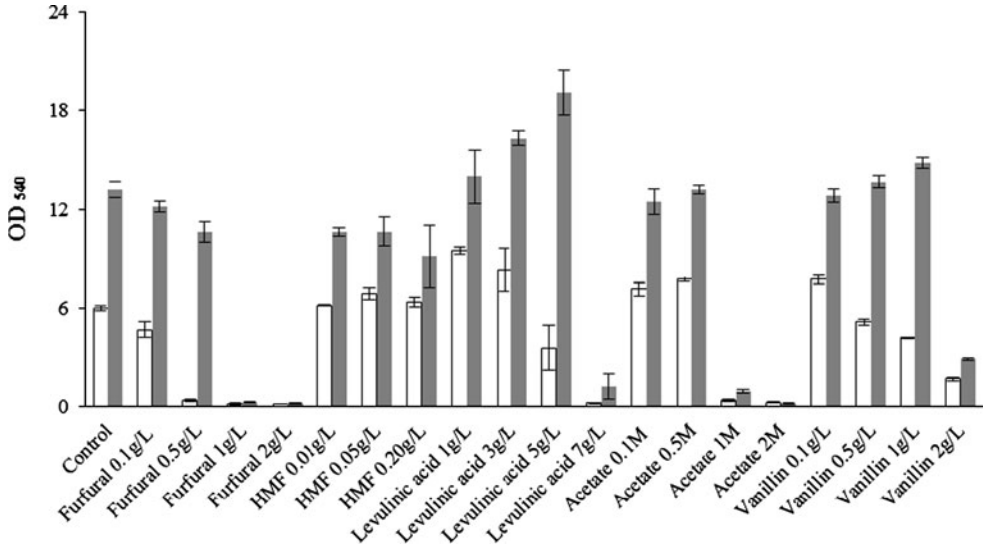

Fig. 2 Growth of *B. cepacia* on 3% xylose in the presence of selected inhibitors (*open bars* represent OD_{540} after 24 h while *closed bars* represent OD_{540} after 48 h)

Table 2 The effects of inhibitory compounds on PHA accumulation and PHA composition by *B. cepacia* grown on 3% xylose

	Control	Furfural 0.1 g/l	Furfural 0.5 g/l	HMF 0.01 g/l	HMF 0.05 g/l	HMF 0.20 g/l	Levulinic acid 1 g/l
PHA content (% of DCW)[a]	42.3 ± 4.0	33.6 ± 0.5	35.8 ± 0.2	45.2 ± 5.6	52.9 ± 4.9	44.1 ± 6.4	54.4 ± 15.6
PHA composition (PHB:PHV)	100:0	100:0	100:0	100:0	100:0	100:0	97.6:2.4
	Levulinic acid 3 g/l	Levulinic acid 5 g/l	Acetate 0.1 M	Acetate 0.5 M	Vanillin 0.1 g/l	Vanillin 0.5 g/l	Vanillin 1 g/l
PHA content (% of DCW)	58.4 ± 5.6	52.4 ± 6.9	46.0 ± 7.7	51.7 ± 2.8	38.7 ± 4.3	38.4 ± 8.8	29.0 ± 3.8
PHA composition (PHB:PHV)	96.9:3.1	93.3:6.7	100:0	100:0	100:0	100:0	100:0

[a] PHA contents were not reported for those cultures that exhibited less than 15 mg biomass after 48 h

polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), as carbon sources [4, 7, 12]. Furthermore, *B. cepacia* has also been shown to grow on aromatic compounds such as lignin derivatives and toluene [20, 23, 36]. In this study, the concentrations of vanillin, furfural, and HMF were monitored by HPLC during the fermentation. The results indicate a gradual catabolism of these inhibitors at low concentrations. The chromatograms showed that vanillin, HMF, and furfural were eventually degraded with the accumulation of biomass until the characteristic peaks for these compounds were completely eliminated (data not shown). A 110-kDa vanillin dehydrogenase has already been purified from *Burkholderia cepacia* TM1, which demonstrated growth on vanillin and vanillic acid [22]. Additionally, genes encoding toluene *ortho*-monooxygenase, hydroxyquinol 1,2-dioxygenase and maleylacetate reductase were identified from the genome of *B. cepacia* for the cleavage and degradation of various aromatic rings [6, 26]. Although a furfural and HMF degradation pathway has not been identified in *B. cepacia* to date, a homology search based on the furfural and HMF catabolic gene cluster of *Cupriavidus basilensis* HMF14 predicted the potential existence of a microbial degradation pathway for furfural and HMF in *Burkholderia* strains [14].

