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Inhibitory Effect of Furanic and Phenolic Compounds on Exoelectrogenesis in a Microbial Electrolysis Cell Bioanode

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ABSTRACT: The objective of this study was to systematically investigate the inhibitory effect of furfural (FF), 5-hydroxymethylfurfural (HMF), syringic acid (SA), vanillic acid (VA), and 4-hydroxybenzoic acid (HBA), which are problematic lignocellulose-derived byproducts, on exoelectrogenesis in the bioanode of a microbial electrolysis cell. The five compound mixture at an initial total concentration range from 0.8 to 8.0 g/L resulted in an up to 91% current decrease as a result of exoelectrogenesis inhibition; fermentative, non-exoelectrogenic biotransformation pathways of the five compounds were not affected. Furthermore, the parent compounds at a high concentration, as opposed to their biotransformation products, were responsible for the observed inhibition. All five parent compounds contributed to the observed inhibition of the mixture. The IC_{50} (i.e., concentration resulting in 50% current decrease) of individually tested parent compounds was 2.7 g/L for FF, 3.0 g/L for HMF, 1.9 g/L for SA, 2.1 g/L for VA and 2.0 g/L for HBA. However, the parent compounds, when tested below their respective non-inhibitory concentration, jointly resulted in significant inhibition as a mixture. Catechol and phenol, which were persistent biotransformation products, inhibited exoelectrogenesis only at high concentrations, but to a lesser extent than the parent compounds. Exoelectrogenesis recovery from inhibition by all compounds was observed at different rates, with the exception of catechol, which resulted in irreversible inhibition.

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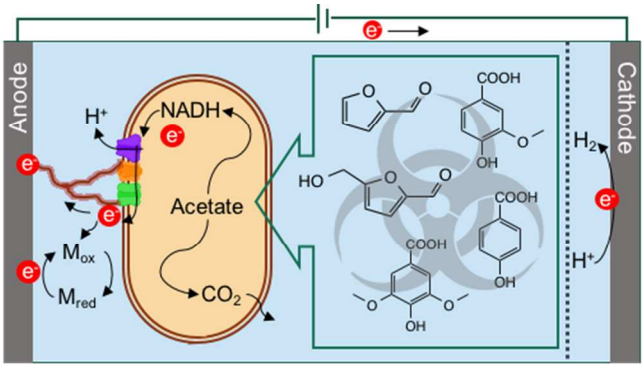

INTRODUCTION

Furanic and phenolic compounds are common byproducts formed during fuel and chemical production from lignocellulosic biomass. In lignocellulosic hydrolysates and pyrolysates, furfural and 5-hydroxymethylfurfural are the primary furan derivatives present at a total concentration up to 5 g/L, while phenolic compounds, including alcohols, aldehydes and acids, are present at a total concentration up to 3 g/L.[1] Furanic and phenolic compounds in biomass pretreatment streams present a significant challenge in biofuel production for a number of reasons. They inhibit growth and activities of hydrogen (H_2)- and ethanol-producing microorganisms, lowering yields of H_2 and ethanol in dark fermentation of lignocellulosic hydrolysates.[2,3] These compounds also make biomass pyrolysates corrosive and unstable, increasing the difficulty of downstream processing.[4] Because some furanic and phenolic compounds have cytotoxic and mutagenic effects,[5] the disposal of waste streams bearing such compounds is of environmental concern.

Microbial electrolysis cell (MEC) is considered as an alternative technology for waste handling and H_2 production, which can be integrated to the overall process of biofuel production from biomass.[6,7] In a MEC, furanic and phenolic compounds are utilized as substrates by the bioanode microorganisms. During microbial metabolism, electrons are extracted from these compounds and then transferred to the anode electrode. With a small voltage supply, the electrons are transferred via an electric circuit to the cathode where reduction of protons takes place (usually abiotically) with the production of H_2. The fact that the MEC technology can achieve simultaneous waste treatment and H_2 production makes it more advantageous than existing methods used to deal with furanic and phenolic compounds, such as solvent extraction to remove these compounds from the biomass pretreatment streams, and use of genetically

engineered, tolerant microorganisms in dark fermentation.[2] MECs have achieved promising conversion of furanic and phenolic compounds (> 98%), H_2 production rate (up to 4.3 L/L-d) and yield (up to 76%) from a mixture of furanic and phenolic compounds up to 1.2 g/L, as well as switchgrass-derived bio-oil aqueous phase components.[6,7]

While furanic and phenolic compounds are well-known inhibitors of dark fermentation for H_2 and ethanol production, their potential inhibitory effect on the bioanode microbial activity has been scarcely reported. A study showed that syringaldehyde, *trans*-4-hydroxy-3-methoxy and 4-hydroxy cinnamic acids at a concentration above 20 mM, as well as benzyl alcohol and acetophenone at 0.2 mM, severely inhibited electricity generation from glucose using a microbial fuel cell (MFC).[10] Our previous study showed that a mixture of furfural (FF), 5-hydroxymethylfurfural (HMF), syringic acid (SA), vanillic acid (VA), and 4-hydroxybenzoic acid (HBA) at a total concentration of 1.2 g/L in a batch MEC bioanode produced significantly lower current and H_2 than at lower concentrations of 0.2 – 0.8 g/L, suggesting inhibition by a high concentration of the mixture of furanic and phenolic compounds.[6] However, neither of these previous studies provided an in-depth analysis of the observed inhibition.

