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# Substituent Effect of Phenolic Aldehyde Inhibition on Alcoholic Fermentation by *Saccharomyces cerevisiae*

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Supporting Information

**ABSTRACT:** Phenolic compounds significantly inhibit microbial fermentation of biomass hydrolysates. To understand the quantitative structure−inhibition relationship of phenolic aldehydes on alcoholic fermentation, the effect of 11 different substituted benzaldehydes on the final ethanol yield was examined. The results showed that the degree of phenolic benzaldehyde inhibition was strongly associated with the position of phenolic hydroxyl groups but not the number of phenolic hydroxyl groups. It was observed that *ortho*-substituted 2-hydroxybenzaldehyde resulted in 15−20-fold higher inhibition than the *meta*- or *para*-substituted analogues of 3- and 4-hydroxybenzaldehydes. From the correlation of the molecular descriptors to inhibition potency in yeast fermentation, we found a strong relationship between the octanol/water partition coefficient (log *P*) of aldehydes and the $EC_{50}$ value. The most inhibitory 2-hydroxybenzaldehyde has the highest log *P* and possesses an *ortho* −OH group capable of forming an intramolecular hydrogen bond, which can potentially increase the cell membrane permeability and toxicity. The results also indicated that the calculated free energy change between phenolic aldehydes and amino acids can be used to predict their structure−inhibitory activity relationship.

## 1. INTRODUCTION

Biochemical conversion of lignocellulosic biomass to ethanol or butanol holds great promise to supplement petroleum-derived fuels, but the process faces several techno-economic challenges. Among these is the release of degradation compounds from biomass pretreatment, which considerably inhibits subsequent enzymatic hydrolysis and microbial fermentation.[1−3]

Phenolic compounds have been identified as key inhibitors in biomass hydrolysates, which significantly inhibit microbial growth and the fermentation rate.[4−6] The phenolic aldehydes and ketones (lignin-derived) have been found to be much more inhibitory than the carbohydrate-derived inhibitors.[6−9] In previous research, 20 aromatic compounds (including 13 phenolic compounds) were evaluated for their inhibition on ethanol fermentation by *Saccharomyces cerevisiae*,[6] and it was concluded that the hydroxyl substitution in hydroxyl-methoxybenzaldehyde had a major effect on inhibition. Franden et al. examined the toxic effect of phenolic acids and aldehydes on the growth of *Zymomonas mobiliz* and concluded that phenolic aldehydes were more toxic than phenolic acids.[10] Ando et al. identified 12 aromatic compounds (including *p*-hydroxybenzaldehyde, cinnamaldehyde, and *p*-hydroxybenzoic acid) from steam-exploded poplar and suggested that the degree of inhibition was associated with the side functional groups of degradation compounds.[11] They indicated that the side groups of unsaturated double bond (C═C), aldehyde (−CHO), and hydroxyl (−OH) contributed significantly to the toxicity of these aromatic compounds.

To date, many lignocellulose-derived phenolic aldehydes and ketones have been identified in biomass hydrolysates and some of them have been evaluated in model systems.[6,8,12] However, the mechanism of inhibition by phenolic aldehydes on microbes has not yet been fully understood. The low concentration and large number of compounds in hydrolysates make determining the contribution of a given degradation compound toward inhibition a difficult task. Indeed, it is not clear whether the inhibition is due to an individual compound or a group of compounds working together. These combined factors complicate the efforts in understanding the mechanism of inhibition, developing cost-effective detoxification methods and improving stress-tolerant yeast and bacteria strains. These challenges may be addressed by the application of quantitative structure−activity relationships (QSARs) as a tool to study the toxicity of inhibitory compounds.[13,14] Structural properties of the inhibitors are the fundamental bases for the chemical reactivities that, in turn, govern their inhibitory actions toward biological cells.[15] Therefore, correlating the inhibitory properties of aromatic aldehydes to their structural features may be used to evaluate or predict their degree of inhibition toward biofuel production. Within the phenolic aldehydes, electrophilicity and hydrophobicity are properties of the aldehyde group and benzene ring, respectively. As such, the mechanism of inhibition could be due to chemical reactions between

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phenolic aldehydes and biological nucleophiles or physical destabilization of cell membranes by phenolic aldehydes. Physicochemical descriptors that can associate the phenolic aldehyde structure to microbial inhibition include the octanol/water partition coefficient (log $P$), dipole moment, energy of the lowest unoccupied molecular orbital ($E_{LUMO}$), energy of the highest occupied molecular orbital ($E_{HOMO}$), and partial charge of the reactive center. Furthermore, to establish relationships related to the chemical mechanism of inhibition, knowledge about the likelihood of the reaction of the aromatic aldehydes with specific biological nucleophiles is necessary.[14]

QSAR has been used in classifying and predicting the biological actions of untested inhibitors in drug discovery and environmental toxicity assessment.[13,14] However, they have received sparse attention in studying inhibitors in biofuel production from lignocellulosic biomass. In this study, the inhibitory effect of 10 phenolic aldehydes (Figure 1) and 1 benzaldehyde on alcoholic fermentation by *S. cerevisiae* was examined to determine (1) if substituents on phenolic aldehydes affect the inhibition, (2) if there are correlations between physicochemical descriptors and inhibition, and (3) if the inhibitory activity can be related to the reaction between the benzaldehydes and biological nucleophiles. The results from this work will provide insights into the inhibition mechanism of the phenolic aldehydes and may be used to guide the development of cost-effective detoxification practices targeting specific inhibitors.

