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Detoxification of Corn Stover and Corn Starch Pyrolysis Liquors by *Pseudomonas putida* and *Streptomyces setonii* Suspended Cells and Plastic Compost Support Biofilms

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Plant biomass can be liquefied into fermentable sugars (levoglucosan then to glucose) for the production of ethanol, lactic acid, enzymes, and more by a process called pyrolysis. During the process microbial inhibitors are also generated. *Pseudomonas putida* (ATCC 17484) and *Streptomyces setonii* 75Vi2 (ATCC 39116) were employed to degrade microbial inhibitors in diluted corn stover (Dcs) and diluted corn starch (Dst) pyrolysis liquors. The detoxification process evaluation included measuring total phenols and changes in UV spectra, a GC-MS analysis, and a bioassay, which employed *Lactobacillus casei* subsp. *rhamosus* (ATCC 11443) growth as an indicator of detoxification. Suspended-cell cultures illustrated limited detoxification ability of Dcs and Dst. *P. putida* and *S. setonii* plastic compost support (PCS) biofilm continuous-stirred-tank-reactor pure cultures detoxified 10 and 25% (v/v) Dcs and Dst, whereas PCS biofilm mixed culture also partially detoxified 50% (v/v) Dcs and Dst in repeated batch culture. Therefore, PCS biofilm mixed culture is the process of choice to detoxify diluted pyrolysis liquors.

KEYWORDS: Corn stover; corn starch; pyrolysis liquors

INTRODUCTION

The pyrolysis process can liquefy agricultural residues into an alternative source of organic chemicals (*1*). It becomes the simplest method for biomass saccharification (*2*). Agricultural crop residues consist of lignocelluloses, which are mainly cellulose, hemicellulose, and lignin. When lignocellulosic material is rapidly heated in the absence of oxygen (a process known as pyrolysis) followed by cooling and condensing, a dark brown mobile liquid with a wide range of oxygenated organic chemical compounds is produced. The liquid can be used in many static fuel applications for power generation and heating; it can be upgraded to conventional transport fuels; and it can be used to drive or manufacture a wide range of chemical specialties and intermediates (*1*, *3*). When the pyrolysis process is carried out under controlled conditions, it is called fast pyrolysis and it produces a high yield of anhydrosugar, levoglucosan (1,6-anhydro-β-D-glucopyranose) (*3*, *4*).

Levoglucosan contains an intermolecular glucosidic bond. It can serve directly or indirectly as a feedstock for the fermentation industries to produce fuels, chemicals (*5*−*7*), a precursor for the synthesis of rare sugars, and herbicidal derivatives or as substrate for microbial fermentations (*8*−*10*). Levoglucosan can be metabolized directly by eukaryotic and prokaryotic microorganisms (*2*) or indirectly through hydrolysis with mild acid to produce glucose (*10*). However, during the fast pyrolysis of biomass, numerous compounds, such as phenols, benzene, furan, furfuryl derivatives, and many other oxygenated compounds, are generated (*3*, *11*). These compounds are inhibitors or toxic to microbial growth (*12*). Therefore, postpyrolysis cleanup via activated carbon and solvent extraction are essentially required (*10*, *12*, *13*). However, these physical/chemical methods result in waste generation with negative environmental and economic impacts, including some loss of levoglucosan. Thus, biological treatments to remove these microbial inhibitors are the method of choice.

Bacteria can degrade many toxic compounds via induced enzymes (*14*). *Pseudomonas* strains were found to be capable of cometabolic degradation of aromatic compounds with at least one of the compounds being used as a carbon source (*15*). However, the degradation of a specific compound in a mixture is affected by other substituents of the mixture (*4*). Gram-negative bacteria are better equipped to cope with solvent tolerance (*16*) because it has an outer membrane compared to the single cytoplasmic membrane in Gram-positive bacteria (*17*). Moreover, the organic-solvent-tolerant bacteria such as *Pseudomonas putida* overcome the toxic and destructive effects of organic solvents by the presence of various adaptive mechanisms (*17*−*19*). On the other hand, most of the organic-solvent-tolerant bacteria, such as *Pseudomonas*, develop biofilms (*20*−*22*).

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The biofilm-associated cells are more resistant to many toxic substances such as antibiotics, chlorine, and detergents (*23*). The reasons for resistance might be due to the decreased bacterial growth rate, cell signaling, decreased diffusion into the biofilm, and formation of biofilm-specific substances such as exopolysaccharide (EPS) (*24*, *25*).

The biofilm is considered to be a natural form of immobilized cells attached by EPS to a variety of surfaces rather than being swept away by the water current (*26*). However, when the biofilm is utilized in fermentation, attention must be paid to the movement between substrate and cell so that it dose not become rate limiting (*27*). At Iowa State University (ISU), we developed a novel biofilm support called plastic composite support (PCS) tubes, which can be customized for the microorganism of choice to produce a PCS biofilm (*26*, *28*, *29*). The PCS tubes have been shown to stimulate biofilm formation and enhance the productivity of ethanol (*30*), lactic acid (*31*), and succinic acid (*32*).

This research aims to demonstrate the ability of suspended-cell and PCS biofilm reactors to biologically detoxify diluted corn stover and diluted corn starch pyrolysis liquors. The detoxification process goal is to remove or reduce microbial inhibitors in the pyrolysis liquors without utilizing the levoglucosan, which can then be used to produce a value-added fermentation product. Two microorganisms, *P. putida* and *Streptomyces setonii*, which are unable to utilize levoglucosan but have the ability to utilize numerous aromatic compounds (*4*, *33*−*37*), were employed as suspended-cell and PCS biofilm pure and mixed-batch cultures to detoxify fast pyrolysis liquors. The mixed-culture PCS biofilm reactor successfully detoxified 25% (v/v) diluted pyrolysis liquors, producing a potential fermentation feed with 3.2 and 7.9% levoglucosan in Dcs and Dst, respectively.

MATERIALS AND METHODS

Microorganisms. *Streptomyces setonii* 75Vi2 (ATCC 39116), *Pseudomonas putida* (ATCC 17484), and *Lactobacillus casei* subsp. *rhamosus* (ATCC 11443) were obtained from American Type Culture Collection (Manassas, VA). *P. putida* was maintained on agar slants containing 1.0 g/L of beef extract, 2.0 g/L of yeast extract, 5.0 g/L of peptone, 5.0 g/L of NaCl, 0.45 g/L of KH_2PO_4, 2.39 g/L of Na_2HPO_4, and 15.0 g/L of agar (Sigma Chemical Co., St. Louis, MO) and stored at 4 °C. The initial pH of the medium was 6.8 (*38*). *S. setonii* was maintained at 4 °C as sporulated culture on agar stock culture slants of yeast extract−malt agar (*39*). *L. casei* was maintained as freeze-dried culture and as working culture by monthly transfers in MRS broth (Difco Laboratories, Detroit, MI) and stored at 4 °C (*40*).

