Mechanisms of Surfactant Effects on Biomass Conversion

A Final Report Submitted to

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Submitted by

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Abstract

Non-ionic surfactants are known to enhance the biochemical conversion of lignocellulosic biomass to bioethanol. Their mechanisms of action, however, are incompletely understood. This research was conducted to elucidate those mechanisms by studying the effects of the surfactant Tween 80 on the interactions of cellulose, lignin, and lignocellulosic substrates with cellulolytic enzymes. Model substrates for adsorption and hydrolysis studies by quartz crystal microbalance with dissipation monitoring were prepared from aqueous cellulose nanocrystal and lignin suspensions or lignin solutions with Kraft lignin, organosolv lignin, and milled wood lignin. Whether substrate-adsorbed or in solution, Tween 80 had no effect on the apparent enzymatic hydrolysis rate of model cellulose substrates. Depending on the type of lignin, Tween 80 caused removal of lignin from model lignin substrates and either increased or decreased adsorption of cellulolytic enzymes onto these substrates. In lignocellulosic substrates, lignin hindered both the adsorption of cellulolytic enzymes onto the substrates and the enzymatic hydrolysis of the substrates. Substrate-adsorbed Tween 80 increased the apparent enzymatic hydrolysis rate of lignocellulosic substrates but the extent of increase depended strongly on the lignin type and substrate morphology. Hence, non-ionic surfactants may mitigate the inhibitory effect of lignin on the enzymatic hydrolysis of lignocellulosic biomass.
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Executive Summary

Surfactants are molecules that contain both an oil-soluble and a water-soluble component. Several studies have shown that non-ionic surfactants have beneficial effects on the biochemical conversion of lignocellulosic biomass, including increased hydrolysis rates and glucose yields, better enzyme recovery, lower required enzyme loadings, increased ethanol yields, and reduced time to attain maximum ethanol concentration. However, the mechanisms by which non-ionic surfactants enhance biomass conversion are incompletely understood. The aim of this project was to elucidate those mechanisms by studying the effects of the non-ionic surfactant Tween 80 on the interactions of model cellulose, lignin, and lignocellulosic substrates with cellulolytic enzymes. Model substrates for adsorption and hydrolysis studies by quartz crystal microbalance with dissipation monitoring were prepared from aqueous suspensions of cellulose nanocrystals and lignin particles and from lignin solutions. Model lignin and lignocellulosic substrates were prepared with three different types of lignin, namely Kraft lignin, organosolv lignin, and milled wood lignin. The presence of lignin in lignocellulosic substrates caused both decreased adsorption of cellulolytic enzymes onto the substrates and decreased apparent hydrolysis rates, relative to those observed for cellulose substrates. Tween 80 was found to adsorb onto all types of model substrates and, depending on the type of lignin, cause lignin removal from model lignin and layered lignocellulosic substrates. Depending on the type of lignin, substrate-adsorbed Tween 80 was found to either increase or decrease adsorption of cellulolytic enzymes on lignin substrates. Whether substrate-adsorbed or in solution, Tween 80 molecules did not significantly affect the apparent enzymatic hydrolysis rate of cellulose substrates. In contrast, the apparent enzymatic hydrolysis rate of lignocellulosic substrates was increased after exposure of the substrates to Tween 80. For layered lignocellulosic substrates, the increase was caused by Tween 80-mediated removal of lignin from the substrate, making the subjacent cellulose layer more accessible to the enzymes. The study has shown that non-ionic surfactants may mitigate the inhibitory effect of lignin on the enzymatic hydrolysis of lignocellulosic biomass.
Problem Statement

The Renewable Fuels Standard of the Energy Independence and Security Act of 2007 mandates that 36 billion gallons of ethanol be used by 2022, with at least 16 billion gallons of that being cellulosic ethanol. However, no commercial-scale cellulosic ethanol plants are currently operating in the United States and predicted production costs ($5.15/gallon of gasoline equivalent (2007 dollars))¹ are still much higher than the market gasoline price.

There are two viable pathways for the conversion of lignocellulosic feedstocks to fermentable sugars: 1) acid hydrolysis, and 2) pretreatment followed by enzymatic hydrolysis. An evaluation of the future potential of each process revealed that the enzymatic pathway offered greater opportunities for ethanol cost reduction in the long run.² Figure 1 shows the different steps involved in biochemical biomass conversion.