In a bioanode, where soluble external terminal electron acceptors (e.g., nitrate and sulfate) are absent, the conversion of high molecular weight, complex compounds occurs via fermentation followed by exoelectrogenesis.[11] Fermentative bacteria first convert the complex compounds to simpler molecules, among which acetate is a key electron donor used in subsequent exoelectrogenesis.[11] There are two sub-steps in exoelectrogenesis: intracellular metabolism of electron donor and extracellular electron transfer to the anode electrode.[12,13] Inhibition of either sub-step can lead to failure of current production. In parallel with exoelectrogenesis, methanogenesis can also occur using acetate and/or H_2/CO_2 resulting from the

fermentation of complex compounds, which, however, results in a decrease of cathodic H_2 production. Our previous study showed that although the current decreased by 64%, as a result of inhibition by the furanic and phenolic mixture at 1.2 g/L, the transformation rate of the individual furanic and phenolic compounds increased by more than two-fold with an increase of the total mixture concentration from 0.8 to 1.2 g/L.[6] Acetate accumulated at high levels with the decreased current, while was not detected with normal current production when the anode was fed with the compounds mixture at a total concentration of less than 1.2 g/L.[6] These results demonstrate that inhibition by the furanic and phenolic compounds in the MEC bioanode primarily impacted the acetate-consuming process (i.e., exoelectrogenesis) as opposed to the acetate-producing process (i.e., fermentation). Thus, the present study focused on the inhibitory effect of furanic and phenolic compounds on exoelectrogenesis.

The following questions were raised in our previous studies: (1) Are the parent compounds or their transformation products responsible for the observed inhibition of exoelectrogenesis? (2) Which compound(s) in the mixture is/are the inhibitor(s)? (3) Do these compounds interact synergistically leading to more severe inhibition? To address these questions, the present study was conducted with the objective of assessing the potential inhibitory effect of two furanic (FF and HMF) and three phenolic compounds (SA, VA and HBA), as well as their biotransformation products, on exoelectrogenesis in a MEC bioanode. The selected compounds are typically found in waste streams generated during the pretreatment of lignocellulosic biomass,[14] and were used in our previous study where inhibition of exoelectrogenesis was first observed.[6]

MATERIALS AND METHODS

Chemicals. FF, HMF, SA, VA and HBA were parent compounds used in this study. Catechol and phenol, which were persistent transformation products, were also tested for their inhibitory effect on exoelectrogenesis. The above-mentioned seven chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA). A mixture of the five parent compounds in a stock solution at a total concentration of 10 g/L (15.6 g COD/L; COD, chemical oxygen demand) was prepared with each compound at equal electron equivalents (3.12 g COD/L) dissolved in the anolyte solution and stored at 4 ºC.

MEC. A dual-chamber (250 mL each) H-type MEC was used in the present study. The anode electrode was a bundle of five stripes of carbon felt (1 cm × 1 cm × 5 cm, each). The cathode electrode was a platinum-coated carbon cloth with a projected surface area of 30 cm^2. The MEC anode was inoculated with electrode-attached biofilm from a MFC anode fed with the above-described mixture of the five compounds for 6 months. The MEC was then fed with the five compounds mixture at a total concentration of 0.2 - 1.2 g/L in batch and then continuous-flow modes for over 2 years. The anolyte was microbial growth medium and the catholyte was 100 mM phosphate buffer, both at pH 7.0, with composition as previously described.[6] A potentiostat (Interface 1000, Gamry Instruments, Warminster, PA) was used to set a voltage of 0.6 V at the anode relative to the cathode. The total anode biomass concentration measured as protein was 352 ± 10 and 471 ± 101 mg/L, before and after the experiments reported here, respectively; approximately 90% of the biomass was biofilm-associated, whereas 10% was in suspension. The established biofilm microbial community mainly consisted of *Geobacter, Anaerovorax, Acetobacterium, Eubacterium* and unclassified *Clostridia* spp., including exoelectrogens, putative furanic and phenolic degraders, and other acetogenic fermentative bacteria, as previously reported.[6,15]

Batch Runs with Parent Compounds Mixture. In order to determine whether the parent compounds or their transformation products are inhibitory to exoelectrogenesis, two experiments were conducted. First, six consecutive 24-h batch runs were performed with an increasing initial total concentration of the parent compounds mixture (0.8, 1.2, 2.4, 3.6, 6.0 and 8.0 g/L). Exogenous acetate was not added. The selection of the initial concentration range was based on a previous observation that 0.8 g/L was non-inhibitory and 1.2 g/L was inhibitory to exoelectrogenesis.[6] The 0.8 g/L run was a non-inhibitory control. In between each batch run, the anode chamber was drained and fresh anolyte added. In these batch runs, the anode microbial community experienced an increasing initial concentration of the parent compounds mixture, and the response of exoelectrogenesis in terms of current and cathodic H_2 production was monitored. The second experiment was conducted in a single batch run with the parent compounds mixture added repetitively every 24 h at a non-inhibitory concentration (0.8 g/L). Unlike the first experiment, the second experiment created a condition where the dose of the parent compounds was kept low, but the concentration of transformation products gradually increased. Based on our previous study,[6] in which complete transformation of the parent compounds mixture at 0.8 g/L occurred within 24 h, the concentration of the mixture was not expected to increase with daily feedings of 0.8 g/L. In addition, the anolyte was not replaced throughout this experiment, thus resulting in the accumulation of transformation products. A control experiment was conducted with repetitive additions of sodium acetate (NaAc) at increasing concentrations (0.1 – 10 g/L) in the bioanode without any furanic or phenolic compounds.