The melting temperature (T_m), glass transition temperature (T_g), decomposition temperature (T_{decomp}), and molecular mass (M_N and M_W) of the extracted PHA polymers produced in the presence of different inhibitors were also determined. No significant differences were observed for T_g (from 3.1 to 7.3°C) and T_m (from 173.0 to 176.2°C) except for a sample obtained while in the presence of 7 g/l levulinic acid, which exhibited a T_g of 9.1°C and T_m of 165.5°C due to the incorporation of 6.7% (w/w) PHV into PHB. T_{decomp} varied from 194.8 to 230.0°C. Samples with higher molecular mass tended to have lower T_{decomp} in this study. This result, combined with the observation that PHA contents in the presence of most concentrations of inhibitors showed no significant difference from that of the control, indicated that inhibitors did not affect PHA synthesis, although reduced cell growth was observed in the presence of high concentrations of inhibitors.

Detoxification of wood hydrolysate

In order to increase the fermentability of wood hydrolysate, several pretreatment methods were evaluated for detoxification purposes. Theoretically, overliming is effective due to precipitation or chemical destabilization of inhibitors [33], activated charcoal could improve the fermentability of hydrolysate by absorbing phenolic compounds [25], and anion and cation exchange resins will remove potentially toxic anions and cations by exchanging with chloride and sodium ions, respectively.

In this study, because no furfural, and only relatively low concentrations of HMF and acetic acid were detected after membrane filtration, the concentration of total phenolics was measured as an indicator of the efficiency of detoxification.

As shown in Table 3, overliming exhibited the greatest removal of phenolic compounds while other treatments removed no more than 10% of total phenolics from the membrane-treated wood hydrolysate. Although overliming was not regarded as efficient in reducing total phenolics of wood hydrolysate [33], this method has been reported as being capable of removing syringic acid, which is one of the most abundant phenolics in the hemicellulosic hydrolysate [21].

Compared to untreated wood hydrolysate, increased concentrations of total phenolics and decreased pH were observed after sterilization for most samples. However, for those treated with low-temperature sterilization (85°C), only a slight increase of total phenolics (less than 8% compared to 33.7% from normal sterilization groups) and a decrease in pH were observed except for samples detoxified by overliming, which exhibited decreased total phenolics. These results indicate that low-temperature sterilization, when compared to normal autoclaving, prevented the release of additional phenolic compounds and other acidic inhibitors from soluble lignin [15]. The combination of overliming and low-temperature sterilization resulted in a 65% removal of total phenolics from the original membrane-treated wood hydrolysate. Other benefits of this approach include lower energy cost and ease of operation as $Ca(OH)_2$ could also be utilized to neutralize wood hydrolysate, which has an initial pH lower than 4. Furthermore, in a related study [21], overliming also removed a significant amount of furfural and HMF from a hemicellulosic hydrolysate of sugar cane bagasse.

Sugar loss, from 0.8 to 30.0%, occurred after detoxification and sterilization. Among all treatments, activated charcoal led to the highest sugar loss, especially after autoclaving. Overliming of wood hydrolysate showed the best sugar recovery after either normal autoclaving or low-temperature sterilization compared to wood hydrolysate samples treated with other methods. However, it was observed in our study that many factors could affect the sugar loss during the detoxification such as temperature, processing time, and the ratio of the reactant to wood hydrolysate, all of which should be precisely controlled to prevent sugar loss.