Chemicals. Yeast extract (Ardamine Z) was obtained from Sensient Flavor (Juneau, WI). Glucose (Cerelose) was obtained from International Ingredients (St. Louis, MO). Standards 2-methoxy-5-methylphenol, 2,6-dimethoxyphenol, 1,2,4-trimethoxybenzene, 2(5*H*)-furanone, and 1,6-anhydro-β-D-glucopyranose (levoglucosan) were obtained from Sigma Chemical Co.

Preparation of Pyrolysis Liquors. The pyrolysis process was carried out in a pilot-scale fluidized pyrolyzer at Iowa State University (*5*). The reactor consists of a 15-cm-diameter bubbling fluidized bed operated at 400−600 °C. It processes 2.5−5.0 kg/h of biomass to produce 1.3−2.6 L/h of pyrolysate oil. The heat required for pyrolysis was provided by burning pyrolytic gas or natural gas in an external burner and directing this hot gas through a heat exchange jacket surrounding the pyrolytic reactor. Recirculated pyrolytic gas serves as the fluidization agent for the bubbling bed, thus avoiding the addition of air to the reactor, which would reduce pyrolysate yield. Products of pyrolysis include gas (a flammable mixture consisting of hydrogen, carbon monoxide, carbon dioxide, and some noncondensable hydrocarbons), particulate matter (ash and char), and condensable vapors. Particulate matter is removed by insulated cyclones designed to keep the gas stream at high temperature. The gases and vapors then enter a series of four water-cooled, shell-and-tube heat exchanges designed to condense water and organic vapors, which forms the pyrolysate used in the fermentation trials. In this research, the pyrolysate was prepared from corn stover, which is composed of 50% cellulose with some hemicelluloses and lignin (*1*). or from commercial corn starch available at local grocery stores (*11*). The pyrolysates generated were called corn stover pyrolysis liquor and corn starch pyrolysis liquor.

The pyrolysis liquors are brown, a distinctive odor, and contain significant yields of levoglucosan and monosaccharides. *P. putida* and *S. setonii* were unable to utilize levoglucosan. To prepare both pyrolysis liquors for fermentation, they were vigorously mixed with deionized water at a 1:2 ratio and then stored overnight at 4 °C. Insoluble precipitates (IP) were removed by centrifugation at 10000*g* for 15 min at 10 °C. The supernatants were neutralized with $CaCO_3$ and then centrifuged at 15000*g* for 20 min at 10 °C. The supernatants of diluted corn stover (Dcs) and diluted corn starch (Dst) pyrolysis liquors were filter sterilized by 0.45-μm PTFE membrane Acrodisc filter (Pall Corp., East Hills, NY) to prevent any further changes in chemical composition that might result from heat sterilization.

Detoxification of Pyrolysis Liquor by Suspended-Cell Culture. To determine the ability of *S. setonii* and *P. putida* suspended cells to detoxify high concentrations of the filter-sterilized pyrolysis liquor, Dcs and Dst at 10, 25, and 50% (v/v) (final concentrations, respectively) were added to a medium consisting of 10 g/L of yeast extract (Ardamine Z), 10 g/L of $CaCO_3$, and 100 mL/L trace elements (*39*) at pH 6.5−7. Each flask was inoculated with a fresh culture of 24-h *S. setonii* or 18-h *P. putida* (A_{620} of 0.5) and evaluated in a replicate of three (n = 3) for each concentration of Dcs and Dst. All inoculated flasks were incubated with shaking at 125 rpm for 10 days at 30 °C for *P. putida* and at 37 °C for *S. setonii*. The control flasks were not inoculated.

PCS Biofilm Formation. The biofilm reactors of *P. putida*, *S. setonii*, or mixed culture were developed on PCS tubes attached to the continuous stirred tank reactor (CSTR) agitator shaft. The PCS blend for this research contained 50% (w/w) of polypropylene to give the PCS high mechanical stability and the ability to withstand sterilization temperatures and high pH, 40% (w/w) ground dried soybean hull (Cargill Soy Processing Plant, Iowa Falls, IA) to create a porous surface, 5% (w/w) dried bovine albumin (American Protein Corp., Ames, IA) to protect micronutrients during high-temperature extrusions (*28*), 5% (w/w) yeast extract (Ardamine Z), and mineral salts (0.6 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of $KH_2 2PO_4$, 1 g/L of sodium acetate, 0.03 g/L of $MnSO_4 \cdot H_2O$, and 0.5 g/l of K_2HPO_4) to reduce PCS hydrophobicity (*28*). These dry ingredients were mixed in a separate container prior to being poured into the extruder hopper. The twin-screw corotating Brabender PL2000 extruder (model CTSE-V; C. W. Brabender Instruments, Inc., South Hackensack, NJ) was operated at a rate of 11 rpm, barrel temperatures of 200, 220, and 200 °C, and a die temperature of 167 °C to form a continuous tube. Composite supports with a wall thickness of 3.5 mm and an outer diameter of 10.5 mm were cut into 10-cm lengths and end cut at an angle to allow the media to flow through the inside of tubes. Six PCS tubes were stacked in rows of two parallel tubes and then bound to the CSTR agitator shaft in a grid-like fashion (*31*).

A computer-controlled New Brunswick Bioflo 3000 (Edison, NJ) benchtop fermentor equipped with pH, temperature, agitation, and oxygen-dissolved controls was employed with batch and continuous cultures. The 1.2-L vessel (1-L working volume) was equipped with filtered-sterilized air in and out, alkali, acid, medium addition, and broth removal ports (*31*). To control pH, acid and alkali were added from graduated burets, which were refilled aseptically with 1 N HCl and 1 N NaOH. The broth removal port was connected with two branched lines to withdraw a sample and to draw off all culture media from the vessel. On the side of the vessel a removal port equipped with a liquid break was used to run continuous culture. The reactor was sterilized with water in the autoclave for 1.25 h at 121 °C. After sterilization, medium was used to dilute water at a dilution rate of 0.6 h^{-1} overnight (*31*).

Except for omitting the $CaCO_3$ from the medium, the same culture medium that was used for suspended-cell culture experiments was employed with pH control runs at 6.5. Biofilm culture media for *P.*