## 2. MATERIALS AND METHODS

**2.1. Materials.** 2-Hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 2,3-dihydroxybenzaldehyde, 2,4-dihydroxybenzaldehyde, and glucose were obtained from Alfa Aesar (Ward Hill, MA). 4-Hydroxybenzaldehyde, 2,3,4-trihydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin), and glycerol were obtained from Acros Organics (Morris Plains, NJ). 3,4,5-Trihydroxybenzaldehyde and 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin) were obtained from TCI (Tokyo, Japan). 3,5-Dihydroxybenzaldehyde was obtained from Matrix Scientific (Columbia, SC). Benzaldehyde was obtained from Aldrich (Milwaukee, WI). Ethanol was obtained from Sigma-Aldrich (St. Louis, MO). All chemical reagents were purchased as chromatographic grade. 2-Hydroxybenzaldehyde, 2,3-dihydroxybenzaldehyde, and *o*-vanillin were prepared in ethanol individually as 1 M stock solutions and stored at 4 °C before use. All other benzaldehydes were added to the fermentation directly on a weight basis. The structures of all benzaldehydes used in this work are as shown in Figure 1.

**2.2. Fermentation and Inhibition Assay.** Baker's yeast (Fleischmann's), *S. cerevisiae* (ATCC 58515), was used in the fermentation experiments and maintained on a yeast extract peptone dextrose (YPD) agar plate containing 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, and 20 g/L agar at 4 °C. Prior to fermentation, the isolated colony was pre-cultured in YDP liquid medium for 12−15 h and washed twice with sterile water. The yeast concentration was determined using an ultraviolet−visible (UV−vis) spectrophotometer at 600 nm, and an inoculum of 2 g/L was used for the fermentation experiments.

Batch fermentations were conducted in 125 mL sterile serum bottles containing 50 mL of 2% (w/w) glucose solution without supplementing additional nutrients. The benzaldehydes were added to the sterilized glucose solution from their stock solutions or as powders directly. Each benzaldehyde was added at four concentrations. After the benzaldehydes were added, the glucose solution was incubated in a temperature-controlled water bath at 60 °C for 30 min to dissolve the benzaldehydes. The fermentation was performed at 30 °C and spun at 150 rpm in a shaking incubator. Aliquots of 0.25 mL were withdrawn and analyzed by high-performance liquid chromatography (HPLC). All fermentations were run in duplicate.

