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# Oxidation of Small Alcohols Using Spinach Photosystem II: Use of a natural catalyst for potential green synthesis

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**Abstract**

Photosystem II (PSII), a protein complex found in plants and an integral part of photosynthesis, uses light to oxidize water to oxygen. PSII may also be capable of oxidizing small alcohols. This light driven biotransformation could be an excellent green oxidation method. PSII was isolated from spinach leaves and its activity verified by quantifying the oxygen produced from water. Methods were developed to identify alcohols and their oxidized products using gas chromatography. The reaction of 2-propanol and *n*-propanol with PSII and light were explored. *n*-Propanol and PSII were also mixed in the dark with $H_2O_2$ as the potential oxidant. While trace amounts of oxidation products were observed in a few experiments, further investigation of this reaction is necessary to determine whether this method can be used as a viable and quantitative green alternative for the oxidation of alcohols in organic synthesis.

## Introduction

Photosystem II (PSII), a protein complex found in plants and an integral part of photosynthesis, uses light to oxidize water to oxygen. PSII is also an essential component of the electron transport mechanism to form NADPH in photosynthesis, and aids in the establishment of the proton gradient to power ATP synthase and its production of ATP. PSII may also be capable of oxidizing small alcohols to ketones or aldehydes. This light-driven biotransformation could be an excellent green oxidation method.

### *Background*

Originally, it was believed that enzymes had evolved only to perform their designated natural metabolic reactions. Thankfully, challenges to this assumption have shown that enzymes can be used effectively to react with a wide range of non-natural substrates (Faber 1997). These reactions, or biotransformations, are carried out using living cells, organs or enzymes. They are unique and desirable because of their regio- and stereo- specificity, as well as their ability to proceed at common pH ranges and temperatures. Much investigation has been done using enzymes from rapidly reproducing microorganisms, but it is believed that the future of biotransformations lies in the plant world (Giri et al 2001). Documented plant biotransformations using in vitro plant cell and organ cultures include reactions such as hydroxylation, glucosylation, reduction of C-C double bonds and oxido-reductions between alcohols and ketones (Faber 1997).

Specifically of interest, enantiomer specific oxido-reductions with alcohols have been done using *N. tabacum*. Also of interest are oxidations of alcohols using the plant *Myrtillocactus*, from the cactus family (Giri et al. 2001). Redox reactions have been explored in some detail, focusing on those used to introduce oxygen into a substrate, rather than the

oxidation of oxygen already incorporated into the compound (Faber 1997). Most of these reactions happen using cumbersome whole cell systems, specifically those with a microbial origin. The Baeyer-Villiger reaction, which further oxidizes ketones into esters or lactones, is carried out with *Acinetobactor calcoaceticus*, a harmful pathogen (Faber 1997). While enzymatic biotransformations that introduce oxygen and perform further oxidations have been reported, few had been documented. The field of biotransformations has enormous potential; certain plants do posses the ability to carry out oxidation reactions.

The study of biotransformations has led to research seeking to mimic the function of biological compounds and enzymes through the design of synthetic catalysts. "Bionic Catalyst Design: A Photochemical Approach to Artificial Enzyme Function," reports on the work done to imitate environmentally benign reactions (Knör 2001). The main goals in this area are the activation of atmospheric dioxygen and the "ecological conversion of alcohols into carbonyl compounds." It is noted that several native enzymes already carry out this reaction and work has been done to create a similar light-driven synthetic enzymatic reaction. Acknowledgement of the power of the natural reaction proposed in this research is encouraging.

The discussion of the challenge of an ecological oxidation of alcohols to ketones includes "Copper-Catalyzed Oxidation of Alcohols to Aldehydes and Ketones: An Efficient Aerobic Alternative," (Markó et al. 1996). Many oxidation reactions require toxic or hazardous oxidizing agents in stoichiometric amounts to perform time consuming reactions. Insufficient attempts to create a catalytic system that could oxidize alcohols motivated this research and creation of such an environmentally friendly catalytic system for this oxidation reaction was attempted. Using a copper-nitrogen organometallic catalytic complex in nonpolar solvents at 70° – 90° C, primary, secondary, allylic and benzylic alcohols were oxidized with good to excellent yields. These

reactions did not necessarily require the use of pure oxygen, but occurred in air over slightly longer periods of time. Lower temperatures were also acceptable, but required a longer reaction time as well. The only by-product of this reaction was water, an environmentally friendly material. Details of the specific reactions are tabled, showing the success of this reaction with many alcohols (Markó et al. 1996). More benign oxidations, such as the proposed oxidation of small alcohols with PSII, would be an asset to the scientific community in this developing field.