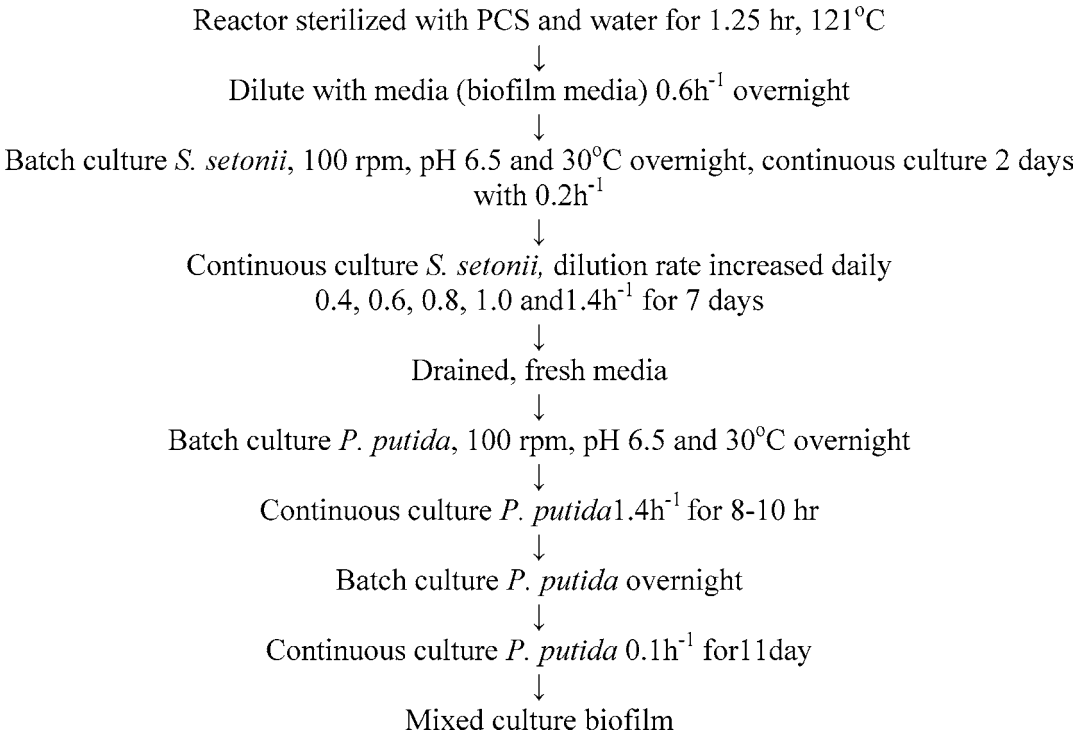

Figure 1. Series of steps, setting, and sterilizations for mixed-culture biofilm production.

Table 1. Bioassay, Ability of *L. casei* To Grow on MRS Medium Amended with Different Concentrations of Zero Time and Bacterial-Treated Filter-Sterilized Diluted Corn Stover Pyrolysis Liquors (Dcs) or Diluted Corn Starch Pyrolysis Liquor (Dst)[a]

			bacterial-treated Dcs						bacterial-treated Dst					
	control[b]		10% (v/v)		25% (v/v)		50% (v/v)		10% (v/v)		25% (v/v)		50% (v/v)	
bacterial treatment	A_{620}	LA[c] (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)
suspended cell culture														
P. putida	NG[d]	ND[d]	2.1	3.2	0.04	1.8	NG	ND	2.2	3.1	NG	ND	NG	ND
S. setonii	NG	ND	1.2	5.3	1.0	3.4	NG	ND	2.5	4.9	NG	ND	NG	ND
biofilm culture														
P. putida	NG	ND	2.1	7.2	1.9	5.1	NG	ND	2.1	6.1	2.2	6.0	0.1	ND
S. setonii	NG	ND	1.6	8.3	2.1	7.2	NG	ND	2.6	6.3	3.4	6.2	NG	NG
mixed culture	NG	ND	2.6	17.7	3.6	16.3	2.9	9.8	3.2	17.9	4.4	13.4	2.2	6.8

[a] The detoxification of Dcs and Dst was carried out by *P. putida* and *S. setonii* suspended cell and mixed culture biofilms. *L. casei* growth on MRS medium alone produces A_{620} of 3.8 and 19 g/L of lactic acid. Each value represents an average of three replicates ($n = 3$). [b] Zero time Dcs or Dst added to MRS medium and inoculated with *L. casei*. [c] Lactic acid production (g/L). [d] NG, no growth; ND, no lactic acid detected.

putida and *S. setonii* were prepared in 90-L batches, sterilized in a B-Braun 100-D fermentor (Allentown, PA) with continuous agitation for 25 min at 121 °C, and adjusted to pH 6.5 with sterile 3 N HCl. The sterilized medium was aseptically transferred into two sterilized 50-L carboys equipped with a carboy-filling port, a medium delivery line with a liquid break, and an air vent capped with a 0.45-μm air filter for storage.

***P. putida* Biofilm.** Biofilm was developed on the PCS via batch and continuous cultures using the previously described media at pH 6.5, constant agitation (100 rpm), and 30 °C. The fermentor was inoculated with 5 mL (A_{620} of 0.5) of an 18-h *P. putidia* culture and then incubated as batch. Continuous culture was started after 24 h with a high dilution rate of 1.4 h^{-1} for 8−10 h. The fermentor was then incubated as batch culture overnight followed by a low dilution rate of 0.1 h^{-1} for 11 days. This unusual sequence of dilution rates was determined empirically to work the best for *P. putida* biofilm development.

***S. setonii* Biofilm.** Biofilm was developed on the PCS via continuous culture using the previously described medium at pH 6.5, constant agitation (100 rpm), and 37 °C. The fermentor was incubated overnight to ensure sterility and inoculated with 5 mL of spore suspension (~1.7 × 10^6 spores/mL) of 24-h *S. setonii* culture. *S. setonii* biofilm was developed via continuous culture by increasing the dilution rates gradually, from 0.2 to 0.4, 0.6, 0.8, 1.0, and 1.4 h^{-1}, for 7 days.

Mixed-Culture Biofilm. Fresh PCS tubes were fixed to the agitator shaft in the reactor, and the same procedures described for single cultures were followed. The *S. setonii* biofilm was allowed to develop first as described previously, and then the reactor was drained. Fresh *P. putida* medium was added to the reactor and inoculated with 5 mL (A_{620} of 0.5) of fresh culture, and then the batch and continuous cultures were performed as described previously. Daily sample was collected and examined by Gram staining. After 18 days of incubation, the mixed-culture biofilm was developed. The series steps to develop the mixed-culture biofilm are summarized in **Figure 1**. Once the biofilm was established, the bioreactor required no further inoculations.

Detoxification of Pyrolysis Liquor by Biofilm Culture. Three concentrations, 10, 25, and 50% (v/v), of filter-sterilized Dcs and Dst were evaluated. The bacterial treatment was run as batch culture with 1 L of working volume including the desired concentration of Dcs or Dst, pH 6.5, constant agitation (100 rpm), and 30 °C for *P. putida*, 37 °C for *S. setonii*, and 30 °C for mixed culture. Samples were recovered on days 0 (control), 4, and 10 for analysis. After 10 days, the reactor was drained, and the bacterial-treated effluents were evaluated. The reactor was refilled with fresh medium and the same concentration of

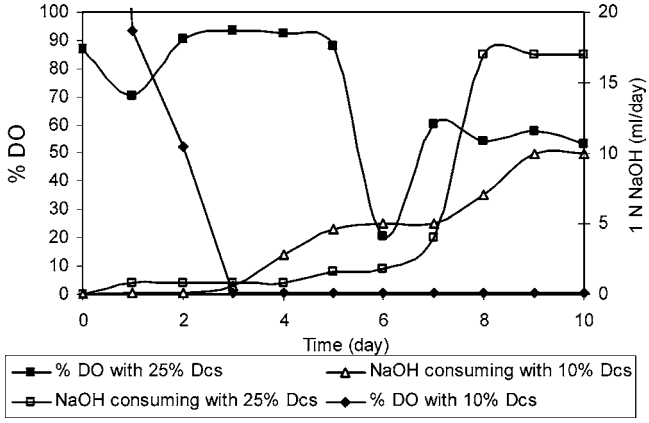

Figure 2. Percentage oxygen uptake and alkali consumption for pH control in bioreactor during the detoxification of 10 and 25% (v/v) Dcs by *P. putida* PCS biofilm. Each value represents an average of three replicates ($n = 3$).