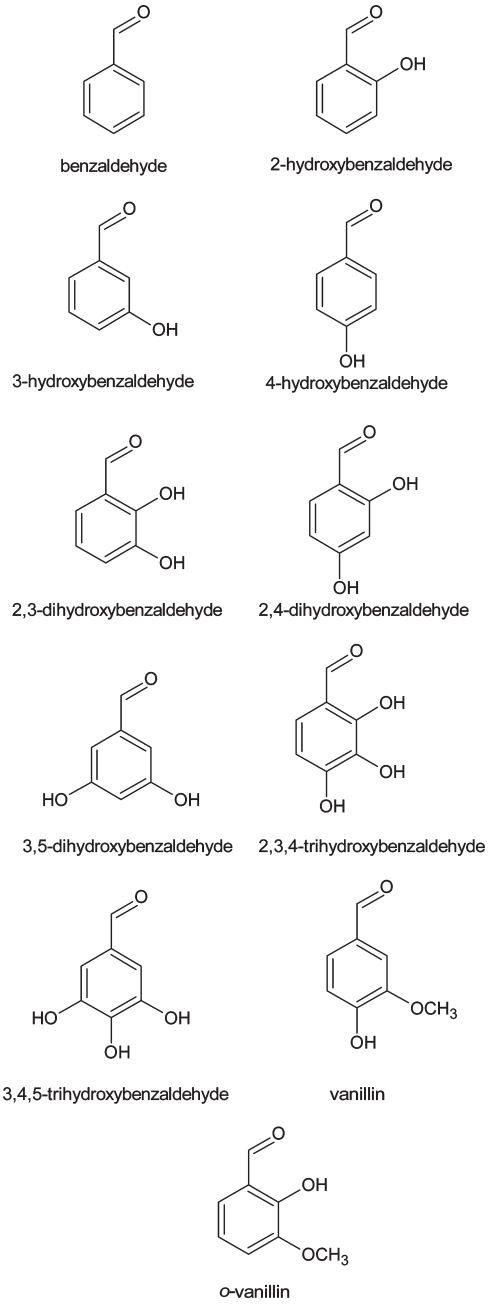


**Figure 1.** Chemical structures of benzaldehyde and phenolic benzaldehydes.

To calculate the half maximal effective concentration ($EC_{50}$), the inhibitory effect of each phenolic aldehyde on the ethanol yield was determined at four concentrations in fermentation. The $EC_{50}$ represents the concentration of phenolic aldehyde resulting in a final ethanol yield of 50% of the control at 48 h. The four concentrations of each phenolic aldehyde were selected to cover a range of the ethanol yield for which 50% was approximately the midpoint. The dose−response results were fitted to a linear relationship from which the $EC_{50}$ value was determined. The lower the $EC_{50}$ value, the higher the inhibitory activity.

**2.3. HPLC Analysis.** Glucose and ethanol were analyzed by HPLC (Shimadzu) using a strong cation-exchange column (Aminex HPX-87H, 300 × 7.8 mm) with a refractive index detector (RID-10A). The mobile phase was 5.0 mM $H_2SO_4$, with a flow rate of 0.6 mL/min and


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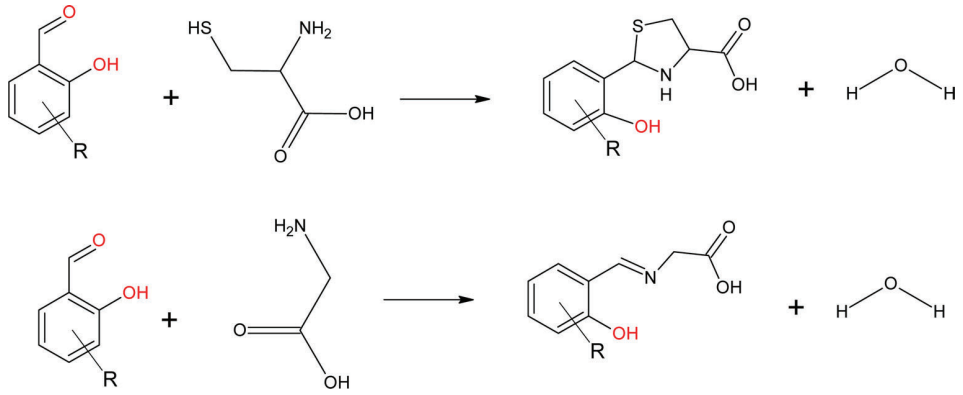

**Figure 2.** Potential reactions between phenolic benzaldehyde and glycine/cysteine.

a column temperature of 45 °C. All compounds were quantified by integrating their peak areas.

**2.4. Calculation of Physicochemical Descriptors and Reaction Parameters.** The octanol/water partition coefficient (log *P*) was calculated for each phenolic aldehyde using Marvin sketch (6.1, VG). $E_{LUMO}$, $E_{HOMO}$, dipole moment, electrophilicity index ($\omega$), and natural bond order (NBO) charges at the reactive center (i.e., the carbonyl group and carbon 1 in the benzene ring) were calculated using Gaussian 09, revision C.01, as implemented on the Alabama Supercomputer Network. The geometry of each benzaldehyde was optimized using the M06-2X density functional method and the 6-311++G(d,p) basis set, with a frequency calculation and ultrafine integration grid. The electrophilicity index ($\omega$) was calculated in following equation:

$$\omega = \frac{\mu^2}{2\eta} = \frac{E_{HOMO}{}^2 + 2E_{HOMO}E_{LUMO} + E_{LUMO}{}^2}{4(E_{LUMO} - E_{HOMO})}$$

where $\mu$ is the molecular chemical potential and $\eta$ is the molecular hardness.

To investigate the reactivity of the phenolic aldehydes with specific biological nucleophiles, the Gibbs free energy change was determined for the reaction of each phenolic aldehyde with cysteine and glycine per the reactions shown in Figure 2. The result was calculated as the difference in free energy between the product and the reactant. It is known that the aldehyde group can participate in covalent bonding with an amine group in the amino acids (free or in proteins) to form Schiff base adducts or react with both amine and thiol groups to form thiazolidine derivatives.[16−18] In the current work, glycine was used to represent amino acids that form Schiff base adducts with hydroxybenzaldehydes and cysteine as an amino acid that forms thiazolidine derivatives with hydroxybenzaldehyde (Figure 2).