Feedstock is delivered to the feed-handling area for storage and size reduction. After size reduction, it is subjected to a pretreatment to disrupt the crystallinity of cellulose and association with lignin and make the pretreated material more accessible to cellulolytic enzymes. Many different pretreatment methods are being considered, but dilute acid treatment (~1% sulfuric) at moderate temperatures (140-190 °C) is currently preferred.⁴ Pretreatment requires a conditioning step to remove by-products that are toxic to the fermenting organism. After pretreatment and conditioning, the biomass (now primarily cellulose) is hydrolyzed to glucose by a mixture of cellulolytic enzymes. After several days in saccharification tanks, the mixture of sugars and unreacted cellulose is transferred to fermentation tanks and subjected to fermenting microorganisms, which ferment all sugars to ethanol. The resulting “beer” is distilled to separate the ethanol from water and residual solids. The ethanol distillate is further purified through vapor-phase molecular sieves and the residual solids from the distillation are combusted in a fluidized bed combustor to produce steam and process heat.

Despite intensive research efforts, the enzymatic hydrolysis of lignocellulosic biomass still presents significant technical challenges. The hydrolysis rate rapidly decreases with conversion, resulting in low yields and long residence times in the saccharification tanks. High enzyme loadings are needed for adequate cellulose conversion, and enzyme recycling is difficult due to adsorption of enzymes on residual solids. Many hypotheses have been presented to explain the rapid decrease in hydrolysis rate, including thermal denaturation of the enzymes,⁵⁻⁸ slowing down/stopping/inactivation of the enzymes,⁶,⁹⁻¹⁶ inhibition by hydrolysis products (glucose and cellobiose),⁷,¹⁰,¹⁷⁻²⁰ transformation of the substrate into a less digestible form,²¹ and/or the
heterogeneous structure of the substrate.\textsuperscript{21,22} Several authors have suggested that a fraction of the enzymes irreversibly and non-productively adsorbs onto residual lignin present in the pretreated and conditioned biomass.\textsuperscript{13,16,23-25} Our understanding of the factors that are responsible for decreasing hydrolysis rate is still very limited.

Several research teams have observed positive effects of non-ionic surfactants on the enzymatic hydrolysis of cellulosic substrates. Castanon and Wilke reported a 14% increase in glucose yield and a more than doubling in enzyme recovery from newspaper saccharification upon the addition of 0.02 g Tween 80 per gram of newsprint.\textsuperscript{26} Helle et al. observed a seven-fold increase in hydrolysis rate of microcrystalline cellulose (Sigmacell 100) after adding 0.16 g Tween 80 per gram of cellulose.\textsuperscript{27} Biosurfactants such as sophorolipid, rhamnolipid, and bacitracin had a similar effect on the saccharification of Sigmacell 100 and addition of 1 g/L of sophorolipid increased the hydrolysis rate of delignified, steam-exploded wood by 67%.\textsuperscript{27} Tween 20 was found to be slightly more effective than Tween 80 in the saccharification of lime-pretreated cornstover. A Tween 20 loading of 0.15 g per gram of dry biomass increased the cellulose and xylan conversion by 42% and 40%, respectively.\textsuperscript{28} The simultaneous saccharification and fermentation of softwood was shown to benefit in multiple ways by the addition of 2.5 g/L of Tween 20: (i) the ethanol yield was increased by 8%, (ii) the amount of enzyme loading could be reduced by 50%, while maintaining a constant yield, (iii) the enzyme activity increased in the liquid fraction at the end of SSF, and (iv) the time required to attain maximum ethanol concentration was reduced.

Different mechanisms have been proposed for the beneficial effect of surfactants on the enzymatic hydrolysis of cellulose. The proposed mechanisms fall into one of three categories: (1) the surfactant changes the ultrastructure of the substrate, making the cellulose more susceptible to enzymatic attack,\textsuperscript{27} (2) surfactants increase enzyme stability, e.g. by reducing thermal denaturation or deactivation by shear forces,\textsuperscript{28,29} and (3) surfactants affect enzyme-substrate interactions, e.g. enhance the wettability of the substrate and reduced irreversible enzyme adsorption.\textsuperscript{26-28,30} For saccharification in the presence of lignin, Eriksson et al. suggested that surfactant adsorption onto lignin prevents non-productive binding of enzymes to lignin.\textsuperscript{30} The multitude of proposed mechanisms demonstrates that our understanding of the effects of non-ionic surfactants on lignocellulosic biomass and its interactions with cellulolytic enzymes are incomplete.