The work to create a catalytic oxidation reaction provided the impetus behind the quest to create catalysts modeled after biological systems. Many photocatalytic artificial enzymes, photozymes, produce ketones or aldehydes as well as $H_2O_2$ (Knör 2001). The process generates $H_2O_2$ in equimolar amounts to the oxidation products. One such artificial reaction, using the designed catalyst SbO(tpp)OH, is quite efficient in comparison to many natural and synthetic processes and is only dwarfed by the reaction of alcohol oxidase, a flavoprotein with specificity to oxidize methanol and ethanol. Biological references, listing all catalysts known to aerobically oxidize ethanol to acetaldehyde under ambient conditions, include a variety of compounds. The oxidation of ethanol occurs in PSII (green plants) in the manganese cluster, with a $K_{cat}$ of 0.38 and a specific activity of 0.47. Attributed to research by Frasch et al. (1988), it is encouraging to see it succinctly stated that the oxygen-evolving complex (OEC) of PSII is considered by others to have these oxidizing capabilities. The oxidation abilities of PSII are realized as others model PSII in the creation of synthetic catalysts.

Because the mechanism of action and structure of the PSII OEC are still not fully known, current research gives attention to this area. Using x-ray crystal studies to continue past work on the determination of the structure and makeup of PSII, much of the structure of the photosynthetic oxygen evolving center has been mapped (Ferreira et al. 2004). The resolution

of the protein structure of PSII, along with ligands such as the OEC, heme groups, plastoquinones, pheophytins, and bicarbonates, increases understanding of the involvement of the protein in photosynthesis (Figure 1).

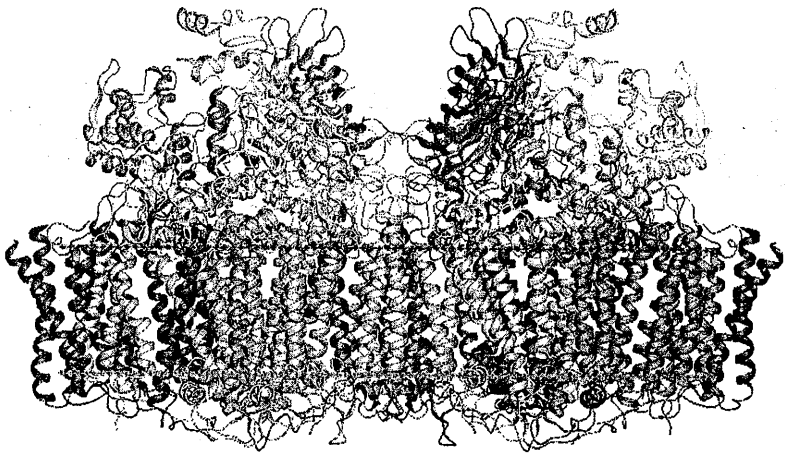

**Figure 1:** Protein structure of Photosystem II. Note the many alpha helices and membranal properties of the protein. From: http://opm.phar.umich.edu/images/proteins/2axt.gif

In light of goals to exploit the OEC for non-natural purposes, the most informative work comes from the proposal of the Mn structure of the OEC, which had been previously unknown. It is now suggested that the OEC consists of five metal ions, in a $Mn_3CaO_4$ cubane cluster connected to a fourth Mn with a mono-μ-oxo-bridge. In the past, the importance of calcium in the OEC was established without knowledge of its direct role in the structure of the complex. It is even possible that the water, or possibly hydroxide, ligates to the $Ca^{2+}$. Although this cannot be shown with the resolution of available techniques, the currently proposed model does allow for sufficient space for this binding to occur. The oxidation carried out by the OEC is not simply dependent upon these catalytic metals. Amino acid residues present in the OEC appear to be necessary components of oxidation. This knowledge is helpful in analyzing the feasibility of the

proposed oxidation of small alcohols, and important in understanding other research involving the mechanisms of oxidation in the OEC of PSII.