**2.5. Statistical Analysis and Structure−Inhibition Relationships.** The data results of molecular descriptors and the $EC_{50}$ value were analyzed using one-way analysis of variance (ANOVA, Excel 2010, Microsoft Office). A $p$ value of <0.05 was considered to be signficant. Statistical calculation of correlations between physiochemical parameters and log $EC_{50}'$ was carried out by regression analysis using Excel 2010 (Microsoft Office). The statistical values include $n$, the number of observations; $s$, the stardard error of the estimate; $r^2$, the coefficient of determination; $F$, Fisher statistic; and $p$, the signficance. A value of $p < 0.05$ indicated that the correlation was significant.

## 3. RESULTS AND DISCUSSION

**3.1. Substitution Effect of Phenolic Aldehyde Inhibition on Alcoholic Fermentation.** The inhibitory effects of different phenolic aldehydes on alcoholic fermentation were examined (Table 1 and Figures 3−5). The results showed that the phenolic hydroxyl group played an important role in inhibition, especially the *ortho* phenolic hydroxyl group.

Benzaldehyde without the −OH group at 20 mM inhibited the ethanol yield to 83% of the reference glucose fermentation (0.42 g/g), with an $EC_{50}$ value of 27.5 mM. The inhibition of phenolic benzaldehydes can be categorized into low, medium, and high levels. The most inhibitory ones were *ortho*-substituted phenolic benzaldehyde, including 2-hydroxybenzaldedyde, 4-hydroxybenzaldehyde, 2,4-dihydroxybenzladehyde, 2,3,4-trihydroxybenzaldehyde, and *o*-vanillin, with an $EC_{50}$ value of 0.9−5.2 mM. The medium level of inhibitors was *para*- or *meta*-substituted phenolic benzaldehyde, including 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde ($EC_{50}$ value of 14.9−18.6 mM). The least inhibitory ones were 3,5-dihydroxybenzaldehyde, 3,4,5-trihydroxybenzaldehyde, and vanillin ($EC_{50}$ value of 25.9−40 mM), which have no *ortho* substitution.

In comparison of benzaldehyde and 2-hydroxybenzaldehyde, the later showed 30-fold higher inhibition activity than the former (Table 1 and Figure 3). The remaining seven phenolic benzaldehydes also showed higher inhibition than benzaldehyde, except for 3,5-dihydroxybenzaldehyde and 3,4,5-trihydroxybenzaldehyde. This indicated that the phenolic hydroxyl group in benzaldehydes was essential for their inhibition on yeast fermentation. To further examine the substitution effect on benzaldehyde inhibition, it was observed that *ortho*-substituted 2-hydroxybenzaldehyde resulted in 15−20-fold higher inhibition than the *meta*- or *para*-substituted analogues of 3- and 4-hydroxybenzaldehydes (Table 1 and Figure 3). This agreed well with previous findings that *ortho*-substituted benzaldehyde showed the highest inhibition against *Aspergillus oryzae* and the other eight fungal strains.[19] Previously, Fredman et al. also reported that the antimicrobial activity of *ortho*-substituted benzaldehyde was higher than 3- and 4-hydroxybenzaldehydes and vanillin in the assay against *Escherichia coli*.[20]

In *o*-substituted phenolic aldehyde, the phenolic hydroxyl group can form an intramolecular hydrogen bond between −OH and carbonyl oxygen. This will stabilize a resonance structure with a positive charge on the carbonyl carbon. Consequently, it increases the carbonyl carbon electrophilicity.

Analysis of isomers of dihydroxybenzaldehyde also suggested that the *ortho* −OH group can influence the inhibition significantly (Table 1 and Figure 4), because 3,5-dihydroxybenzaldehyde was much less inhibitory ($EC_{50}$ > 40 mM) than 2,3- and 2,4-dihydroxybenzaldehydes ($EC_{50}$ of 0.9−2.1 mM). Similar observations have been reported previously that 3,4-dihydroxybenzaldehyde was less active against bacteria



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**Table 1. Inhibition of Phenolic Benzaldehydes on Fermentation by *S. cerevisiae***