In order to further investigate the effects of detoxification methods, two sets of shake-flask experiments were performed for 48 h using 40% of membrane-treated wood hydrolysate as the control and detoxified wood hydrolysate sterilized by both normal autoclaving and low-temperature sterilization. PHA contents were determined after 48 h. However, as precipitates could not be well separated from biomass, xylose consumption was employed in our study to monitor cell growth.

Compared to normal autoclaving, low-temperature sterilized hydrolysate showed better fermentability based on xylose consumption after 48 h (Fig. 3). Specifically, overliming resulted in the best performance (86.4% xylose consumption for autoclaved wood hydrolysate fermentation and 90.3% for low-temperature sterilization). Great improvement in fermentability was also observed with the control group under low-temperature sterilization (52.2 vs. 89.0%). The use of cation exchange resin was less desirable with autoclaving but a significant difference was observed when using low-temperature sterilization (21.0 vs. 84.9%). By comparison, no significant difference was observed using activated charcoal-processed hydrolysate under two sterilization methods (81.0 and 80.1%, respectively). Compared to the control, the anion exchange resin only improved fermentability after normal autoclaving (61.4% xylose consumption).

Table 3 Comparison of xylose, total phenolics, and pH of wood hydrolysate samples treated by different methods of detoxification and sterilization to the original wood hydrolysate

	MTH[a]	OL[b]	AC[c]	Cation[d]	Anion[e]
Wood hydrolysate before and after detoxification					
Xylose (g/l)	71.9	71.3	64.8	66.6	68.6
Total phenolics (g/l GAE)	0.60	0.26	0.55	0.56	0.59
pH	7.10	7.20	7.10	7.08	6.99
After normal sterilization (121°C for 20 min)					
Xylose (g/l)	70.5	68.6	50.3	65.8	58.9
Total phenolics (g/l GAE)	0.96	0.28	0.83	0.65	0.79
pH	5.53	5.88	5.38	5.46	5.58
After low-temperature sterilization (85°C for 45 min)					
Xylose (g/l)	65.1	69.9	64.8	64.4	67.9
Total phenolics (g/l GAE)	0.69	0.21	0.70	0.60	0.64
pH	6.82	6.32	6.35	6.99	6.38

[a] Membrane-treated hydrolysate (MTH)

[b] MTH processed by overliming

[c] MTH processed by activated charcoal

[d] MTH processed by cation exchange resin

[e] MTH processed by anion exchange resin

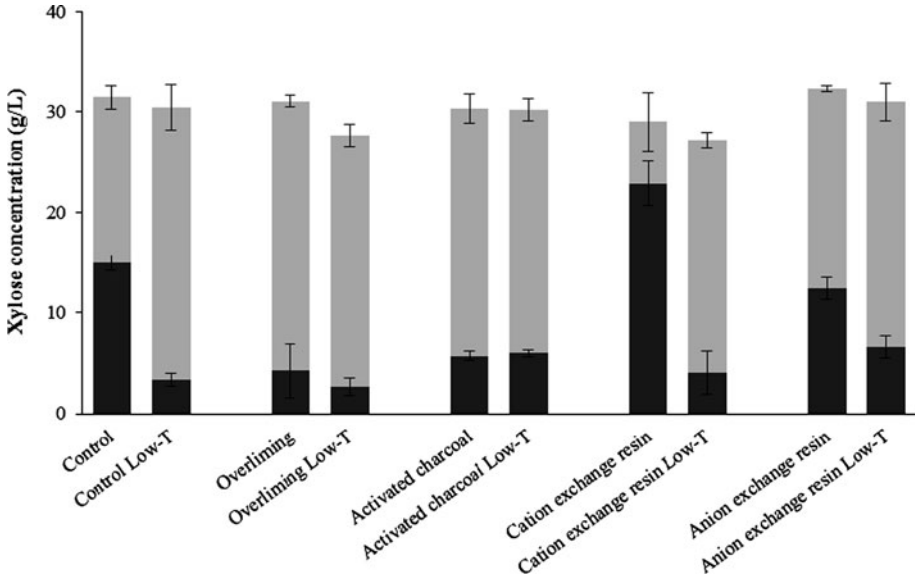

Fig. 3 Xylose consumption during growth of *B. cepacia* on detoxified wood hydrolysate after a 48 h fermentation (*gray bars* represent xylose consumed during fermentation, *black bars* represent residual xylose after 48-h fermentation)