Batch Runs with Individual Parent Compounds. In order to determine which compounds contributed to the observed inhibition of exoelectrogenesis, individual parent compounds (FF, HMF, SA, VA and HBA) were tested in separate batch runs. For each

compound, a batch run was initiated with fresh anolyte and 1 g/L NaAc as the exogenous exoelectrogenic substrate. Exogenous NaAc was added in excess to avoid differences in current production due to the difference in the biotransformation extent of each of the furanic and phenolic compounds tested. After 1 h of incubation with NaAc, but without any furanic or phenolic compound added (non-inhibited, control condition), one of the five compounds was added to the anode chamber at increasing levels of 0.8, 1.2, 2.4 and 3.6 g/L at 1, 3, 5 and 7 h, respectively (unless otherwise stated). The goal of sequential additions of each compound at increasing levels resulted in more than 50% inhibition based on current production. Based on the results of the compounds mixture, higher than 50% inhibition was observed with the mixture at 3.6 g/L. Thus, same concentrations as the mixture in the range of 0.8 - 3.6 g/L were selected for the individual compounds. The exoelectrogenic response was monitored by the current, recorded every 5 min, and cathodic H_2 production measured before each subsequent compound addition. A control run with 1 g/L NaAc, in the absence of any inhibitor, was conducted prior to each batch run for all the compounds tested.

Batch Runs with Catechol and Phenol. Catechol and phenol, which were persistent transformation products of the five compounds mixture, were further determined to be transformation products of VA and HBA, respectively. The potential inhibitory effect of catechol and phenol was evaluated in batch runs following the same procedure as above-described for the individual parent compounds. The levels of catechol or phenol added to the bioanode were pre-selected as 0.8, 1.2 and 2.4 g/L, based on the fact that VA or HBA at 3.6 g/L (the highest total level added) can produce 2.4 g/L of catechol or 2.5 g/L of phenol at most (1:1 molar ratio). In the batch run conducted with catechol, where a low extent of inhibition was observed after a total addition of 2.4 g/L, another 1.2 g/L was added to result in a significant inhibition.

Analytical Methods. The parent compounds and transformation products were quantified using a high performance liquid chromatography (HPLC) unit equipped with a HPX-87H column as previously described.[6] pH was measured following procedures outlined in Standard Methods.[16] Gas production was measured by acid brine solution displacement in glass burets, equilibrated to 1 atm. Gas composition (H_2, CO_2 and CH_4) was quantified using a gas chromatography unit equipped with two columns and two thermal conductivity detectors.[6] The anode biomass was quantified by protein measurement using the Pierce™ BCA assay kit after extraction as previously described.[15] Cyclic voltammetry was conducted under the maximum inhibition and non-inhibited conditions (i.e., control) for each individual compound, at a scan rate of 5 mV/s from - 0.5 to + 0.3 V, with the anode as the working electrode, cathode as the counter electrode, and a Ag/AgCl electrode as the reference electrode inserted in the anode chamber.

Calculations. The extent of inhibition was calculated based on the ratio of the decreased current under inhibition (I_{inh}) to that under non-inhibited, control conditions ($I_{non-inh}$) (eq 1). The inhibitory concentration at 50% inhibition (IC_{50}) is defined as the inhibitor concentration at which the current decreases by 50% compared to the current under non-inhibited conditions.

$$Inhibition\ (\%)\ = \left(1 - \frac{I_{inh}}{I_{non-inh}}\right) \times 100 \qquad (eq\ 1)$$

Potential synergistic inhibition was evaluated by comparing the observed effect of the compounds mixture to the additive effect of its components. The additive effect was calculated from the experimental data of individual compounds using three methods:[17] effect summation, concentration addition, and independent action as described in detail in Text S1.

RESULTS AND DISCUSSION

Inhibitory Effect of the Furanic and Phenolic Mixture. In the six batch runs conducted with 0.8, 1.2, 2.4, 3.6, 6.0 and 8.0 g/L of the furanic and phenolic mixture (FF, HMF, SA, VA and HBA), the maximum current in each batch run decreased with increasing initial total concentration of the mixture (Figure 1A). The first batch run at 0.8 g/L served as a non-inhibited control based on the findings of our previous study in which the furanic and phenolic mixture at 0.8 g/L did not have any negative impact on exoelectrogenesis.[6] Higher initial concentrations of 1.2 - 8.0 g/L resulted in a current decrease, with a more than 50% decrease at 3.6 g/L (4^{th} batch run) and 91% decrease at 8.0 g/L (last batch run; Figure 1A). Similar to the current, the H_2 production rate decreased from 0.18 L/L-d to non-detected in the last batch run conducted with 8.0 g/L. For the batch runs with high initial mixture concentrations (3.6 – 8.0 g/L), 1.0 N NaOH was added upon each substrate addition to adjust the pH to 7.0, thus compensating for the acidity of the phenolic compounds added. The anolyte pH slightly decreased from 7.0 and remained at 6.7 or above during all batch runs. Such pH values are above the generally accepted inhibiting pH value (6.0) for *Geobacter* spp..[18] In addition, the control experiment conducted with 0.1 – 10 g/L NaAc showed that the current increased with increasing NaAc concentration, even with a decreasing pH from 7.0 to 6.0 (Figure S1). Thus, the decreased current in the batch runs conducted with the furanic and phenolic mixture is attributed to the inhibitory effect of these compounds and not to potential transfer limitations of electrons and protons. Compared to our previous study conducted one year before the present study with the same MEC,[6] in which exoelectrogenesis was inhibited by more than 66% by the same furanic and phenolic mixture at 1.2 g/L, the present study showed a higher tolerance of the bioanode to these compounds (11 and 69% inhibition at 1.2 and 3.6 g/L, respectively). The increased tolerance is partially attributed to a 3-fold increase of biofilm-associated biomass during the 1-year MEC operation. Dominant