| compound | concentration (mM) | $Y_{EtOH}$[a] (g/g) | $EC_{50}$[b] (mM) |
|---|---|---|---|
| glucose control | | 0.42 ± 0.00 | N/A |
| benzaldehyde | 40.0 | 0.03 ± 0.00 | 27.5 |
| | 20.0 | 0.35 ± 0.02 | |
| | 10.0 | 0.47 ± 0.01 | |
| | 5.0 | 0.46 ± 0.01 | |
| 2-hydroxybenzaldehyde | 5.0 | 0.01 ± 0.00 | 0.9 |
| | 2.5 | 0.02 ± 0.00 | |
| | 1.0 | 0.22 ± 0.03 | |
| | 0.5 | 0.42 ± 0.00 | |
| 3-hydroxybenzaldehyde | 40.0 | 0.03 ± 0.00 | 14.9 |
| | 20.0 | 0.10 ± 0.00 | |
| | 10.0 | 0.44 ± 0.00 | |
| | 5.0 | 0.44 ± 0.01 | |
| 4-hydroxybenzaldehyde | 40.0 | 0.05 ± 0.00 | 18.6 |
| | 20.0 | 0.18 ± 0.00 | |
| | 10.0 | 0.46 ± 0.00 | |
| | 5.0 | 0.44 ± 0.00 | |
| 2,3-dihydroxybenzaldehyde | 5.0 | 0.05 ± 0.00 | 0.9 |
| | 2.5 | 0.06 ± 0.00 | |
| | 1.0 | 0.11 ± 0.00 | |
| | 0.5 | 0.45 ± 0.00 | |
| 2,4-dihydroxybenzaldehyde | 10.0 | 0.06 ± 0.00 | 2.1 |
| | 5.0 | 0.07 ± 0.00 | |
| | 2.5 | 0.18 ± 0.01 | |
| | 1.0 | 0.44 ± 0.00 | |
| 2,3,4-trihydroxybenzaldehyde | 10.0 | 0.08 ± 0.00 | 5.2 |
| | 5.0 | 0.17 ± 0.00 | |
| | 2.5 | 0.44 ± 0.00 | |
| | 1.0 | 0.44 ± 0.01 | |
| 3,5-dihydroxybenzaldehyde | 40.0 | 0.44 ± 0.00 | >40 |
| | 20.0 | 0.44 ± 0.01 | |
| | 10.0 | 0.44 ± 0.00 | |
| | 5.0 | 0.44 ± 0.00 | |
| 3,4,5-trihydroxybenzaldehyde | 40.0 | 0.43 ± 0.00 | >40 |
| | 20.0 | 0.43 ± 0.00 | |
| | 10.0 | 0.43 ± 0.00 | |
| | 5.0 | 0.43 ± 0.01 | |
| vanillin | 40.0 | 0.13 ± 0.00 | 25.9 |
| | 20.0 | 0.22 ± 0.00 | |
| | 10.0 | 0.43 ± 0.00 | |
| | 5.0 | 0.42 ± 0.00 | |
| *o*-vanillin | 5.0 | 0.05 ± 0.00 | 1.5 |
| | 2.5 | 0.07 ± 0.01 | |
| | 1.0 | 0.43 ± 0.00 | |
| | 0.5 | 0.43 ± 0.00 | |

[a] $Y_{EtOH}$ represents the ethanol yield at 48 h based on original glucose. [b] $EC_{50}$ represents the concentration of phenolic aldehydes resulting in a final ethanol yield of 50% of the control at 48 h.

compared to *ortho*-substituted dihydroxybenaldehydes.[20] Fitzgerald et al. also suggested that the closer side groups to the aldehyde moiety resulted in the greater antimicrobial activity for phenolic aldehydes. This was evidenced by our observations, especially for the side −OH group. The trihydroxybenzaldehydes also showed the importance of the *ortho* −OH group in benzaldehyde inhibition, but the extra −OH group did not increase the inhibition. However, previous research has reported that the antibacterial activity of trihydroxybenzaldehyde was higher than those of di- and monohydroxybenzaldehydes. This probably was because different microbes (bacteria versus yeast) were used in the evaluation system.