**Approach**

The goal of this project was to develop a comprehensive understanding of the mechanisms by which surfactants enhance the enzymatic hydrolysis of lignocellulosic biomass. The non-ionic surfactant Tween 80 (Figure 2) was chosen as a model substance.

![Figure 2. Molecular structure of Tween 80.](image-url)
The specific objectives of the project were to:

(1) Develop model cellulose, lignin, and lignocellulosic substrates for adsorption and hydrolysis studies by quartz crystal microbalance with dissipation monitoring (QCM-D);

(2) Study the adsorption behavior of Tween 80 on the model substrates;

(3) Determine the effects of Tween 80 on the adsorption of cellulolytic enzymes on model lignin substrates; and

(4) Determine the effects of Tween 80 on the enzymatic hydrolysis rates of model cellulose and lignocellulosic substrates.

**Methodology**

**Model Cellulose Substrates**

Model cellulose substrates for adsorption and hydrolysis studies by QCM-D were prepared from cellulose nanocrystals. Cellulose nanocrystals are rod-like nanoparticles of cellulose generally obtained by acid hydrolysis of a cellulose starting material, such as bleached wood pulp or cotton. In this project, the cellulose nanocrystals were prepared by sulfuric acid hydrolysis of dissolving-grade softwood sulfite pulp. As a consequence of the use of sulfuric acid, the obtained cellulose nanocrystals had sulfate ester groups on their surface, which had to be removed prior to use of the cellulose nanocrystals in model cellulose substrates. Two desulfation methods were evaluated, namely hydrolytic desulfation with hydrochloric acid and solvolytic desulfation in dimethyl sulfoxide via the pyridinium salt. The acid hydrolytic method achieved only partial desulfation and produced gradually decreasing sulfate contents upon successive repetition. The solvolytic method achieved nearly complete desulfation in a single step. Model cellulose substrates were obtained by spin coating aqueous suspensions of solvolytically desulfated cellulose nanocrystals onto QCM-D sensors.

**Model Lignin Substrates**

Model lignin substrates for Tween 80 and enzyme adsorption studies by QCM-D were prepared from three different types of lignin, namely Kraft lignin, organosolv lignin, and milled wood lignin. The lignins were dissolved in aqueous ammonia and spin coated onto QCM-D sensors.

**Model Lignocellulosic Substrates**

Two different types of model lignocellulosic substrates for adsorption and hydrolysis studies by QCM-D were prepared. The first type, referred to as composites lignocellulosic substrates, were prepared by spin coating mixed aqueous suspensions of lignin particles and cellulose nanocrystals onto QCM-D sensors. Aqueous suspensions of lignin particles were obtained by dialysis of lignin solutions in aqueous ammonia against deionized water. The nominal lignin content of composite lignocellulosic substrates ranged from 10 to 30%. The second type, referred to as layered lignocellulosic substrates, were prepared by spin coating a lignin layer from aqueous lignin suspensions onto a model cellulose substrate. Two lignin concentrations, specifically 0.5 and 1 wt %, were tested.
Substrate Characterization

All model substrates were characterized in terms of root-mean-square (RMS) surface roughness by atomic force microscopy. The model cellulose and composite lignocellulosic substrates were also characterized in terms of atomic composition by X-ray photoelectron spectroscopy. The surface energy of the model lignin substrates was measured by contact angle measurements.

QCM-D Experiments

A quartz crystal microbalance is a device that applies alternating current to a sensor, comprising a quartz crystal. By monitoring the oscillation amplitude and phase lag of the crystal, it can detect weight changes of less than 1 ng·cm$^{-2}$ on the crystal’s surface. The decay rate of the oscillation after termination of the current provides information about the viscoelastic properties of the adsorbed layer on the crystal’s surface.

The adsorption behavior of Tween 80 on model cellulose, lignin, and lignocellulosic substrates was studied by exposing the substrates in a QCM-D to Tween 80 solutions and monitoring the changes in the oscillation of the subjacent quartz crystals upon Tween 80 adsorption or erosion of the substrate.

