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Quantifying second generation ethanol inhibition: Design of Experiments approach and kinetic model development

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HIGHLIGHTS

- Novel DoE methodology was used to guide kinetic model development.
- High levels of ethanol masked the effects of other inhibitors.
- Removal of ethanol from the DoE design enabled identification of other effects.
- A simple kinetic model accounting for DoE-identified inhibition was developed.
- DoE was also used to identify significant effects on HMF and furfural reduction.

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ABSTRACT

While softwoods represent a potential feedstock for second generation ethanol production, compounds present in their hydrolysates can inhibit fermentation. In this study, a novel Design of Experiments (DoE) approach was used to identify significant inhibitory effects on *Saccharomyces cerevisiae* D_5A for the purpose of guiding kinetic model development. Although acetic acid, furfural and 5-hydroxymethyl furfural (HMF) were present at potentially inhibitory levels, initial factorial experiments only identified ethanol as a significant rate inhibitor. It was hypothesized that high ethanol levels masked the effects of other inhibitors, and a subsequent factorial design without ethanol found significant effects for all other compounds. When these non-ethanol effects were accounted for in the kinetic model, $\bar{R}^2$ was significantly improved over an ethanol-inhibition only model ($\bar{R}^2$ = 0.80 vs. 0.76). In conclusion, when ethanol masking effects are removed, DoE is a valuable tool to identify significant non-ethanol inhibitors and guide kinetic model development.

1. Introduction

Ethanol production from lignocellulosic material such as wood or agricultural wastes is becoming increasingly important due to the environmental effects, extensive land use, and limited potential of first generation biofuels. Although second generation biofuels offer the potential for increased quantities of more environmentally friendly fuels, the recalcitrant nature of biomass makes conversion more challenging. To break the complex structure of lignocellulose and produce fermentable sugars, a harsh pretreatment step is often required before enzymatic hydrolysis. Several compounds created during pretreatment are inhibitory to yeasts, including organic acids, aldehydes, and phenolics. Inhibitors slow cell growth and can negatively affect ethanol production rates and yields. If the hydrolysate is then concentrated (a potential strategy to improve ethanol titers), individual inhibitor concentrations also change. For example, recent results show that evaporation can remove 100% of furfural, 10.8% of acetic acid and 8.9% of 5-hydroxymethyl furfural (HMF) from a pine hydrolysate at pH 5.0 (Gurram and Menkhaus, 2013); however, the 3.4-fold reduction in total volume still resulted in a 3.1-fold increase in acetic acid and HMF concentrations. Low pH evaporation can remove additional acetic acid, but higher pH adjustment costs are then incurred, and overall acetic acid concentrations still increase (Cox et al., 1993). Similarly, membrane separations such as reverse osmosis or nanofiltration can be used to selectively concentrate sugars while removing inhibitory compounds, but membrane foul-

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ing can become problematic (Gautam and Menkhaus, 2014; Leberknight and Menkhaus, 2013). It is also possible to use specific operations that target the removal of inhibitory compounds from biomass slurries, such as polyelectrolyte flocculation/adsorption, but these can add complexity and cost to the overall process (Burke et al., 2011; Carter et al., 2011a,b). Because inhibitor removal from concentrated hydrolysates is costly, it is important to establish which inhibitors are most detrimental, if they interact synergistically, and the upper limits for hydrolysate concentration and subsequent fermentation.

Organic acids like acetic acid affect *Saccharomyces cerevisiae* by diffusing through the plasma membrane and lowering the intracellular pH, which must be neutral for optimal cell function. Low acetic acid concentrations can actually stimulate ethanol production rates and yields (Palmqvist et al., 1999; Palmqvist and Hahn-Hägerdal, 2000); however, higher concentrations are inhibitory, slowing growth and ethanol production. Acetic acid tolerance in *S. cerevisiae* is strain dependent; for one strain, concentrations as low as 6 g/l reduced ethanol production by 74% (Phowchinda et al., 1995), while higher concentrations (up to 9 g/l) improved ethanol productivity for another strain (Palmqvist et al., 1999). The medium pH also plays a role in inhibition, as only the un-dissociated acid diffuses through the cell membrane, lowering the intracellular pH (Palmqvist and Hahn-Hägerdal, 2000).

Furfural and HMF inhibit glycolytic enzymes and furfural may also affect cell membrane integrity. These compounds cause a lag in cell growth until they are metabolized by the organism (Banerjee et al., 1981; Palmqvist and Hahn-Hägerdal, 2000). Furfural inhibition is also strain dependent, with concentrations as low as 2 g/l reported as inhibitory (Boyer et al., 1992). Larger inocula tend to decrease aldehyde inhibition due to aldehyde metabolism at faster rates. In batch experiments with 2 g/l furfural, inhibition was not observed with a relatively high cell concentration (3 gDW/l), but was significant for a cell concentration of 0.2 gDW/l (Palmqvist et al., 1999). HMF is not as well studied; it is reported to be less inhibitory than furfural, but is metabolized more slowly (Taherzadeh et al., 2000). For one *S. cerevisiae* strain, HMF concentrations of 3 g/l were reported to be inhibitory (Keating et al., 2006), while another strain maintained 50% of its inherent ethanol production capacity at HMF concentrations up to 8 g/l (Clark and Mackie, 1984).