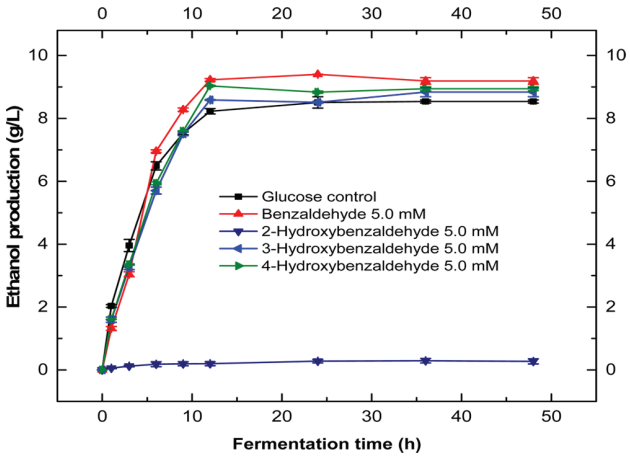


**Figure 3.** Effect of 2-, 3-, and 4-hydroxybenzaldehydes on ethanol production by *S. cerevisiae*.

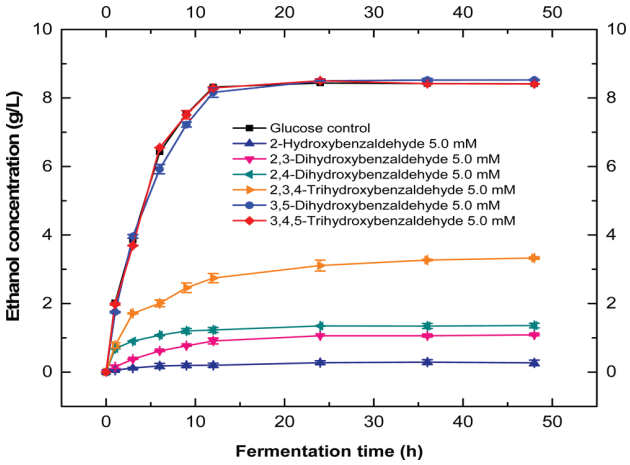


**Figure 4.** Effect of di- and trihydroxybenzaldehydes on ethanol production by *S. cerevisiae*.

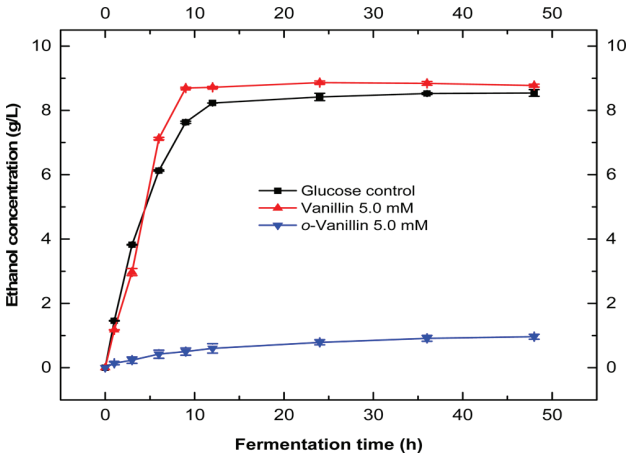


**Figure 5.** Effect of vanillin and *o*-vanillin on ethanol production by *S. cerevisiae*.

The comparison of vanillin and *o*-vanillin with 2-hydroxybenzaldehyde revealed that the presence of a methoxyl group


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Table 2. Calculated Physicochemical Descriptors of Phenolic Benzaldehydes and Their Inhibition

| compound | $C_{carb}$ | $O_{carb}$ | $C_1$ | log $P$ | $E_{HOMO}$ (au) | $E_{LUMO}$ (au) | dipole (debye) | $\omega$ | log $EC'_{50}$ [a] |
|---|---|---|---|---|---|---|---|---|---|
| benzaldehyde | 0.435 | 0.529 | 0.177 | 1.69 | −0.3255 | −0.0394 | 3.380 | 0.116 | 4.439 |
| 2-hydroxybenzaldehyde | 0.427 | 0.510 | 0.224 | 2.03 | −0.2990 | −0.0322 | 4.331 | 0.103 | 2.954 |
| 3-hydroxybenzaldehyde | 0.436 | 0.525 | 0.154 | 1.38 | −0.3011 | −0.0407 | 4.372 | 0.112 | 4.173 |
| 4-hydroxybenzaldehyde | 0.433 | 0.539 | 0.209 | 1.38 | −0.3015 | −0.0305 | 4.481 | 0.102 | 4.270 |
| 2,3-dihydroxybenzaldehyde | 0.428 | 0.508 | 0.213 | 1.73 | −0.2872 | −0.0318 | 5.711 | 0.100 | 2.954 |
| 2,4-dihydroxybenzaldehyde | 0.425 | 0.519 | 0.252 | 1.73 | −0.2950 | −0.0220 | 4.959 | 0.092 | 3.322 |
| 3,5-dihydroxybenzaldehyde | 0.437 | 0.520 | 0.134 | 1.08 | −0.2940 | −0.0410 | 4.138 | 0.111 | 4.602 |
| 2,3,4-trihydroxybenzaldehyde | 0.426 | 0.515 | 0.235 | 1.43 | −0.2888 | −0.0218 | 6.474 | 0.090 | 3.716 |
| 3,4,5-trihydroxybenzaldehyde | 0.434 | 0.531 | 0.163 | 0.78 | −0.2911 | −0.0323 | 5.630 | 0.101 | 4.602 |
| vanillin | 0.433 | 0.547 | 0.190 | 1.22 | −0.2860 | −0.0299 | 2.286 | 0.097 | 4.413 |
| *o*-vanillin | 0.428 | 0.511 | 0.218 | 1.87 | −0.2820 | −0.0276 | 5.939 | 0.094 | 3.114 |

[a] Log $EC'_{50}$ represents log($EC_{50}$ × 1000), in which the concentration unit of $EC_{50}$ was changed from millimolar to micromolar.