Dcs or Dst for reevaluation. Prior to the addition of a new concentration of Dcs or Dst, the reactor was drained and refilled with fresh media and run as a continuous culture for 2−3 h at a dilution rate of 0.4 h^{-1} to remove any residual spent medium that might be remaining. In mixed-culture biofilm, samples were recovered after each run and examined with Gram stain to ensure the mixed-culture biofilm was maintained (**Figure 1**).

Sample Analysis. The overall reduction of aromatic compounds in Dcs and Dst samples was monitored by ultraviolet (UV) absorption spectrum. The absorption spectrum was determined in 200−300 nm via a Beckman UD 40 from Beckman Instruments, Inc. (Fullerton, CA).

For GC-MS analysis, 25 mL of bacterial-treated and nontreated Dcs and Dst samples was acidified to pH 4.0 with HCl and then extracted three times with ethyl acetate, three times with ethyl acetate/acetone 2:1, and three times with ethyl ether (*41*). The pooled organic phases were dehydrated with anhydrous sodium sulfate, and the solvents were removed via rotoevaporation. The dried extract was resuspended in 25 mL of ethyl acetate and filtered via a syringe filtration unit with 0.45-μm cellulose acetate plus filters (GE Osmonics, Trevose, PA). The analysis was performed by using an Agilent 6890 series GC system with an Agilent autosampler 7683 series injector and equipped with a micromass GCT mass spectrometer and a column of JW DB.5 MS (30 m by 0.25 mm by 0.25 μm; Agilent Technologies, Palo Alto, CA). The sample was injected at a split ratio of 1 to 100. For the quantitative analysis, five compounds in Dcs samples including phenol, 2-methoxy-5-methylphenol, 2,6-dimethoxyphenol, 1,2,4-trimethoxybenzene, and 1,6-anhydro-β-D-glucopyranose (levoglucosan) and two compounds in Dst including 2(5*H*)-furanone and levoglucosan were used as standards to perform quantitative analysis in zero time and bacterial-treated Dcs and Dst samples. The levoglucosan is partially dissolved in ethyl acetate. For levoglucosan GC-MS quantitative analysis, 25 mL of zero time and bacterial-treated samples of Dcs and Dst was dehydrated, and then the dried extract was resuspended in 25 mL of methanol (*42*).

Phenolic compounds were extracted as above for GC-MS analysis and then resuspended in 25 mM phosphate buffer (pH 6.0) instead of ethyl acetate. The phenolic assay is based on the oxidation of phenolate ion, where ferric ions are reduced to the ferrous state, which was detected by the formation of the Prussian blue complex $[Fe_4[Fe(CN)_6]_3]$ with a potassium ferricyanide-containing reagent (*43*). The assay mixture contained 25 mL of deionized water, 250 μL of sample, 3 mL of ferric chloride reagent, and 3 mL of potassium ferricyanide reagent. The sample absorbance was measured at 720 nm. The phenolic contents in the samples were expressed as syringic acid equivalents (10 μg mL^{-1} gives A_{760} of 0.377/mL) (*44*). Total phenolic assay was performed on Dcs samples and not Dst samples, which contained no lignin. Furfurals found in Dst were capable of being oxidized by the reagents of the total phenolic assay, which will reduce the reagents, causing a color change and the appearance of a phenolic (*43*).

The samples collected for the bioassay were centrifuged at 10000*g* for 20 min at 20 °C and then filter sterilized with a 0.45-μm PTFE filter membrane. The bioassay was carried out in 250-mL flasks by mixing 2:1 (v/v) zero time or bacterial-treated Dcs or Dst samples and concentrated MRS medium, keeping the MRS concentration constant. The flasks were inoculated with 0.5 mL (A_{620} of 0.5) of 18-h *L. casei* culture and incubated at 37 °C as a static culture for 24 h. The control was incubated under the same conditions with zero and different concentrations of bacterial-treated Dcs or Dst sampels. The samples were analyzed for cell density by absorbance at 620 nm using a Spectronic 20 and for lactic acid production by using a Hewlett-Packard (San Feranando, CA) high-pressure liquid chromatograph (HPLC) 1100 equipped with a Waters model 2410 refractive index detector, column heater, autosampler, and computer controller. Lactic acid was separated on a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad

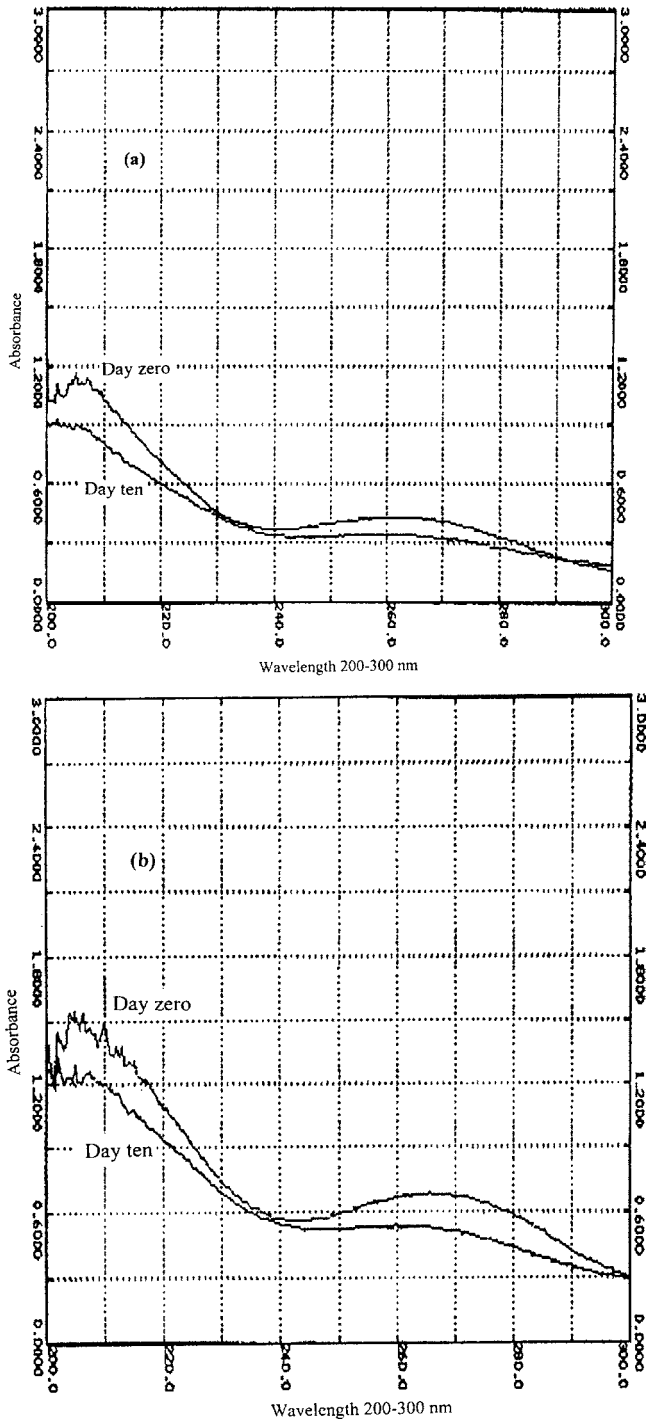

Figure 3. UV absorbance change of 10% (v/v) Dcs amended medium on day 0 and after 10 days of incubation with (**a**) *S. setonii* and (**b**) *P. putida* suspended cells.