The effect of alcohols on the OEC of PSII was explored to see how alcohols could inhibit the oxygen evolution that normally occurs in PSII (Frasch 1988). The most successful reactions were run with alcohols and $H_2O_2$ in the dark with the OEC of PSII as a catalyst. In the reaction involving only PSII and varying concentrations of alcohols, increasing concentrations of ethanol, glycerol, and propargyl alcohol progressively inhibited $O_2$-evolution. While ethanol and glycerol were slight inhibitors, propargyl alcohol proved drastically more effective than either of these. In experiments run with $H_2O_2$ and the OEC as a catalyst, the presence of alcohols also impaired $O_2$-evolution. Glycerol was by far the most effective inhibitor, while propargyl alcohol was still quite effective. Both of these alcohols appeared to compete with $H_2O_2$ and successfully displace it from the catalytic site of the OEC.

Low concentrations of alcohols impaired the OEC reversibly, while higher concentrations, especially those in the presence of $H_2O_2$ caused complete inactivation of the OEC and release of the catalytic manganese. This work showed propargyl alcohol could be converted to an aldehyde in the presence of $H_2O_2$. This oxidation was done with less success using ethanol and glycerol, but nevertheless, these three alcohols were shown to be oxidized. By inactivating the OEC with a Tris/EDTA wash prior to adding the alcohols, it was verified that the OEC is essential to this reaction, as no aldehydes were formed. Conclusively, with the exception of glycerol, it was shown that alcohols inhibited PSII $O_2$-evolution in the same relative proportions that they were oxidized to aldehydes. Glycerol was a better inhibitor, but a less effective oxidative substrate. This pivotal paper gives evidence that the oxidation of alcohols to ketones using PSII may be possible (Frasch et al. 1988).

ESEEM as well as EPR were used to determine whether small alcohols were able to bind to the manganese cluster in the OEC of PSII (Force et al. 1998). Intrigue concerning the OEC ligation sphere and the proposal that as analogs of water, alcohols could be useful as molecular probes, motivated this research. A range of $^2$H-labeled alcohols of different size and bulkiness were used to gain information about the shape of the binding site with these magnetic resonance techniques, as well as its binding characteristics. Initially 1.0 M concentrations of methanol, ethanol, 2-propanol and *n*-propanol were used. These concentrations had no effect on the $O_2$-evolution rates of PSII. When concentration rates were increased, $O_2$-evolution rates varied greatly in the presence of 2-propanol and *n*-propanol and only slightly in the presence of methanol (Figure 2).

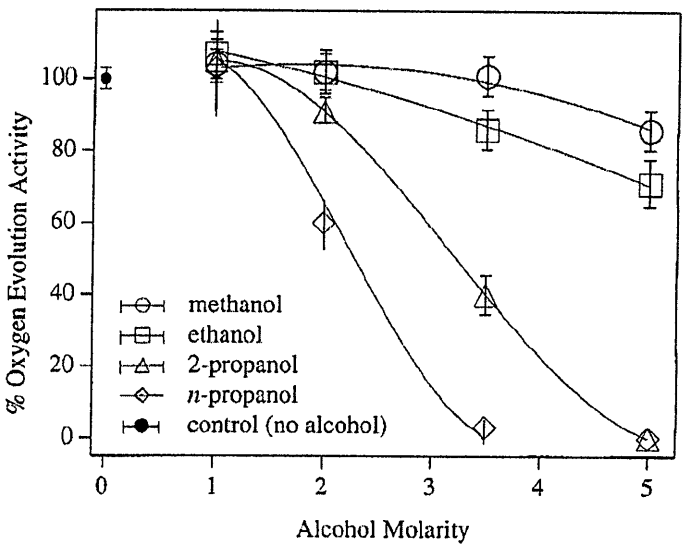


**Figure 2:** Chart from Force et al. (1998) showing decrease in oxygen evolution activity as alcohol molarity increases. Note especially the effect of *n*-propanol and 2-propanol.

This inhibition was shown to be reversible. Notably, the electron paramagnetic resonance (EPR) spectra were affected by alcohol concentrations considerably lower than the concentrations affecting $O_2$-evolution.