Table 3. Regression Analysis between Log $EC'_{50}$ and Molecular Descriptors

| regression | $n$ | $r^2$ | $S$ | $F$ | $p$ |
|---|---|---|---|---|---|
| log $EC'_{50}$ = −53.19 + 132.36$C_{carb}$ | 10 | 0.73 | 0.37 | 24.70 | <0.001 |
| log $EC'_{50}$ = −17.97 − 41.75$O_{carb}$ | 10 | 0.60 | 0.45 | 13.69 | 0.005 |
| log $EC'_{50}$ = 6.57 + 13.79$C_1$ | 10 | 0.57 | 2.56 | 11.79 | 0.007 |
| log $EC'_{50}$ = 6.10 − 132.36 log $P$ | 10 | 0.69 | 0.40 | 19.58 | 0.002 |
| log $EC'_{50}$ = 2.01 − 19.89$E_{HOMO}$ | 10 | 0.12 | 0.66 | 1.25 | 0.293 |
| log $EC'_{50}$ = 2.26 + 50.55$E_{LUMO}$ | 10 | 0.25 | 0.61 | 2.99 | 0.118 |
| log $EC'_{50}$ = 5.11 − 0.265dipole | 10 | 0.23 | 0.62 | 2.68 | 0.136 |
| log $EC'_{50}$ = −0.17 − 39.79$\omega$ | 10 | 0.25 | 0.61 | 3.01 | 0.117 |
| log $EC'_{50}$ = −31.71+ 85.50$C_{carb}$ − 0.86 log $P$ | 10 | 0.87 | 0.27 | 25.88 | <0.001 |

appeared to be not important in benzaldehyde inhibition (Table 1 and Figure 5). However, the position of the −OH group was critical in the contribution of a higher inhibitory activity of *o*-vanillin. These results are consistent with the previous observations[6] that 1.3 mM *o*-vanillin completely inhibited both cell growth and ethanol production, while vanillin and isovanillin had no inhibitory activity.

We hypothesize that the inhibitory activity of phenolic aldehydes are governed by the chemical reactivity of carbonyl groups (C═O), which, in turn, was affected by the *ortho*-substituted −OH group. Previously, various atomic and molecular descriptors have been widely used to characterize chemical reactivity of quinones, unsaturated esters, and phenols.[21,22] These descriptors include atomic charge, hydrophobicity (octanol/water partition coefficient, log $P$), energy of the lowest unoccupied molecular orbital ($E_{LUMO}$), energy of the highest occupied molecular orbital ($E_{HOMO}$), dipole moment, and electrophilicity index ($\omega$).[14,23]

**3.2. QSAR Analysis.** The $EC_{50}$ value of phenolic benzaldehydes on yeast fermentation was correlated with atomic and molecular descriptors, as summarized in Table 2. A strong correlation ($r^2$ = 0.73; $p$ < 0.001) has been observed between the partial charge on carbonyl carbon ($C_{carb}$) and the $EC_{50}$ value of aldehydes (eq 1 in Table 3). Within the carbonyl group, the electronegative oxygen draws electrons away from the carbonyl carbon, making it partially positive and electrophilic. However, the higher partial charge of $C_{carb}$ resulted in lower inhibition with a higher $EC_{50}$ value. This suggested that the calculated partial charge of $C_{carb}$ probably cannot be used to associate with the electrophilicity of phenolic aldehydes in this study. Strong association was also observed between log $P$ and the $EC_{50}$ value. Log $P$ is a global parameter, which measures the hydrophobicity of a molecule.[24] It indicates the ability of the compound to diffuse through the cell membrane to reach intracellular targets.[25] The strong positive relationship between log $P$ and inhibition suggests that more hydrophobic phenolic aldehydes may diffuse through the cell membrane and, therefore, be more likely to react with biological nucleophilic sites in the yeast cell, causing inhibition. Recently, Over et al. found that an intramolecular hydrogen bond can improve the membrane permeability in small-molecule drug development.[26] Ashwood et al. also reported to use an intermolecular hydrogen bond to increase the lipophilivity of an antagonist compound and enhance its central nervous system penetration and pharmacological activity.[27] It was interesting to notice that the most inhibitory 2-hydroxybenzaldehyde has the highest log $P$, which possesses a *ortho* −OH group capable of forming an intramolecular hydrogen bond, which can potentially remove one donor hydrogen and one acceptor oxygen from benzaldehyde. Thus, the intramolecular hydrogen bond in *ortho*-substituted benzaldehydes increased the cell membrane permeability and toxicity. These results are in agreement with previous reports that the toxicity of aldehydes toward ethanologenic *E. coli* and *S. cerevisiae* was related to their hydrophobicity.[8,12] Linear regression analysis revealed that $E_{LUMO}$, $E_{HOMO}$, dipole moment, and $\omega$ were not correlated to the fermentation inhibitory (Table 3).