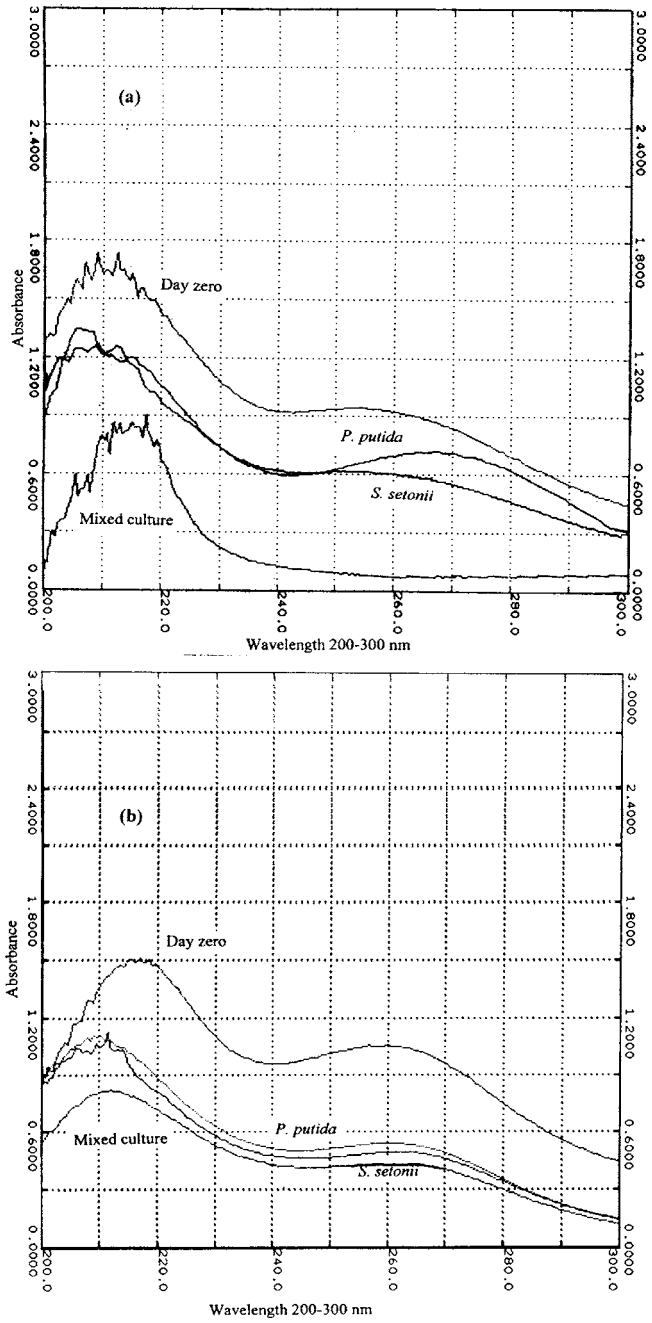

Figure 4. UV absorbance change of 25% (v/v) amended medium (**a**) Dcs and (**b**) Dst on day 0 and after 10 days of incubation with *P. putida*, *S. setonii*, and mixed-culture biofilm.

Chemical Division, Richmond, CA) with 0.12 N sulfuric acid as the mobile phase at a flow rate of 0.8 mL/min with a 20 μL injection volume and a 65 °C column temperature.

RESULTS

Detoxification with Suspended-Cell Culture. *S. setonii* and *P. putida* grew on media containing yeast extract and $CaCO_3$ and different concentrations of Dcs or Dst. With 10% (v/v) Dcs the A_{620} for *P. putida* was 1.9 and that for *S. setonii* 2.3, whereas with 10% (v/v) Dst the A_{620} was 1.1 for *P. putida* and 1.5 for *S. setonii*. On 25% (v/v) Dcs and Dst A_{620} values were 0.3 and 0.1 for *S. setonii*, respectively, whereas *P. putida* did not grow. Neither *S. setonii* nor *P. putida* grew on 50% (v/v) Dcs and Dst. Bioassay results for 10 and 25% (v/v) Dcs or Dst treated

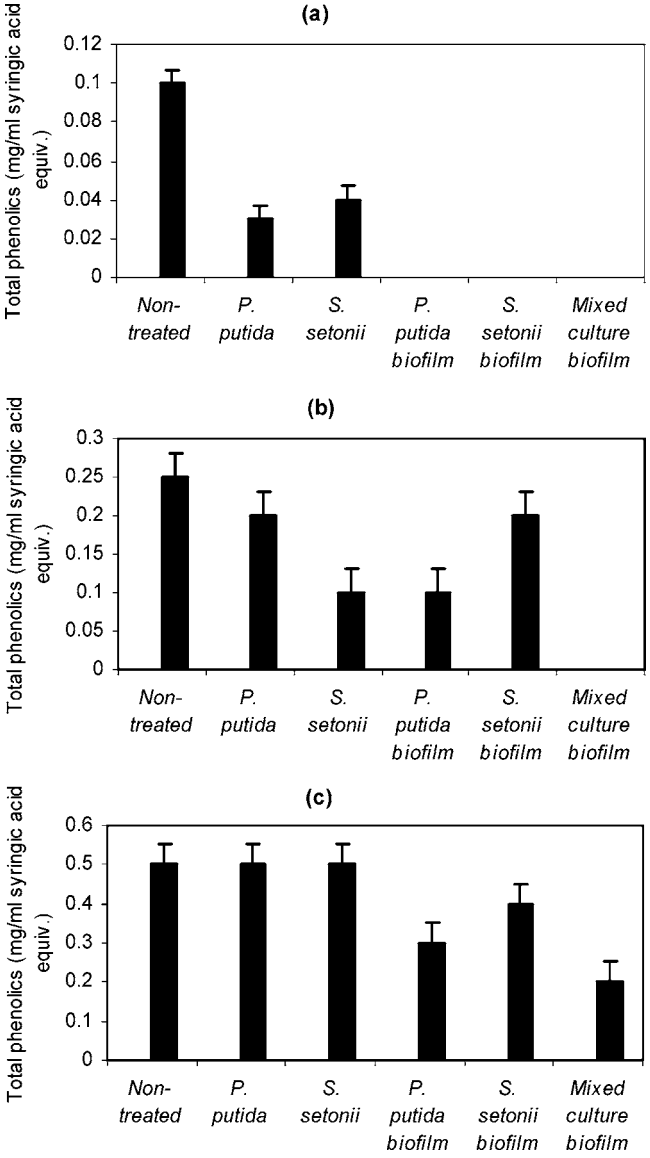

Figure 5. Reduction of total phenolics assay in (**a**) 10% (v/v) Dcs, (**b**) 25% (v/v) Dcs, and (**c**) 50% (v/v) Dcs, after 10 days of incubation. Each value represents an average of three replicates ($n = 3$).

with *P. putida* or *S. setonii* were illustrated by *L. casei* growth and production of lactic acid (**Table 1**).