Although after low-temperature sterilization, the control group showed only slight difference on xylose consumption compared to overliming, certain detoxification treatments are still considered as necessary for PHA production from wood hydrolysate since the concentrations of inhibitors vary in a wide range among different hydrolysate samples depending on the production conditions, neutralization, and pretreatment methods. In addition, undiluted wood hydrolysate, which is preferred in the commercial PHA production, contains high amounts of inhibitors and could be very toxic to fermenting microorganisms without any treatment.

PHA production on detoxified wood hydrolysate in a 1-l fermentor

In order to scale-up PHA production to provide sufficient quantities for further studies, a 1-l fermentation was performed using 40% of wood hydrolysate processed by overliming combined with low-temperature sterilization. In addition, 7.5 g/l ammonium sulfate was employed as the nitrogen source. Samples were taken for PHA content and microbial biomass as shown in Fig. 4.

After the lag phase, the highest biomass together with the lowest PHA content (% PHA of dry cell weight) was observed at 32 h fermentation (with the addition of 200 ml undiluted wood hydrolysate at 24 h), where the nitrogen and carbon source were still sufficient to support excess cell growth. With the depletion of both carbon and nitrogen sources, both biomass and PHA production decreased until more carbon source (200 ml undiluted hydrolysate) was added after 48 h to raise the xylose concentration. A high C/N ratio in the fermenter then triggered PHA accumulation during the second stage of fermentation. Highest PHA content was observed at the end of the fermentation (51.4% PHA of DCW, 8.72 g PHA/L broth after 96 h). Similar trends for PHA and biomass accumulation were observed after the addition of more wood hydrolysate. The yields of final PHA and biomass from xylose are 0.19 g PHA/g xylose and 0.37 g biomass/g xylose.

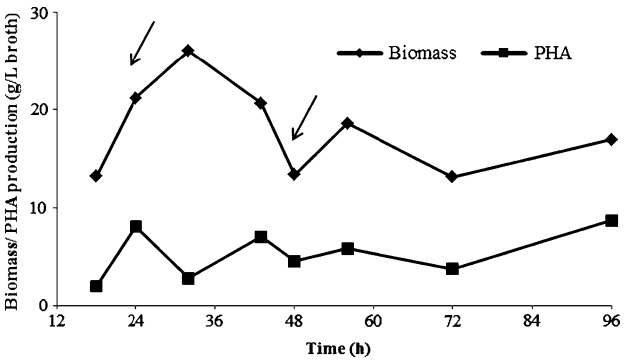

Fig. 4 PHA production and biomass growth of *B. cepacia* on wood hydrolysate detoxified by overliming and low-temperature sterilization in a 1-l fermenter for 96 h; 40% of detoxified wood hydrolysate containing 31.9 g/l xylose plus 7.5 g/l $(NH_4)_2SO_4$ were added initially. Undiluted detoxified wood hydrolysate (200 ml each time) was added after 24 and 48 h, as shown by the *arrows*

This approach allows *B. cepacia* to gradually metabolize the initial inhibitors present in diluted wood hydrolysate and thus achieve a higher concentration of the hydrolysate in the fermentor by adding undiluted hydrolysate when the biomass accumulates. Furthermore, the fermentation could be divided into two stages: one for microbial growth at low C/N and the other for PHA production at preferred high C/N when more carbon source is added. Higher yields of biomass and PHA from wood hydrolysate could be expected after further optimization of feeding strategy.