220 microbial species in the biofilm were almost identical to those previously reported,[6,15] but a
221 higher relative abundance of the fermentative species over exoelectrogens was observed after the
222 1-year MEC operation. Thus, a more robust and tolerant biofilm can be developed through
223 prolonged exposure of the microbial community to the five furanic and phenolic compounds.
224 Consistent with our previous study,[6] the transformation rate of the parent compounds did
225 not decrease when exoelectrogenesis was inhibited (Figure 1B). In fact, the transformation rate
226 of the parent compounds in the 3.6 - 8.0 g/L runs was more than 1.5-fold higher than in the 0.8
227 g/L run (3.4-3.9 vs. 2.0 g/L-d), although the current decreased by more than 69%. The detected
228 transformation products were catechol, phenol and acetate, whose concentration increased as the
229 parent compounds were transformed during each batch run (Figure 1C). Catechol and phenol
230 were transformation products of VA and HBA, respectively. The highest observed concentration
231 of catechol (0.32 g/L) and phenol (0.34 g/L) represents 82% of VA and 86% of HBA
232 transformed, respectively, based on 1:1 molar ratio between the parent compounds and their
233 transformation products. The acetate accumulation rate was higher in the 3.6 - 8.0 g/L runs than
234 in the 0.8 g/L run (0.25 - 0.29 vs. 0.1 g/L-d), which indicates that the acetate consumption by the
235 exoelectrogens was slower than its production by the fermenters in the 3.6 – 8.0 g/L runs. The
236 increased transformation rate of the parent compounds and acetate accumulation in contrast to
237 the decreased current, confirm that non-exoelectrogenic activities (e.g., fermentation) were not
238 impacted in the bioanode, but instead, exoelectrogenesis was mainly inhibited. Although not
239 observed in the relatively short duration experiments conducted in the present study, severe
240 inhibition of exoelectrogenesis in MEC systems operated over long periods may result in the
241 accumulation of high levels of fermentation products and lead to feedback inhibition of
242 fermentation.

The fermentative process in the MEC bioanode used in this study involves different reactions and microbial species from those found in H_2 and ethanol dark fermentation studies, which are usually based on sugar degradation. In the present study, fermentation in the MEC anode refers to the transformation of furanic and phenolic compounds to mainly acetate, carried out by furanic and phenolic degraders (such as *Acetobacterium* and *Eubacterium* spp.), as mentioned above.[6,15] H_2 and ethanol dark fermentation refers to the conversion of sugars to H_2 and ethanol by certain *Clostridium* spp. and yeasts. The furanic and phenolic fermenting bacteria, which can grow on these compounds, are presumably less susceptible to inhibition than microbial species involved in H_2 and ethanol fermentations, as well as exoelectrogenesis.

Gas in the bioanode headspace consisted of CO_2 and CH_4; H_2 was not detected in the anode. CO_2 produced by both fermentation and exoelectrogenesis varied from 10 to 20 mL per run, without a clear correlation to the exoelectrogenic response in these batch runs. However, a lower CH_4 production was observed while exoelectrogenesis was inhibited in the 3.6 - 8.0 g/L runs, compared to the other batch runs conducted with lower initial mixture concentrations (2 - 3 mL vs. 9 - 12 mL). Thus, the observed decrease in current production was not caused by competition for electron equivalents with methanogens. It has been reported that methanogenesis in mixed cultures was inhibited significantly by FF and HMF at 5-10 g/L, as well as phenolic compounds at 1.2 g/L, and that more severe inhibition was observed with mixtures.[3,19] In addition, methanogenesis could also be impacted by low pH. A desired pH range for methanogenesis is 6.5 – 7.6.[20] In the above-discussed batch runs, the lowest anolyte pH value observed in each run was 6.7 - 6.9. However, a small proton concentration gradient may exist at the anolyte-biofilm interface and within the depth of the biofilm, resulting in a slightly lower pH value inside the biofilm.

Inhibition by Parent Compounds vs. Transformation Products. In the second experiment, when the furanic and phenolic mixture was added daily to the anode at an initial, non-inhibitory concentration of 0.8 g/L, the current remained at 3.5 mA throughout five mixture additions (Figure 2A), while the cathodic H_2 production rate was stable at 0.18 ± 0.02 L/L-d. The pH decreased from 7.0 to 6.5 over the course of the experiment, although it was adjusted to 7.0 by 1.0 N NaOH upon each addition of the 0.8 g/L mixture. The resulting steady current and H_2 production rate are in contrast to those observed in the first experiment conducted with increasing initial total concentration of the five compounds mixture. The parent compounds were totally transformed within 1 d before the addition of the next mixture (Figure 2B). Thus, the total concentration of the parent compounds mixture remained below 0.8 g/L at all times in the second experiment. The five additions of the parent compounds mixture led to an accumulation of transformation products reaching a concentration comparable to that resulting from the addition of 3.6 - 8.0 g/L parent compounds mixture (Figure 1C and 2C). Five daily additions of 0.8 g/L are equivalent to a cumulative addition of 4.0 g/L. Thus, the results of the second experiment can be compared to those of the 3.6 g/L batch run in the first experiment. The fact that the transformation products were at comparable concentrations in both experiments, and that the current decreased only in the first experiment (but not in the second), indicates that the transformation products were not the cause of the observed inhibition. Instead, the initial concentration of the parent compounds, which was the main difference between the two experiments, had a clear impact on the current. As discussed above, in the first experiment the current decreased with an increasing dose of the parent compounds mixture from 0.8 to 3.6 g/L and higher; the second experiment avoided one-time, high doses of the parent compounds by distributing a total 4.0 g/L dose in five days (0.8 g/L per day), and the current was not impacted.

289 Therefore, it is concluded that the observed inhibition of exoelectrogenesis was due to the parent
290 compounds, rather than to their transformation products.

