Recombinant Manganese Peroxidase Production and Recovery

Chris Child, James Patterson, and Regina Pimentel
School of Chemical, Biological, and Environmental Engineering

Issue

Issue: The lignin in plant cell walls obstructs hemi-cellulose and cellulose polymers contained in plant biomass, which prevents their conversion to fermentable sugars used in biofuel production.

Project Objectives

- Develop an assay to monitor MeOH levels in the broth
- Produce recombinant manganese peroxidase (MnP, Figure 1) at ≥ 2000 U/L
- Maintain dissolved oxygen (DO) levels at ≥ 20% liquid saturation

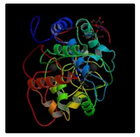


Figure 1: MnP structure.
Source: Marc Stephenson, CSUN.

Lignin Degradation

The key components of plant cell walls are the polymers lignin, hemi-cellulose, and cellulose. Both hemi-cellulose and cellulose are readily converted into fermentable sugars, but lignin hinders this conversion.

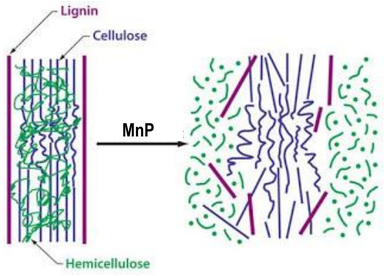


Figure 2: Diagram of plant cell wall components disrupted by MnP oxidation.
Source: Gary Brodeur et al., 2011.

MnP is an enzyme naturally produced by the white rot fungus *Phanerochaete chrysosporium*. The gene encoding MnP has been cloned into a *Pichia pastoris* yeast vector for large-scale production. MnP oxidizes lignin, increasing hemi-cellulose and cellulose availability for biofuel production.

Bioreactor Setup

P. pastoris yeast cells were cultivated in a bioreactor (Figure 3) for a batch time of 24 hours followed by 56 hours of fed batch growth. Initial growth was mediated by glycerol substrate then switched over to methanol substrate to promote rMnP production.

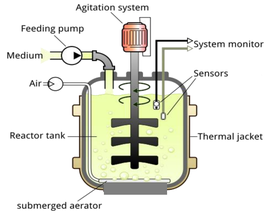


Figure 3: Simplified diagram of a bioreactor.
Source: Yassine Mrabet, 2009.

Cell and Enzyme Assays

Sample absorbance at 600 nm was related to cell density with Equation 1 (Figure 4). Supernatant absorbance at 469 nm was related to enzyme activity with Equation 2 (Figure 5).

Density *[g/L]* =

$$0.3 \cdot A_{600\,nm} \cdot Dilution\ Factor \quad \textbf{(1)}$$

Activity *[U/L]* =

$$\frac{A_{469\,nm} \cdot Vol_{assay} \cdot 10^6}{49{,}600\ M^{-1}cm^{-1} \cdot Vol_{sample}} \quad \textbf{(2)}$$

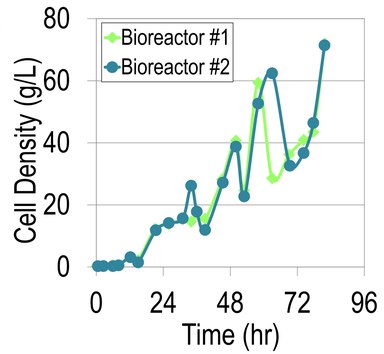


Figure 4: Cell density during batch and fed-batch phases. Growth is dependent on both substrate (glycerol/methanol) and DO levels.

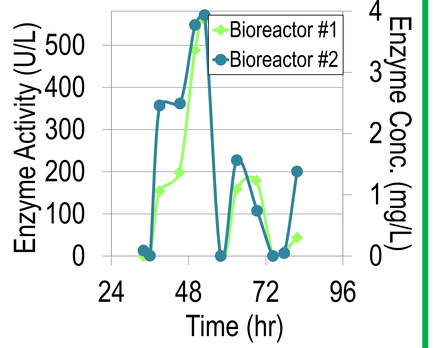


Figure 5: Enzyme production begins during methanol fed-batch phase and is dependent on methanol concentration.