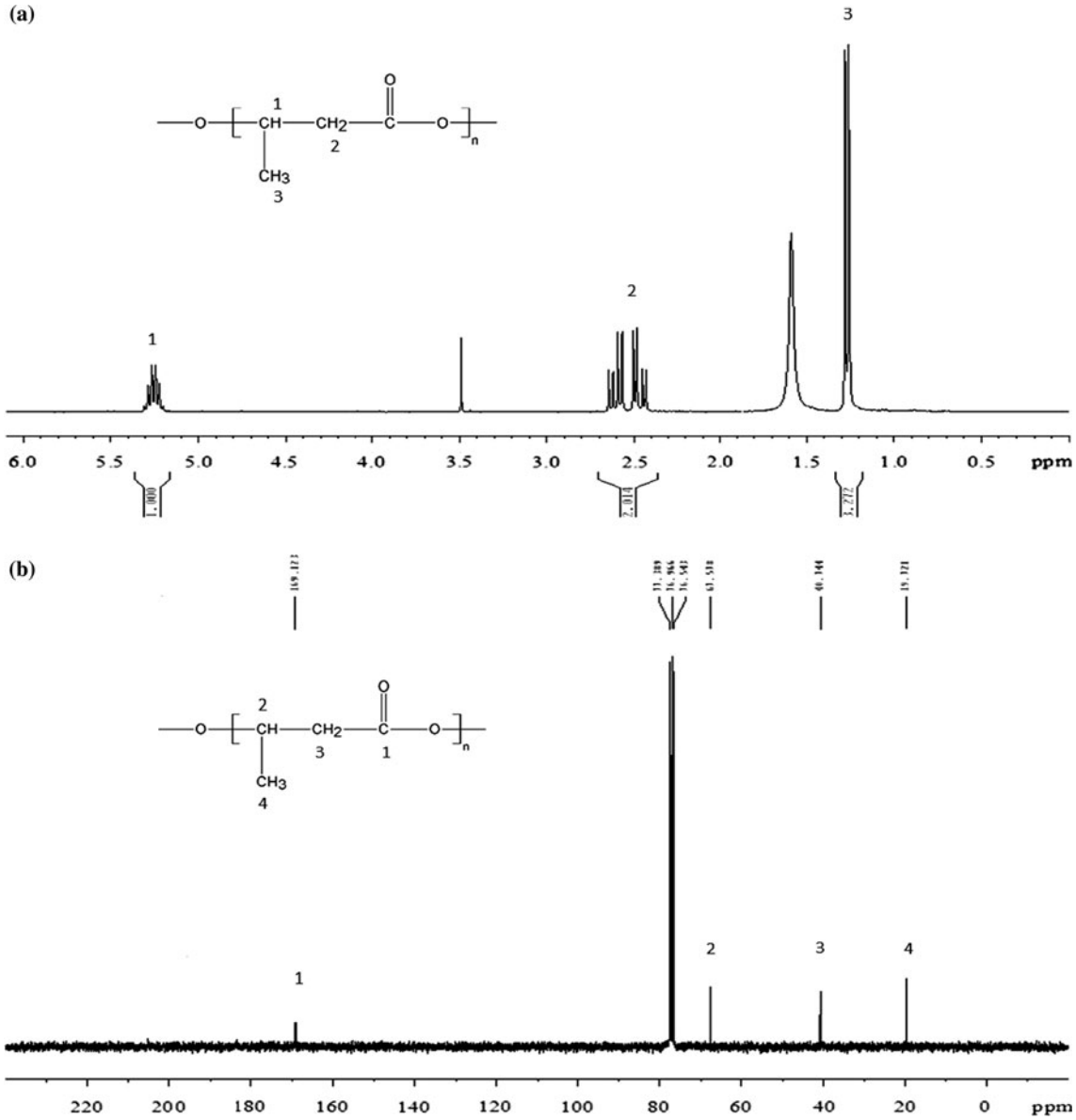

Fig. 5 300 MHz ^{1}H (**a**) and ^{13}C (**b**) NMR spectra obtained from PHA produced on detoxified wood hydrolysate in a 1-l fermentation. The resonance *peaks* (*1*–3 in **a** and *1*–*4* in **b**) were labeled to correspond with the appropriate hydrogen and carbon atoms present in the PHA monomers