Biofilm Formation. The optimal pH for *P. putida* was 6.5, whereas *S. setonii* grew well in a pH range from 6.5 to 7.2. The *P. putida* did not grow well at 37 °C, but both *P. putida* and *S. setonii* grew well at 30 °C. Therefore, pH 6.5 and 30 °C were employed for PCS biofilm reactors. *S. setonii* PCS biofilm was defined on the PCS after an overnight batch culture followed by 2 days of continuous culture at a dilution rate of 0.2 h^{-1}. Biofilm increased visibly with increasing dilution rates. The thick biofilm, which covered all of the PCS, was recorded on day 7 with the dilution rate of 1.4 h^{-1} (**Figure 1**). *P. putida* PCS biofilm development required an overnight batch culture, followed by 8−10 h of continuous culture at 1.4 h^{-1}, followed by 24 h of batch culture and then 0.1 h^{-1} of continuous culture for 11 days. Mixed-culture biofilm consisted of *S. setonii* and *P. putida* biofilm. *S. setonii* biofilm was cultured first on PCS followed by *P. putida* biofilm (**Figure 1**). Gram stain of samples collected from the mixed-culture biofilm media illustrated the presence of filamentous Gram-positive and Gram-negative rods,

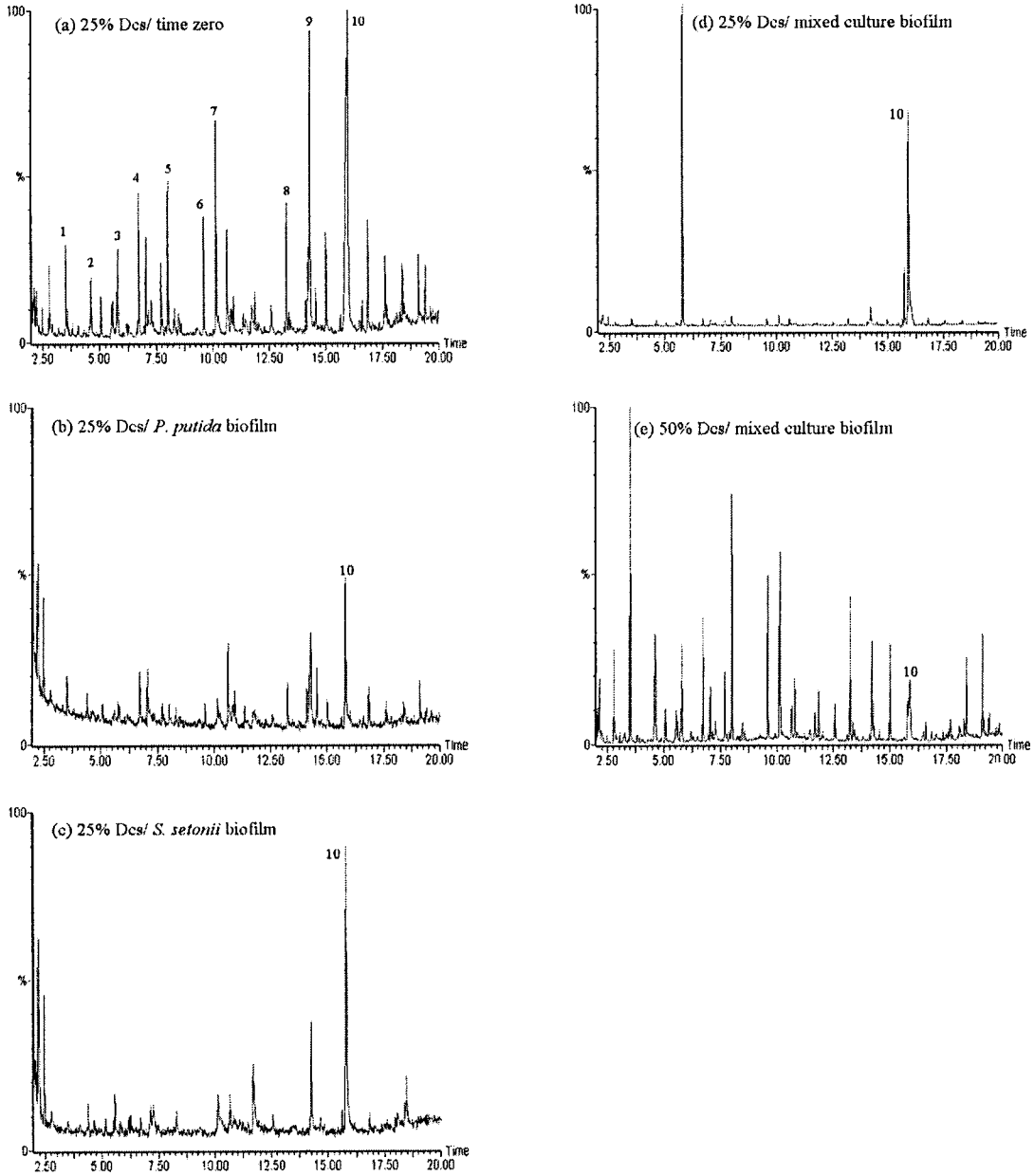

Figure 6. Chromatographic profiles of zero time Dcs (**a**); 25% (v/v) Dcs treated by *P. putida* biofilm (**b**), *S. setonii* biofilm (**c**), and mixed-culture biofilm (**d**); and 50% (v/v) Dcs treated by mixed-culture biofilm (**e**) after 10 days of incubation. Identified peaks: phenol (1), 1–2-furanyl (2), 2-furancarboxaldehyde (3), 2-hydroxy-3-methyl-2-cyclopentene (4), 2-methoxyphenol (5), 4-ethylphenol (6), 2,6-dimethoxyphenol (7), 1,2,4-trimethoxybenzene (8), 2-methoxy-5-methylphenol (9), and 1,6-anhydro-β-D-glucopyranose (10).

for *S. setonii* and *P. putida*, respectively. The mixed biofilm appeared to be thick and viscous.

Detoxification of Pyrolysis Liquor by Biofilm Culture. During the bacterial treatment of 10% (v/v) Dcs and Dst the DO_2 decreased rapidly within the first 2 days and then remained at a low level until the end of the study (**Figure 2**), whereas with the 25% (v/v) Dcs and Dst the DO_2 remained high (>90%) until day 6 and then decreased rapidly with a corresponding consumption of alkali (1 N NaOH) beginning on day 7 (**Figure 2**). Thus, O_2 was not limiting. The bioassay confirmed that the bacterial biofilm cultures were effective in detoxification of 10 and 25% (v/v) Dcs and Dst (**Table 1**). Neither *S. setonii* nor *P. putida* biofilm was able to detoxify 50% (v/v) Dcs or Dst, whereas the mixed-culture biofilm did (**Table 1**).