The effects of Tween 80 on the adsorption of cellulolytic enzymes on model lignin substrates were determined by exposing untreated and Tween 80-treated lignin substrates to solutions of cellulolytic enzymes from the fungus *Trichoderma reesei* (Celluclast 1.5 L, Novozymes) and monitoring the changes in the oscillation of the subjacent quartz crystals.

Similarly, the effects of Tween 80 on the enzymatic hydrolysis rates of model cellulose and lignocellulosic substrates were determined by exposing untreated and Tween 80-treated substrates to solutions of cellulolytic enzymes and monitoring the changes in the oscillation of the subjacent quartz crystals.

Findings

Model Substrates for QCM-D Studies

Model cellulose substrates had an RMS surface roughness of 3.6 nm and a carbon-to-oxygen ratio of 1.38. Model lignin substrates were much smoother, with RMS surface roughnesses between 0.8 and 1.1 nm. The polar surface energy component of model lignin substrates ranged from $14.9 \pm 1.4$ to $23.1 \pm 2.9\%$ $(8.1 \pm 0.7$ to $13.4 \pm 1.4$ mN·m$^{-1}$) and the dispersive surface energy component ranged from $85.1 \pm 4.6$ to $76.9 \pm 6.7\%$ $(46.1 \pm 1.6$ to $44.7 \pm 2.5$ mN·m$^{-1}$), depending on the type of lignin. Model lignin substrates from organosolv lignin had the smallest polar component and the largest dispersive component, whereas model lignin substrates from milled wood lignin had the largest polar component and the smallest dispersive component. Composite lignocellulosic substrates had RMS surface roughnesses between 2.4 and 3.3 nm and carbon-to-oxygen ratios between 1.38 and 1.81, depending on the type of lignin and nominal lignin content. Layered lignocellulosic substrates had RMS surface roughnesses between 2.3 and 5.6 nm, depending on the type of lignin and the concentration of the lignin suspension used for spin coating.

Adsorption Behavior of Tween 80 on the Model Substrates

The adsorption behavior of Tween 80 on model cellulose substrates followed the Langmuir adsorption model. The maximum surface excess concentration of Tween 80 on the model
substrate was 8.35 mg·m⁻². Comparison of the maximum surface excess concentration with one
determined by surface plasmon resonance showed that the Tween 80 layer on the cellulose
substrate was highly hydrated and had a coupled buffer content of 91%.

Tween 80 adsorbed also onto lignin substrates but caused a weight loss, indicating substrate
erosion. The weight loss, which was greatest for organosolv lignin substrates and smallest for
milled wood lignin substrates, was most likely due to Tween 80-mediated removal of lignin from
the substrate’s surface.

Tween 80 adsorbed to a greater extent onto composite lignocellulosic substrates than onto model
cellulose substrates. Substrate erosion, as observed for model lignin substrates, was not observed
for composite lignocellulosic substrates. The extent of Tween 80 adsorption on composite
lignocellulosic substrates depended on lignin chemistry, being greatest on substrates containing
Kraft lignin and smallest on substrates containing milled wood lignin.

Layered lignocellulosic substrates showed substrate erosion after Tween 80 adsorption, similar to
that observed for model lignin substrates, except for substrates containing milled wood lignin. As
for the composite lignocellulosic substrates, the extent of Tween 80 adsorption was greatest on
substrates containing Kraft lignin and smallest on substrates containing milled wood lignin.

Effects of Tween 80 on the Adsorption of Cellulolytic Enzymes on Model Lignin
Substrates

The effects of Tween 80 on the adsorption of cellulolytic enzymes on model lignin substrates
depended strongly on lignin chemistry. For Kraft lignin and organosolv lignin, pre-adsorption of
Tween 80 on the substrates resulted in greater enzyme adsorption, whereas for milled wood
lignin it caused decreased adsorption of cellulolytic enzymes. For untreated model lignin
substrates, the surface excess concentration of cellulolytic enzymes after 4 h of adsorption was
highest on milled wood lignin substrates (17.5 ± 0.9 mg·m⁻²) and lowest on Kraft lignin
substrates (9.5 ± 0.4 mg·m⁻²), whereas for Tween 80-treated substrates it was highest on
organosolv lignin substrates (16.5 ± 1.2 mg·m⁻²) and lowest on milled wood lignin substrates
(6.8 ± 0.1 mg·m⁻²).