291 **Inhibitory Effect of Individual Parent Compounds.** To further investigate which of the
292 parent compounds was responsible for the observed inhibition, each compound (FF, HMF, SA,
293 VA and HBA) was added at increasing levels to the bioanode which was also amended with 1
294 g/L NaAc. During the control runs with 1 g/L NaAc, which were performed before testing every
295 compound, the current was steady for 9 h in each run, ranging from 2.0 to 2.5 mA (Figure S2
296 shows a representative control run). The concentration of NaAc, which was the exogenous
297 exoelectrogenic substrate, did not show a significant decrease in 9 h ($P = 0.57$), because a
298 relatively high concentration of NaAc was used to avoid current drop due to exoelectrogenic
299 substrate limitation. This observation also demonstrates that the acetate consumption rate would
300 not have been a sufficiently sensitive parameter to describe the inhibitory effect on
301 exoelectrogenesis. Instead, current was used as the primary parameter to evaluate the rate of
302 exoelectrogenesis.

303 While increasing levels of the individual furanic and phenolic compounds were added to
304 the bioanode, active biotransformation of the compounds resulted in concentrations lower than
305 the pre-specified amounts (Figure S3). Meanwhile, higher than 50% current decrease was
306 observed for all compounds with a total addition up to 3.6 g/L (in 9 h), except for HMF (Figure
307 S3). It is noteworthy that the attained highest concentration of HMF at 7 h upon a total addition
308 of 3.6 g/L was significantly lower than that of FF as a result of fast transformation (Figure S3),
309 which resulted in lower inhibition. In order to achieve a comparable concentration of HMF to
310 that of FF, an extra 1.8 g/L HMF was added at 9 h to make the highest attained concentration of
311 HMF equal to 3.0 g/L. The current decreased upon this last HMF addition (Figure S3).

The extent of inhibition at increasing concentrations of the five parent compounds is shown in Figure 3A and B. The reported concentration of each compound is that measured immediately after each addition. At concentrations of 0.8 g/L and below, no inhibition was observed with any of the tested compounds. Thus, 0.8 g/L was a non-inhibitory concentration for all five compounds. At concentrations from 0.8 to 1.8 g/L, low inhibition (< 20%) was observed for these five parent compounds. However, when the individual compounds were present at 2.1 - 3.0 g/L, the current decreased by more than 50%. The suppressed exoelectrogenic activity was further confirmed by cyclic voltammetry (Figure S4). The current under inhibited conditions was lower than under non-inhibited conditions at all applied potential values (-0.5 to +0.3 V vs. Ag/AgCl). The fact that the current under inhibited conditions did not increase with increasing anode potential to a level comparable to that under non-inhibited conditions rules out the possibility that the observed decrease in current production at inhibition was caused by a limited anode potential. In addition, oxidation peaks observed under non-inhibited conditions disappeared under inhibited conditions. Similar to the current, the H_2 production rate showed a generally decreasing trend in response to the increasing furanic and phenolic compounds concentrations attained immediately after addition (Figure S5). However, the H_2 production rate was not as reliable and sensitive as the current. The reported H_2 production rates are mean values at each time interval (1 or 2 h). In addition, H_2 back diffusion to the anode may have occurred as previously reported and discussed.[6]

The IC_{50} values of FF, HMF, SA, VA and HBA and the extent of inhibition observed at the highest concentration of each parent compound attained in the batch runs are shown in Table 1. The reported IC_{50} values are system-specific, particularly dependent on bioanode biomass concentration, and thus may not be generally applicable to all MEC systems. Nevertheless, it is

noteworthy that FF and HMF had higher IC_{50} values (2.7 and 3.0 g/L) than SA, VA and HBA (1.9, 2.1 and 2.0 g/L), indicating a higher tolerance of the exoelectrogens to the furanic compounds. In addition, a higher extent of inhibition was observed with the phenolic compounds (65 – 80%) than with the furanic compounds (51 – 53%). Thus, overall, the phenolic compounds were more inhibitory to the exoelectrogenesis than the furanic compounds. A plot of IC_{50} versus log K_{ow} values of the five compounds (Figure S6) shows that the furanic compounds, which have considerably lower log K_{ow} values, have significantly higher IC_{50} values than the phenolic compounds, which have higher log K_{ow} values. Such a distinct difference between the furanic and phenolic compounds suggests that hydrophobicity differences may correspond to different modes of inhibition, as further discussed below.

The pH at the end of the batch runs conducted with individual phenolic compounds (6.3 - 6.5) was lower than that with the furanic compounds (6.7), which is attributed to the sequential additions of the acidic phenolic compounds. To assess potential contribution of the low pH to the observed inhibition, SA, VA and HBA were tested again with anolyte at an increased phosphate buffer strength from 50 mM to 100 mM. The pH remained above 6.7 in the runs conducted with a 100 mM buffer. The current under non-inhibited conditions ($I_{non\text{-}inh}$) in the 100 mM buffer runs was approximately 1.5-fold higher than that in the 50 mM runs, which is attributed to the higher ionic and buffer strength (Figure S7).[18,21] However, the current decrease ($I_{non\text{-}inh} - I_{inh}$) was comparable under these two conditions at the same SA, VA and HBA concentrations (Figure S7). Thus, the phenolic compounds can result in significant inhibition even at a nearly neutral pH.