The effects of high sugars and ethanol concentrations on *S. cerevisiae* are also important. Glucose becomes inhibitory at concentrations above 100 g/l through osmotic stress effects (Pratt et al., 2003; Shuler and Kargi, 2002), but high gravity concentrations (up to 330 g/l) are still fermentable by industrial strains (Pereira et al., 2010). As sugars are converted to ethanol, osmotic effects are replaced with ethanol inhibition. Ethanol decreases membrane fluidity, leading to increased proton flux and a lower intracellular pH (Ma and Liu, 2010). Ethanol tolerance has also been shown to be strain dependent; in high gravity batch experiments, laboratory strain CEN.PK 113-7D was limited to a final ethanol titer of 130 g/l while an industrial strain (PE-2) was able to produce 147 g/l (Pereira et al., 2010).

When multiple inhibitors are present in a fermentation system, the net inhibition may be greater than the additive effects of the individual compounds (synergistic effect). Acetic acid and furfural are reported to interact synergistically to inhibit the cell yield, ethanol yield, and specific growth rate in *S. cerevisiae* even at low concentration combinations (0.5 g/l furfural and 5 g/l acetic acid) (Palmqvist et al., 1999). Furfural and HMF can also synergistically inhibit *S. cerevisiae*, completely stopping growth at levels of 30 mmol/l each (2.9 g/l Furfural and 3.8 g/l HMF) (Liu et al., 2004). These synergisms become increasingly important in lignocellulose hydrolysates which may contain all of these compounds at inhibitory levels. Because inhibition is strain dependent, synergism reported for one *S. cerevisiae* strain at one set of concentrations may not be observed in another strain; therefore, it is important that a kinetic model be strain and concentration specific to accurately predict fermentation performance.

To accurately quantify inhibition in a kinetic model, each significant effect needs to be accounted for. Using the traditional one factor at a time (OFAT) approach, trial and error is required, either experimentally by varying one inhibitor at a time (some of which may not be important), or mathematically by adding and deleting model terms to find a best fit to experimental data. In either case, some experiments or kinetic model guesses will be unnecessary or wrong, making the process time consuming and inefficient. In addition, many OFAT experiments are required to check for synergistic effects.

An alternative to OFAT is the Design of Experiments (DoE) approach. DoE is a rigorous statistical method used to determine the significant effects of multiple variables on a given system response. In contrast to OFAT, DoE determines significance by running carefully designed experiments in which all possible effects are present and varied from run to run. By simultaneously checking for all possible main effects and synergisms, DoE can reduce the number of experimental runs and amount of trial and error required. DoE methodology has been previously utilized in fermentation experiments for growth medium design, inhibitor identification, fermentation optimization, and identification of variables important to fermentor operation (Graves et al., 2007; Laluce et al., 2009; Palmqvist et al., 1999; Pereira et al., 2010; Unrean and Nguyen, 2012). Specifically relevant to our study is another DoE fermentation study (Palmqvist et al., 1999) in which combinations of acetic acid, furfural and p-hydroxybenzoic acid were varied for their effect on different *S. cerevisiae* strains. DoE methodology identified several main effects as well as a synergistic effect between acetic acid and furfural; a comparable study with OFAT methodology would have required additional runs and may not have identified this important synergism.

Our current study uses DoE methodology to identify which lignocellulose hydrolysate inhibitors and/or combinations of inhibitors significantly impact *S. cerevisiae* D_5A fermentation. Significant DoE responses will then be used to guide development of a strain-specific kinetic model that can predict hydrolysate fermentation performance under stress from multiple inhibitors. The major softwood hydrolysate inhibitors (acetic acid, furfural and HMF), were studied for their effect on our organism. Glucose and ethanol were also included to account for possible substrate and product inhibition, both of which are important in high gravity fermentations. Predicting continuous fermentation with biocatalyst recycle performance was of special interest for follow-on studies; high cell density continuous cultures have been shown to improve ethanol productivity substantially and quickly metabolize aldehydes. To capture ethanol effects present in continuous fermentation systems at steady state, starting ethanol concentrations of 17–50 g/l (DoE #1) and 25–75 g/l (DoE #2) were screened in the initial factorial designs. Unique to this work is the simultaneous DoE screening of acetic acid, aldehydes, glucose and ethanol to guide rigorous kinetic model development.

2. Methods

2.1. Experimental design

Design-Expert® 8 software (Stat-Ease) was used to design factorial experiments to study the inhibitory effects of glucose, ethanol, acetic acid, furfural and HMF on *S. cerevisiae* D_5A; the specific rates of cell growth (μ), glucose consumption (dS'/dt), and ethanol production (dP'/dt) were quantified as responses. Later, the overall fer-