**3.3. Gibbs Free Energy Change of a Reaction between Benzaldehydes and Cysteine/Glycine.** To examine the substitution effect of phenolic aldehydes on the reaction free energy change ($\Delta G$), we calculated the $\Delta G$ of a reaction between benzaldehydes and cysteine/glycine (Table 4). As shown in Figure 2, we propose that benzaldehydes react with cysteine/glycine to form a Schiff base by nucleophilic addition. Similar reactions between aldehydes and biological nucleophiles have been suggested to lead to the inhibition of important protein/enzyme functions or even loss of cell activity.[21,28] The Gibbs free energies of reaction values are strongly correlated



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**Table 4. Calculated Gibbs Free Energy Change of a Reaction ($\Delta G$) between Phenolic Benzaldehydes and Cysteine/Glycine**

| compound | reaction with cysteine ($\Delta G$, kcal/mol) | reaction with glycine ($\Delta G$, kcal/mol) |
|---|---|---|
| benzaldehyde | 7.04 | 15.97 |
| 2-hydroxybenzaldehyde | 3.34 | 12.92 |
| 3-hydroxybenzaldehyde | 6.65 | 16.61 |
| 4-hydroxybenzaldehyde | 8.38 | 17.01 |
| 2,3-dihydroxybenzaldehyde | 3.96 | 13.37 |
| 2,4-dihydroxybenzaldehyde | 4.74 | 13.97 |
| 3,5-dihydroxybenzaldehyde | 6.15 | 15.56 |
| 2,3,4-trihydroxybenzaldehyde | 4.37 | 13.2 |
| 3,4,5-trihydroxybenzaldehyde | 7.05 | 15.83 |
| vanillin | 9.03 | 18.64 |
| *o*-vanillin | 3.57 | 12.87 |

with $EC_{50}$ (Figure 6). The most inhibitory compound of 2-hydroxybenzaldehyde has the lowest $\Delta G$ (3.34 kcal/mol) when

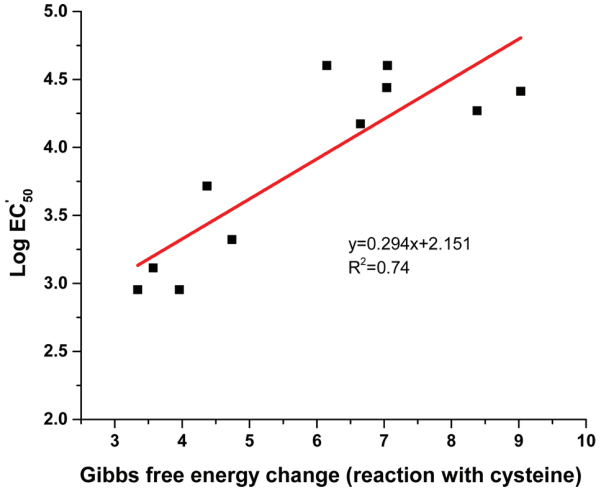


**Figure 6.** Correlation between Gibbs free energy change and log $EC'_{50}$.

reacts with cysteine. The Gibbs free energy change measures the driving force of a reaction, in which a lower $\Delta G$ indicates a higher tendency for a reaction to occur. This means that 2-hydroxybenzaldehyde can readily react with biological nucleophiles in cells and decrease the cell viability compared to other benzaldehydes. The results also indicated that the calculated free energy change between benzaldehydes and amino acids can also be used to predict their structure−activity relationship. For all tested benzaldehydes, the $\Delta G$ values for reaction with cysteine were lower than those for reaction with glycine, suggesting that this nucleophilic addition has a higher reactivity with cysteine. This is probably due to the higher nucleophilicity of the thiol group in cysteine as opposed to the amine group in glycine.[29,30]

## 4. CONCLUSION

The effects of phenolic aldehydes with different substitution on ethanolic fermentation have been examined in this study. With an attempt to better understand the structure−inhibitory activity relationship of phenolic aldehydes on fermentation, we have found that the *ortho*-substituted hydroxyl group played an important role in phenolic aldehyde inhibition. The presence of the *ortho* −OH group can form an intramolecular hydrogen bond within phenolic aldehydes, which probably increase the membrane permeability and toxicity. A strong correlation was observed between log *P* and inhibition, which suggested that compounds with higher hydrophobicity had higher toxicity. The results also indicated that the calculated free energy change between phenolic aldehydes and amino acids can be used to predict their structure−inhibitory activity relationship. Understanding the substitution effect of phenolic aldehydes on fermentation is important if we are to reveal the inhibition mechanism of the phenolic aldehydes in biomass hydrolysates and to develop a cost-effective detoxification process in biofuel production. Future work is needed to confirm if the presence of the *ortho* −OH group can increase the membrane permeability.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.energyfuels.5b03034.

Subsitution effect of benzaldehydes on ethanol production by *S. cerevisiae* (Figure S1) (PDF)

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### Notes

The authors declare no competing financial interest.

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