The results obtained with the individual compounds indicate that all five parent compounds (FF, HMF, SA, VA and HBA) contributed to the inhibition observed with the compounds mixture. Whether these compounds interact synergistically or not was further

assessed by comparing the observed extent of inhibition of the mixture to the additive effect calculated from the extent of inhibition of the five components. As shown in Figure S8, the observed mixture inhibition was higher than the additive effect predicted by the effect summation method. Such an increased combined inhibition has been widely reported for furfural, aromatic compounds, and acetic acid in dark fermentation.[22-24] It is noteworthy that at a mixture concentration below 3.6 g/L, the five compounds were below their non-inhibitory concentration (i.e., 0.8 g/L as discussed above), but jointly resulted in significant inhibition up to 69% (Figure S8). This result indicates that these compounds, even at low, non-inhibitory concentrations, are of concern for a mixture containing a large number of individual compounds. While the effect summation method can provide important information, it is unreliable for synergy quantification with non-linear dose-response curves.[25] Instead, the concentration addition and independent action methods were used for synergy assessment. These two methods assume similar and dissimilar modes of action, respectively.[26] The calculated additive inhibition based on the concentration addition method matched the observed inhibition of the mixture at a concentration range of 0.8 - 2.4 g/L (Figure S8), indicating additivity in this concentration range, beyond which calculations were not possible due to the lack of experimental data of individual compounds at higher concentrations. The independent action method, on the other hand, predicted an additive inhibitory effect higher than the observed inhibition of the mixture (Figure S8). Therefore, based on both the concentration addition and independent action methods, there was no synergistic interaction between the five compounds.

At a concentration of up to 1.8 g/L, none of the compounds resulted in higher than 20% inhibition of exoelectrogenesis (Figure 3), whereas 1 g/L is typically reported to be highly inhibitory to dark fermentation for H_2 and ethanol production.[27] Thus, compared to H_2 and

ethanol fermenters, the exoelectrogens in the present study were more tolerant to furanic and phenolic compounds. However, the following two factors should be taken into account. First, in contrast to dark fermentative cultures, which are mostly suspended, the exoelectrogens in the present study resided mostly in the biofilm of the anode electrode, as discussed above. Biofilm formation can create concentration gradients of toxic substances due to mass transfer limitations. Also, sensitive species in the biofilm can be protected from toxic substances by more resistant species which can even detoxify such compounds.[28,29] Second, the bioanode used in this study had been exposed to the five parent compounds for over 2 years before the inhibition experiments were conducted. As mentioned above, prolonged exposure to the furanic and phenolic compounds led to increased tolerance to these compounds of the bioanode microbial community, including the exoelectrogens.

Inhibitory Effect of Catechol and Phenol. Catechol and phenol were persistent transformation products in MEC runs conducted with the five compounds mixture. However, as discussed above, the transformation products were not responsible for the observed inhibition by the parent compounds mixture, in which case catechol and phenol were present at a concentration 50% lower than that of each parent compound. It is possible that catechol and phenol are intrinsically inhibitory, but their inhibition was not detected at the low concentrations attained in these tests (below 0.35 g/L). When catechol and phenol were added individually to the bioanode, they caused a current decrease of 56 and 36% at 3.2 and 2.3 g/L, respectively (Figure 3C). Cyclic voltammetry under inhibition also showed decreased current and disappearance of oxidation peaks as compared to that conducted under non-inhibited (i.e., control) conditions (Figure S4). Therefore, catechol and phenol, tested as representatives of the transformation products, can be inhibitory if present at a high concentration. However, the extent

of inhibition by catechol and phenol was lower than that of the three phenolic parent compounds at comparable concentrations (Figure 3B, C). While VA and HBA at 2.4 and 2.2 g/L resulted in 80 and 70% inhibition, respectively (Figure 3B), the maximum concentrations of catechol and phenol that could be produced from the above-mentioned VA and HBA concentrations (1.6 and 1.5 g/L, respectively) resulted in less than 20% inhibition (Figure 3C). This observation is another evidence supporting the conclusion that the parent compounds were responsible for the observed inhibition by the mixture. Nevertheless, given the possibility of an increased, combined inhibitory effect of compounds at their respective non-inhibitory concentrations, as discussed above, a combination of transformation products (known and unknown) even at relatively low concentrations could exacerbate the inhibitory effect of the parent compounds. Catechol and phenol are widely found in lignocellulose-derived waste streams.[3,19] Thus, their potential inhibitory effect on exoelectrogenesis should be considered when lignocellulosic residuals are used in bioelectrochemical systems.

Reversibility of Inhibition. The current, which was decreased significantly in the batch runs conducted with the parent compounds mixture at 3.6 g/L, fully recovered in 6 h after the anode was drained and fresh anolyte added along with the parent compounds mixture at 0.8 g/L. However, after the extended and more severe inhibition during the subsequent batch runs at 6 and 8 g/L, the current did not recover in 6 d. In our previous study, inhibition of exoelectrogenesis by the parent compounds mixture at 1.2 g/L was reversed.[6] Thus, the reversibility of the inhibition may be related to the compound's concentration and the duration of inhibition.

With the only exception of inhibition by catechol, higher than 80% current recovery was observed for all compounds with a recovery period varying from 2 to 12 h, after the anode was

427 washed three times and replenished with fresh anolyte and 1 g/L NaAc (Figure S9). No residual
428 inhibitors were detected after the anode was washed. Exoelectrogenesis inhibited by SA, VA and
429 phenol recovered quickly in about 2 h, but it took about 12 h to recover from inhibition by FF,
430 HMF and HBA (Figure S9). Exoelectrogenesis after inhibition by catechol, however, did not
431 recover in 12 h or an extended duration of 7 d (data not shown). The anode was then amended
432 with an electroactive inoculum, and the current increased gradually in the following days. Thus,
433 unlike the other compounds, catechol caused an irreversible inhibition of exoelectrogenesis,
434 which did not recover in 7 d. The difference in reversibility of inhibition and rate of recovery
435 may be related to different modes of inhibition of these compounds.