Characterization of PHA produced on wood hydrolysate by *B. cepacia*

Although containing a high concentration of xylose, levulinic acid, another major byproduct in the hemicellulosic wood hydrolysate, could function as the precursor for PHV synthesis in *B. cepacia*. In order to further elucidate the composition of PHA produced from wood hydrolysate, PHA samples from the 1-l fermentation were purified and then analyzed by ^{1}H and ^{13}C NMR spectroscopy, as shown in Fig. 5. The ^{1}H NMR spectrum showed typical chemical shifts of hydrogens of methyl, methylene, and methine groups of PHB at 1.25, 2.55, and 5.25 ppm in a ratio of 3:2:1, respectively (Fig. 5a). The resonances of corresponding carbons presented chemical shifts at 19.7 ppm for the methyl carbon, 40.7 ppm for the methylene carbon, 67.6 ppm for the methine carbon, and 169.2 ppm for the carbonyl carbon (Fig. 5b). Except for peaks resulting from solvent and water, no other characteristic peaks were identified. These results indicate that only PHB was produced by fermentation of wood hydrolysate by *B. cepacia* because the concentration of levulinic acid contained in wood hydrolysate was probably insufficient for PHV production and was preferably consumed as an energy source during the fermentation.

Molecular mass, T_g, T_m, and T_{decomp} of the PHB samples are listed in Table 4 with the comparison of PHB produced

Table 4 Thermal properties and molecular weight distribution of PHA produced on different carbon sources

	T_m (°C)	T_g (°C)	T_{decomp} (°C)	M_W (kDa)	M_N (kDa)	M_W/M_N
PHA from detoxified wood hydrolysate	174.4	7.3	268.6	1,014.8	450.8	2.25
PHA from 2.2% xylose as sole carbon source[a]	177.0	4.0	273.4 (0.8% HV)	N.A.	469.0	N.A.
PHA from 3% glycerol as sole carbon source[b]	181.9	1.6	281.5	173–304	87–162	N.A.

N.A. not available

[a] From Keenan et al. [11]

[b] From Zhu et al. [38]

on other carbon sources by *B. cepacia* [11, 38]. PHB from just xylose and wood hydrolysate showed very similar molecular mass while PHB from glycerol exhibited the lowest molecular mass due to the shorter polymer chain resulting from the early termination of polymer elongation by glycerol [38]. T_m and T_{decomp} of all listed PHB ranged from 174.4 to 181.9°C and 268.6 to 281.5°C, respectively. The T_g measured from PHB produced from wood hydrolysate was relatively higher compared to PHB from glycerol.

Although the brittleness and thermal properties of PHB could limit its applications, biosynthesis of the copolymer (PHB-co-PHV) could overcome these drawbacks by using levulinic acid as a co-substrate [11]. Based on this study and previous research [11], incorporation of PHV into PHB could lead to changes in thermal properties such as decreased T_m, which would benefit the industrial processing of bioplastics [35]. Besides thermal properties, incorporation of PHV monomers into PHB (PHB-co-20%PHV) could improve the elongation to break from 5% of PHB to 50% and decrease the crystallinity from 70 to 56% [19].

Conclusions

This study investigated the potential of producing PHB from detoxified hemicellulosic wood hydrolysate by *B. cepacia* ATCC 17759. Vanillin, vanillic acid, syringaldehyde, and syringic acid were determined as major inhibitors in the membrane-treated wood hydrolysate by GC-MS. Several detoxification methods were evaluated and overliming combined with low-temperature sterilization demonstrated the highest removal of total phenolics (65%). PHB was produced by a 1-l fermentation after 96 h with the final production of 8.72 g/l broth and 51.4% of DCW. These results indicated that hemicellulosic wood hydrolysate could be utilized as a suitable and inexpensive feedstock for PHB production by *B. cepacia*. However, care must be taken to eliminate potential inhibitors from fermenting microorganisms.

Further characterization of the PHB demonstrated a M_W of 1,014.8 kDa, M_N of 450.8 kDa. T_g, T_m and T_{decomp} were determined to be 7.3, 174.4, and 268.6°C, respectively.

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