Changes in UV Absorption Spectra. Reduction of aromatic compounds was monitored over all by the change in UV absorption spectrum of the Dcs and Dst amended media. Samples treated with suspended cells indicated the presence of aromatic compounds, and the change in $A_{200-300}$ spectra for 10% (v/v) Dcs was less significant (**Figure 3**). Samples treated with biofilms such as the detoxified 25% (v/v) Dcs and Dst showed a significant decrease in $A_{200-300}$ spectra, especially with the mixed-culture biofilm (**Figure 4**).

Phenolic Compounds Reduction for Bacterial-Treated Dcs Pyrolysis Liquors. Bacterial reduction of phenolic compounds by suspended-cell culture was less significant than that shown by the biofilm culture (**Figure 5**). The initial total phenolics for 10, 25, and 50% (v/v) Dcs were 0.1, 0.25, and 0.5 mg/mL syringic acid. For the bacterial treatment of 10% Dcs the pure- and mixed-culture biofilms removed all of the detectable phenolics, whereas for the bacterial treatment of 25% Dcs only the mixed-culture biofilm removed all. For the bacterial-treated 50% (v/v) Dcs the suspended-cell cultures removed none of the detectable phenolics, whereas the pure-culture biofilms

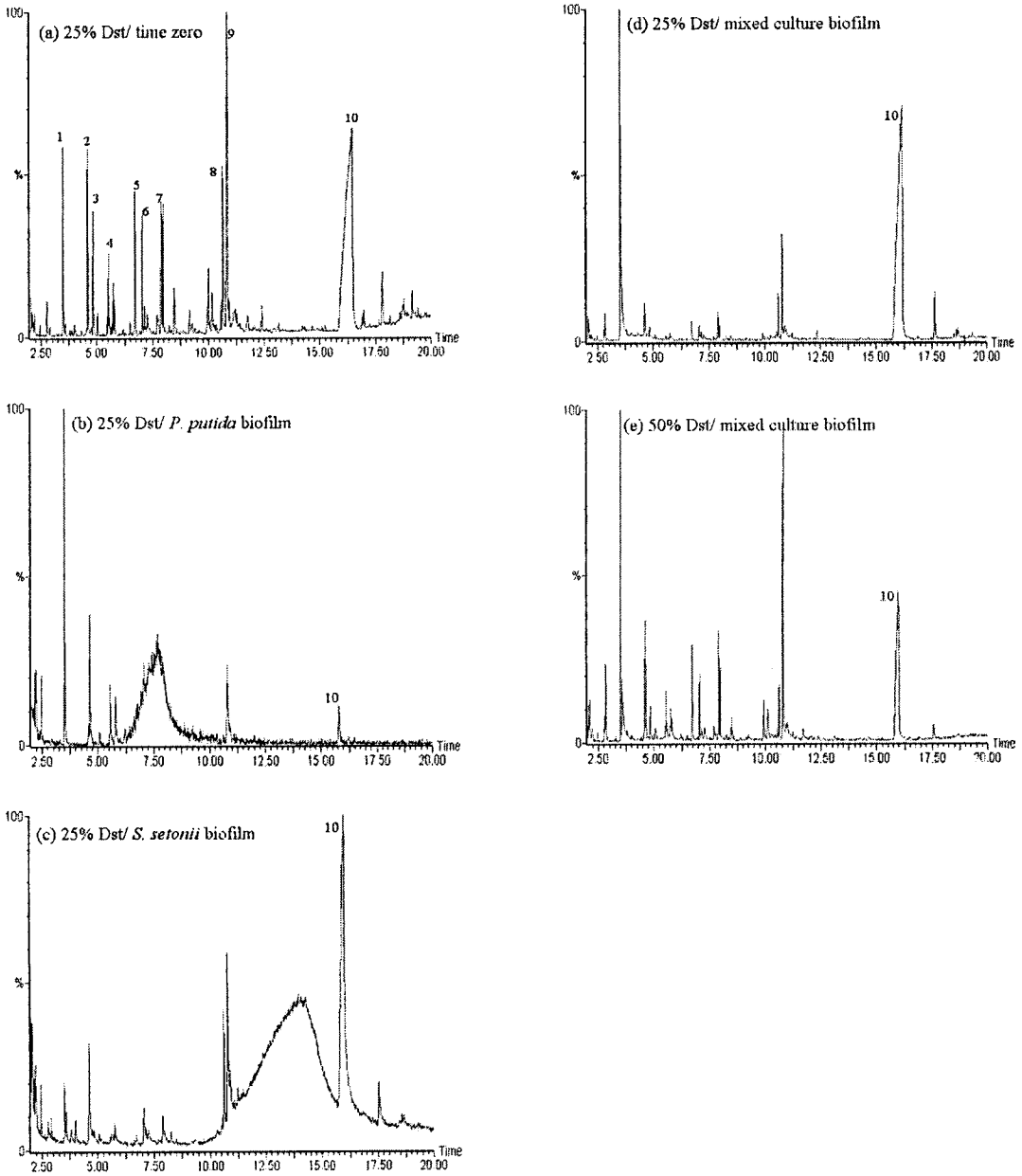

Figure 7. Chromatographic profiles of zero time Dst (**a**); 25% (v/v) Dst treated by *P. putida* biofilm (**b**), *S. setonii* biofilm (**c**), and mixed-culture biofilm (**d**); and 50% (v/v) Dst treated by mixed-culture biofilm (**e**) after 10 days of incubation. Identified peaks: 2-furancarboxaldehyde (1), 2(5*H*)-furanone (2), 1,3-cyclopentanedione (3), 5-methyl-2-furancarboxldehyde (4), 2-hydroxy-3-methyl-2-cyclopentene (5), 3,6-dianhydro-β-D-glucoyranose (6), furyl hydroxymethyl ketone (7), 3,6-dianhydro-D-glucopyranose (8), 5-hydroxymethyl-2-furancarboxaldehyde (9), and 1,6-anhydro-β-D-glucopyranose (10).

removed some (40 to 20%) and the mixed-culture biofilm removed the most (>50%).

GC-MS Analysis of Dcs and Dst Pyrolysis Liquors. The analysis of zero time Dcs and Dst samples showed several major peaks (**Figures 6** and **7**). The bacterial-treated Dcs and Dst samples demonstrated reductions in some peaks. The reduction was more noticeable in 10 and 25% (v/v) Dcs and Dst (**Figures 6** and **7**) compared to the reduction in 50% (v/v) Dcs and Dst. **Table 2** includes molar concentrations of zero time and bacterial-treated Dcs and Dst samples after 10 days of incubation. Again, the mixed-culture biofilm illustrated the best overall reduction of these detected compounds.