436 Little is known about the modes of inhibition of furanic and phenolic inhibitors to
437 exoelectrogenesis, but the inhibitory effects of these compounds on dark fermentation have been
438 reported and may shed light on the observed exoelectrogenesis inhibition. FF and HMF are
439 known to inhibit glycolytic enzymes essential to central metabolism of H_2 and ethanol
440 fermenters.[5] They also lead to significant NADH consumption used in reductive transformations,
441 thus decreasing intracellular levels of NADH.[30] FF has been reported to damage yeast DNA.[5] In
442 contrast to FF and HMF, which primarily target intracellular sites, phenolic compounds
443 compromise cell membrane integrity and functionality by partitioning into cell membranes,
444 changing membrane permeability and viscosity.[3,19]

445 Based on exoelectrogenesis principles, several of the above-mentioned modes of
446 inhibition may have affected different aspects of exoelectrogenesis, which warrants further
447 investigation at the cellular/molecular level. As mentioned above, the first step in
448 exoelectrogenesis is intracellular metabolism of the electron donor (i.e., acetate in the present
449 study), during which electrons are transferred to NADH. The second step is extracellular electron

transfer. The NADH formed in the first step passes through cytochromes in the cell membrane and eventually transfers the electrons to outer membrane proteins, which are able to deliver the electrons to the anode electrode via direct contact, conductive pili or soluble mediators in the anolyte.[12,13] Because FF and HMF can cause increased intracellular NADH consumption,[30] they could negatively impact the NADH yield in the first step of exoelectrogenesis, resulting in less NADH available for current production. As the second step of exoelectrogenesis relies greatly on membrane-bound proteins to perform electron transfer to the anode electrode,[12,13] exoelectrogenesis can be highly susceptible to phenolic inhibition, which targets cell membranes.

It was suggested that aromatic compounds with a log K_{ow} value in the range 1-5 can partition into the inner layer of biological membranes and cause membrane swelling, leading to loss of physiological functions.[31] Compounds with a log $K_{ow} < 1$ or > 5 can typically cause different types of inhibition other than cell membrane disruption.[31] Thus, SA, VA, HBA and phenol, with log K_{ow} values of 1.04, 1.43, 1.58 and 1.46,[32] falling within log K_{ow} range of 1 - 5, have great potential to accumulate in cell membranes and risk disrupting membrane functions. In contrast, catechol has a log K_{ow} of 0.88,[32] (i.e., lower than 1), indicating that cell membrane may not be the primary target of catechol. In fact, catechol has been reported to damage cell DNA and inactivate proteins.[33] The possibility that catechol has a different mode of inhibition than the other phenolic compounds is consistent with the above-discussed difference in the reversibility of inhibition. Thus, with log K_{ow} values lower than 1, catechol, FF and HMF might act similarly, inhibiting intracellular sites, whereas other phenolic compounds with higher log K_{ow} values (1.04 - 1.58) may disrupt cell membranes. Although the mechanism(s) of exoelectrogenesis inhibition warrant(s) further exploration at the cellular/molecular level, such activity was beyond the scope of the present study.

Integration of bioelectrochemical systems to the overall process of biofuel production for water management and byproduct conversion has received increasing attention. Understanding potential inhibitory effects of furanic and phenolic compounds on exoelectrogenesis is relevant to improve the performance of bioelectrochemical systems. The present study shows that exoelectrogens had a relatively high tolerance to individual furanic and phenolic compounds. However, a combination of these five compounds at non-inhibitory concentrations in a mixture resulted in significant inhibition, which was primarily caused by the parent compounds, as opposed to their transformation products. Furanic and phenolic compounds caused a different extent of inhibition, and different modes of inhibition may be at play, which can be related to the physico-chemical properties of these compounds. In complex lignocellulose-derived streams, presence of suitable exoelectrogenic substrate (e.g., acetate) and other readily biodegradable components (e.g., sugars), at high levels relative to inhibitory substances, may help ease inhibition. However, waste streams with complex chemical composition may exacerbate inhibitory effects. To overcome such problems, enhanced tolerance of the bioanode microbial community can be achieved by bioanode microbial pre-enrichment in the presence of potential inhibitors. Recognizing that the biotransformation products are much less inhibitory than the parent compounds, continuous-flow MEC bioanode operation can be a means to reduce the inhibitory effect of such compounds.

SUPPORTING INFORMATION

Text S1 and Figures S1 - S9 (PDF). The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org.

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586 **Table 1.** Inhibitory concentration (IC_{50}) and extent of inhibition observed at the highest
587 concentration of each parent compound attained in batch runs[a,b].

Compound	IC_{50}		Highest concentration		Inhibition[c] (%)
	g/L	mM	g/L	mM	
Furfural (FF)	2.7	28	2.8	29	53
5-Hydroxymethylfurfural (HMF)	3.0	24	3.0	24	51
Syringic acid (SA)	1.9	9.6	2.1	11	65
Vanillic acid (VA)	2.1	12	2.4	14	80
4-Hydroxybenzoic acid (HBA)	2.0	14	2.2	16	70