CARB Methanol Assay

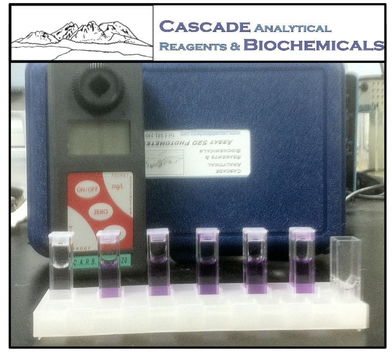
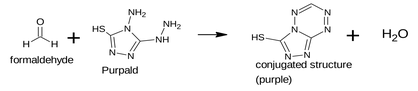


Figure 6: CARB methanol assay kit with Purpald-reacted samples.

The CARB methanol assay quantitatively measures methanol concentration (Figure 6). Alcohol oxidase (AOX) oxidizes methanol to formaldehyde (Equation 3). Purpald reagent then reacts with the formaldehyde to form a conjugated, purple ring-structure (Equation 4).

(3)



(4)

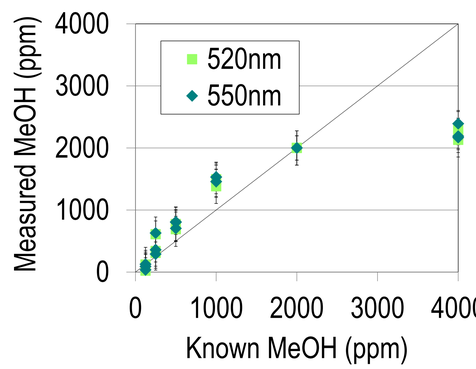


Figure 7: Methanol assay calibration curve (R^2 = 0.9864). The linear range at 520 nm is 1000 ppm to 2000 ppm.

Sample absorbance at 520 nm was compared with a 2,000-ppm methanol standard absorbance to calculate methanol concentration (Equation 5). A calibration curve was created using known concentrations of methanol (Figure 7) and fit to a line (Equation 6).

$$\%\ MeOH = 0.2 \cdot \frac{sample\ absorbance}{standard\ absorbance} \quad \textbf{(5)}$$

$$Measured\ \%\ MeOH = 1.5\ (Known\ \%\ MeOH) \quad \textbf{(6)}$$

Off-gas Analysis

Henry's Law states that at a gas-liquid interface, the partial pressure of the gas is proportional to the gas concentration in the liquid (Figure 8). This law relates headspace O_2 and CO_2 concentrations to the liquid concentrations in the bioreactor at constant temperature (Figure 9).

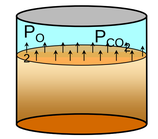


Figure 8: Partial pressure at a gas-liquid interface at equilibrium.

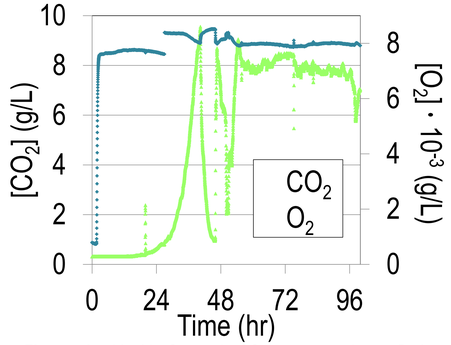


Figure 9: Liquid O_2 and CO_2 concentrations. Both concentrations plateau when cell growth equals cell death.

Future Work

Overall, the assays used for this project proved to be effective methods for in-line analysis of samples during bioreactor runs. The methanol assay reagents are both heat and time sensitive, so they must be prepared prior to each sample analysis. The off-gas analysis setup provided a real-time view of the off-gas concentrations, which can be correlated to concentrations in the liquid. Suggestions for future investigation are provided below.

- Recalibrate the DO probe
- Transport AOX on ice
- Continue testing the validity of in-line methanol testing
- Use off-gas analysis instead of DO probe data
- Test Purpald shelf-life for refrigeration at 4 C vs. freezing at -20 C vs. room temperature at 20 C

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