DISCUSSION

In this study, the ability of two aerobic bacteria, *P. putida* and *S. setonii*, to detoxify diluted corn stover, Dcs, and diluted corn starch, Dst, pyrolysis liquors produced by fast pyrolysis process was demonstrated. The goal was to develop a biological process that would permit the bio-detoxification of pyrolysis liquors for industrial microbial fermentation. To our knowledge, this is the first study aimed to employ microorganisms to detoxify the toxic compounds in this effluent. Previous research employed activated carbon or organic solvents to extract the recalcitrant toxic compounds from pyrolysis liquors (*6*, *12*).

Preliminary work with Dcs and Dst revealed high-viscosity, low-acidity, and toxic compounds, which inhibit microbial growth (*42*). Analytical studies of various pyrolysis liquors have identified a wide range of chemical compounds present in pyrolysis liquors, with most of them toxic to microbial growth (*11*, *12*, *42*). The compounds found in the cellulose pyrolysate are monosaccharides, anhydrosugars, carbonyl compounds, furans and lactones, pyrans, phenols, acids and acid esters, and other compounds (*11*). The phenolic compounds in Dcs pyrolysis liquors are derived from lignin (*11*, *42*). The furan and

Table 2. GC-MS Quantitative Analysis Results of Some Compounds in Different Concentrations of Diluted Corn Stover Pyrolysis Liquors (Dcs) and Diluted Corn Starch Pyrolysis Liquors (Dst) before and after Bacterial Treatment by *P. putida*, *S. setonii*, and Mixed-Culture Biofilms[a]

concn of dilute pyrolysis liquor/ bacterial biofilm treatments[b]	compounds in mM concn in Dcs					compounds in mM concn in Dst	
	phenol	2-methoxy-5-methylphenol	2,6-dimethoxy-phenol	1,2,4-trimethoxy-benzene	levoglucosan	2(5*H*)-furanone	levoglucosan
25% (v/v)/zero time	0.6	2.3	2.6	0.14	120	11	240
25% (v/v)/*P. putida*	0.05	0.062	0.2	0.007	110	3.2	240
25% (v/v)/*S. setonii*	0.038	ND[c]	0.062	ND	110	2.9	240
25% (v/v)/mixed-culture biofilm	0.018	ND	ND	NtagnD	110	2.1	220
50% (v/v)/zero time	1.3	4.7	5.3	0.28	240	22	490
50% (v/v)/mixed-culture biofilm	6.2	0.76	0.78	0.0033	200	62	490

[a] Quantitative analysis was determined according to pure standard for each compound. [b] All bacterial-treated samples were after 10 days of batch culture incubation. [c] ND, compound was not detected.

furfural derivative compounds in Dst are derived from starch and its potential modifications (*33*, *42*). The purpose of the water dilution of the corn stover and the corn starch pyrolysis liquors was twofold: (1) to remove the insoluble portion and (2) to collect the supernatant (the water-soluble compounds), which is more biodegradable than the non-water-soluble compounds (*45*). The supernatants of Dcs and Dst were neutralized with lime and filter sterilized, because heat sterilization hydrolyzes levoglucosan to glucose (*12*).

P. putida and *S. setonii* suspended-cell cultures have the ability to oxidize different aromatic compounds (*4*, *14*, *39*, *46*, *47*), and they were able to grow on 10% (v/v) Dcs and Dst. The microbial toxicity of Dst seems to be from the furfural derivates (**Figure 7**), which once removed detoxified the broth (**Table 1**). There was a difference in toxic compounds present in each diluted liquor when *S. setonii* suspended-cell culture detoxified 25% (v/v) Dcs but not Dst (**Table 1**), whereas *P. putida* was unable to detoxify either diluted pyrolysis liquors. It has been noted that microbial degradation of compound mixtures is strongly affected by other substituents in the mixture, and the rate of consumption is also affected by the presence of these other compounds (*4*). However, the mixture contains low concentrations such as 0.3 mM and can be degraded by pure culture or mixed culture (*34*). Finally, the UV absorption spectra (**Figures 3** and **4**), GC-MS data (**Figures 6** and **7**; **Table 2**), and bioassay (**Table 1**) corroborate and validate our observation that *P. putida* and *S. setonii* were able to biodegrade some toxic compounds in the Dcs and Dst.

Biofilms are generally more resistant to toxic compounds and harsh conditions (*23*, *25*), but not all microorganisms can form biofilm. PCS stimulates biofilm formation and enhances productivity of end products (*30*−*32*). Interestingly, *P. putida* biofilm was developed on the PCS using cycles between batch culture and high to low dilution rates. *Pseudomonas* has been reported to form biofilm under different conditions (*48*−*50*). However, *P. putida* was slow to form PCS biofilm, requiring 11 days to develop a good thick biofilm, whereas *S. setonii* PCS biofilm was developed in 2 days at low dilution rates with even thicker biofilm formation with increasing dilution rates. The mixed-culture PCS biofilm was allowed to develop on the basis of *S. setonii*'s ability to rapidly adhere to the PCS in 2 days and *P. putida* on day 7. Fortunately, both *S. setonii* and *P. putida* grew well at pH 6.5 and 30 °C. The advantage of developing mixed-culture biofilm is to biodegrade compound mixtures through cooperative activity (*34*), and the biodegradation rate by mixed immobilized cells is generally better than that of suspended-cell cultures (*37*).

P. putida, *S. setonii*, and the mixed-culture biofilms were more effective in detoxifying the 10 and 25% (v/v) Dcs and Dst than the 50% (v/v). Again, for Dcs the UV spectra (**Figure 4**), the phenolic compound assay (**Figure 5**), and the GC-MS data (**Figure 6**; **Table 2**) demonstrated removal and reductions in these aromatic compounds, which correlated to detoxification as determined by the bioassay (**Table 1**). Furthermore, biological activity, as observed by changes in DO_2 and alkaline consumption, in the CSRT biofilm reactor were rapid for 10% (v/v) Dcs or Dst, whereas they were diauxic for the 25% (v/v) Dcs and Dst (**Figure 2**). Thus, DO_2 and alkaline consumption, as benchmarks for biological activity, decreased after day 2 until day 5, and were followed by increased biological activity, suggesting a diauxic growth pattern with a lag phase for enzyme induction.

The 50% (v/v) Dcs and Dst represented the upper limit for biological activity by *S. setonii*, *P. putida*, and mixed-culture biofilm reactors. For example, for the 50% (v/v) Dcs, total phenolic illustrated some reduction (**Figure 5**) for all three culture conditions, whereas the bioassay demonstrated no growth for both pure-culture biofilms and growth for the mixed-culture biofilm. These differences were further exhibited by the different levels of lactic acid produced, which demonstrated the mixed-culture had preference for Dcs over Dst (9.8 and 6.8 g/L, respectively) (**Table 1**).

In conclusion, the Dcs and Dst demonstrated that pyrolysis liquor mixture of chemicals in low concentrations can be biologically detoxified using a PCS biofilm reactors. The PCS mixed-culture biofilm was the preferred method. This alternative approach to traditional physical/chemical methods is worth further investigation.

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