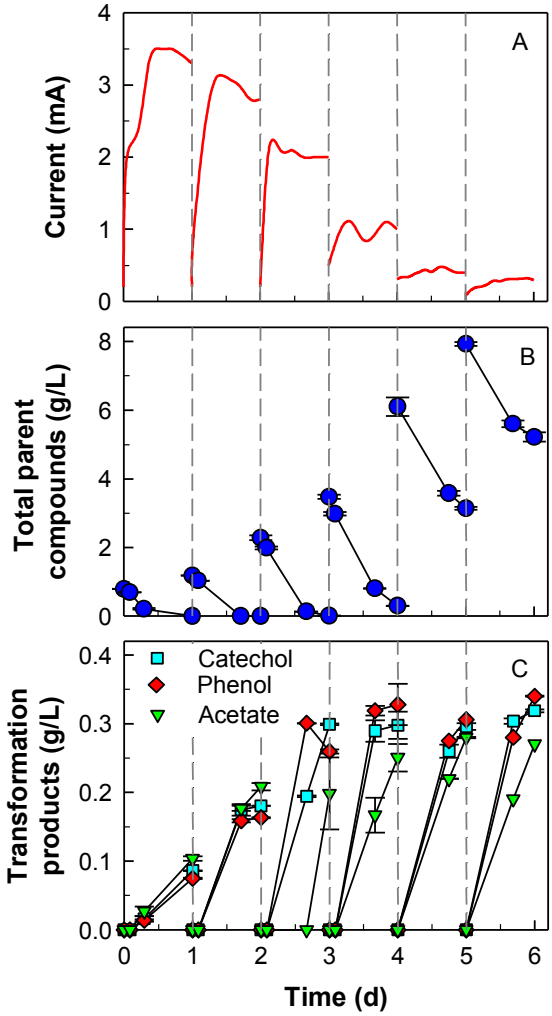
588 [a] Runs conducted with sequential additions of individual compounds at increasing concentrations.
589 [b] All data are mean values with a coefficient of variation lower than 5% (n = 2).
590 [c] Inhibition at the highest concentration attained (column 4 and 5).

LIST OF FIGURES

Figure 1. Current (A), total concentration of the parent compounds (B), and transformation products (C) in consecutive batch runs conducted with increasing initial total concentrations of the parent compounds mixture from 0.8 to 8 g/L. Error bars represent standard deviations (n = 2).

Figure 2. Current (A), total concentration of the parent compounds (B), and transformation products (C) in a single batch run conducted with repetitive additions of 0.8 g/L parent compounds mixture. Error bars represent standard deviations (n = 2).

Figure 3. Inhibitory effect of individual furanic (A) and phenolic compounds (B), as well as transformation products (C) on exoelectrogenesis based on current decrease in batch runs conducted with sequential additions of individual compounds at increasing levels. Error bars represent standard deviations (n = 6). Abbreviations: furfural (FF), 5-hydroxymethyl furfural (HMF), syringic acid (SA), vanillic acid (VA), and 4-hydroxybenzoic acid (HBA).



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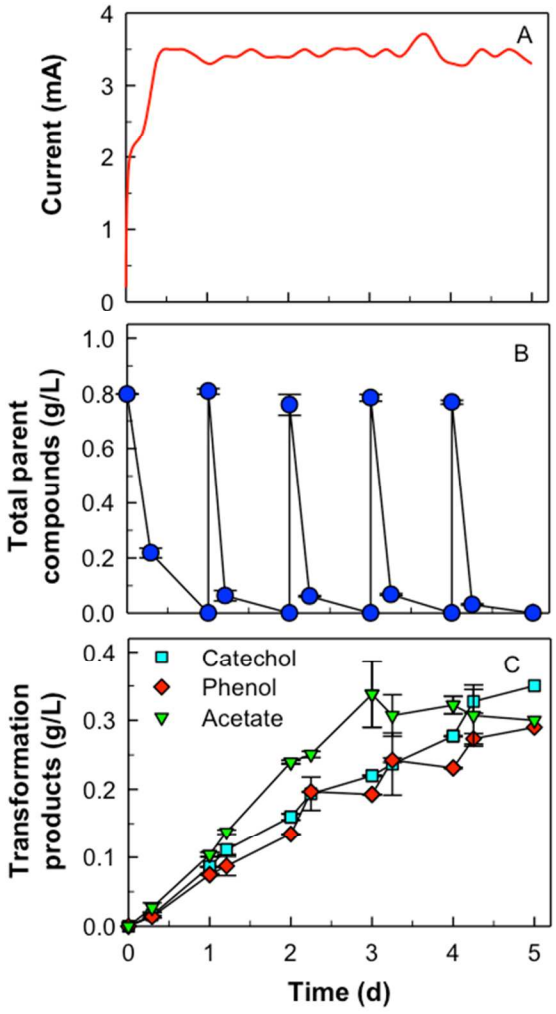

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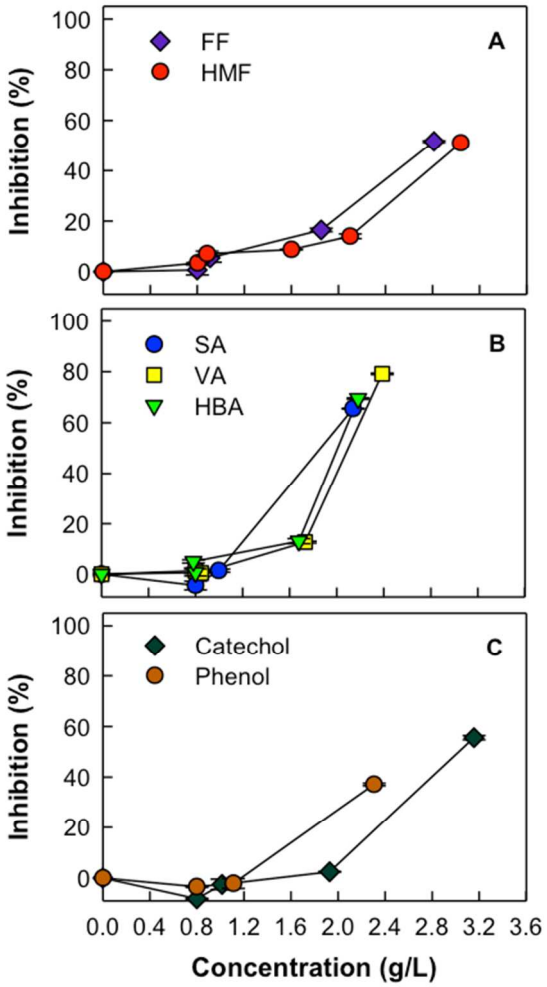

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