Effects of Tween 80 on the Enzymatic Hydrolysis Rates of Model Cellulose and
Lignocellulosic Substrates

Pre-adsorption of Tween 80 from solutions with Tween 80 concentrations of up to 5 mM had no
significant effect on the apparent enzymatic hydrolysis rate of model cellulose substrates.
Similarly, the apparent hydrolysis rate of model cellulose substrates was not affected by the
presence of Tween 80 molecules at concentrations of up to 5 mM in the enzyme solution.

The presence of lignin in lignocellulosic substrates caused both decreased adsorption of
cellulolytic enzymes onto the substrates and decreased apparent hydrolysis rates, relative to those
observed for cellulose substrates. The extent of the inhibitory effect of lignin depended on lignin
type and increased with the amount of lignin present. The lignin surface layer of the layered
lignocellulosic substrates had less of an inhibitory effect on cellulose hydrolysis than the
embedding lignin matrix of the composited lignocellulosic substrates.

Treatment of composite lignocellulosic substrates with a Tween 80 solution prior to hydrolysis
resulted in a significant increase in their apparent hydrolysis rates. The extent of the increase
depended strongly on the amount and type of lignin present in the substrate. The largest increase
in apparent hydrolysis rate (70 times) upon Tween 80 treatment was observed for composite lignocellulosic substrates with a nominal Kraft lignin content of 30%, followed by substrates with a nominal organosolv lignin content of 30% (10 times). Substrates with a nominal lignin content of 10% showed increases in apparent hydrolysis rate of 1.8–2.8 times, with the milled wood lignin-containing substrate exhibiting the largest. The mechanism by which substrate-adsorbed Tween 80 increased the apparent hydrolysis rates of composite lignocellulosic substrates was not obvious from the experiments.

Layered lignocellulosic substrates also showed an increase in apparent hydrolysis rate upon Tween 80 treatment. As for composite lignocellulosic substrates, the extent of the increase depended on the amount and type of lignin present in the substrate. The largest increase (18 times) was observed for substrates prepared from 1 wt % Kraft lignin suspensions, followed by substrates prepared from 0.5 wt % Kraft lignin suspensions (3 times). Substrates prepared from organosolv and milled wood lignin showed increases between 1.4 and 1.9 times. The reason for the increased apparent hydrolysis rates after Tween 80 treatment of the layered lignocellulosic substrates was most likely Tween 80-mediated removal of lignin from the substrate, making the subjacent cellulose layer more accessible to the enzymes.

**Conclusions**

Model cellulose, lignin, and lignocellulosic substrates for adsorption and hydrolysis studies by QCM-D can be prepared by spin coating of aqueous suspensions of cellulose nanocrystals and lignin particles or aqueous lignin solutions. In lignocellulosic substrates, lignin hinders both the adsorption of cellulolytic enzymes onto the substrates and the enzymatic hydrolysis of the substrates.

Tween 80 adsorbs onto cellulose substrates and forms highly hydrated surface layers. The interactions of Tween 80 with lignin substrates depend strongly on lignin chemistry. The interactions of Tween 80 with lignocellulosic substrates depend on both lignin chemistry and substrate morphology. Tween 80 may cause removal of lignin from lignin and layered lignocellulosic substrates.

Depending on lignin chemistry, substrate-adsorbed Tween 80 molecules can either increase or decrease adsorption of cellulolytic enzymes on lignin substrates.

Whether substrate-adsorbed or in solution, Tween 80 molecules do not significantly affect the apparent enzymatic hydrolysis rate of cellulose substrates. In contrast, the apparent enzymatic hydrolysis rate of lignocellulosic substrates is increased after exposure of the substrates to Tween 80. For layered lignocellulosic substrates, the increase is caused by Tween 80-mediated removal of lignin from the substrate, making the subjacent cellulose layer more accessible to the enzymes.

The study has shown that non-ionic surfactants may mitigate the inhibitory effect of lignin on the enzymatic hydrolysis of lignocellulosic biomass.

**Publications and Presentations**

**Publications**


Presentations


(13) Esker, A. R. Probing Polysaccharide and Enzyme Interactions with Cellulose. Biopolymers Program, Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden, February 11, 2011.


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