Using electron spin-echo envelope modulation (ESEEM), results were interpreted to suggest that water or hydroxides have direction ligation to Mn in the $S_2$-state, one of five states in the Kok cycle from $S_0$ to $S_4$ during PSII reaction. Differences among the alcohols resulted in differences in their ability to bind to the Mn cluster. Smaller alcohols such as methanol and ethanol bound better, showing higher $^2H$ peaks in the ESEEM data, while *n*-propanol showed less peak intensity and 2-propanol showed almost none. Thus, smaller alcohols are shown to bind near the Mn cluster, while larger alcohols and DMSO do not. Since methanol and ethanol bind much better than even *n*-propanol, it was theorized that there must be an access channel of limited size restricting access to the Mn cluster. This could be based upon sterics or simply upon size and bulkiness as limiting factors. It was even estimated that the "effective Mn cluster 'access-channel' diameter" to be in the range of 3-4 Å. Further investigation of ESEEM data led to the conclusion that small alcohols bind directly to the Mn cluster. Surprisingly, at low concentrations these alcohols have no effect on $O_2$-evolving activity. This suggests that in relation to the desired ligand, water, alcohols choose different non-interfering binding sites.

This work was continued to determine the extent that water directly binds to the Mn in the OEC (Evans et al. 2004). Even as it was shown that small alcohol binding in the $S_2$ state did occur (Force et al. 1998), this work was repeated to find that this interaction could only be detected in freshly prepared $S_2$ samples (Evans et al. 2004). After storage at cold temperatures, the ESEEM signal decayed in a short period, although some signals did remain. It is interesting to note that this research was not as confident in the binding abilities of small alcohols as had previously been reported (Evans et al. 2004). Continued work is being done with ESEEM and EPR to attempt to find evidence that water does directly bind to the Mn through oxygen. Though

important, this research is more concerned with how the oxidation of alcohols might take place, rather than simply whether it is possible or not.

Other extensive ESEEM studies involving the binding of water and small alcohols to the OEC of PSII have been done (Åhrling et al. 2006). Because it is known that water does bind to the OEC in PSII, these researchers wanted to reevaluate whether or not methanol or even ethanol would bind. Finding a site for the binding of methanol, it was determined that it did not displace water. Signals appeared in the $S_2$ and $S_0$ states from deuterons from both *d*-methanol and $D_2O$. It is unclear though as to why *d*-methanol binding sometimes appeared to have stronger signals than the $D_2O$ binding. It was concluded that methanol does indeed bind to the Mn cluster. In addition, methanol and water both appear to bind non-competitively with similar strengths to the same Mn cluster. Evidence of this binding is important, yet it is unclear whether these binding capabilities are unique to methanol or could be inherent to larger alcohols as well.

Other associated chemical activities of the PSII complex, such as PSII membrane-associated catalase and polyphenol oxidase have also been explored (Sheptovitsky and Brudvig 1996). More than 27 polypeptides and protein subunits are presently a part of the PSII/ OEC complex. Experiments have shown that PSII can mediate other enzymatic activities, such as catalyzing the decomposition of $H_2O_2$ in the dark. Attempts were made to determine whether this activity is due to corresponding proteins or directly correlated to the OEC catalytic site, as well as investigate the polyphenol oxidase (PPO) activity associated with PSII samples. PPO is a copper enzyme found in plants that oxidizes hydroxyphenols to quinones. These proteins were extracted from spinach using polyacrylamide gel electrophoresis, which was also used to determine the distinct molecular weights of the proteins.

When an inhibitor of these catalase enzymes was introduced into some samples; catalase activity was less than 5% of original activity, while $O_2$-evolution activity was only slightly inhibited. This shows that these catalases are not directly contained within the OEC centers and their realm of activity. PPO was extracted from PSII membranes into a supernatant. When heat-treated twice, the PPO activity almost completely disappeared, while $O_2$-evolution was not inactivated. (Sheptovitsky and Brudvig 1996) This further demonstrates that the activities of this enzyme are independent of the OEC. PSII does not only function as an $O_2$-evolving complex. Associated proteins are rich with enzymatic activity and biotransformative potential.

The oxidation of alcohols by the OEC of the PSII may indeed be possible. Biotransformations are viable methods to carry out chemical reactions in laboratories. There is a need for more benign and environmentally friendly oxidation mechanisms, and work has been done to mimic the reactions carried out by nature in artificial substrates.

## Materials and Methods

*Spinach PSII preparation*

Fresh, organic spinach was purified using the BBY prep method (Berthold et al. 1981). A variety of buffers were used in the procedure. All solutions were buffered using 6.0 M HCl and 6.0 NaOH. Grind buffer contained 400 mM NaCl (23.2 g), 2 mM $MgCl_2$ (0.41 g), 1 mM EDTA (0.38 g), 50 mM HEPES (11.9 g), and 2 mg/mL bovine serum albumin fraction V (2.0 g), in deionized water to make 1 liter and buffered to pH 7.5. Wash buffer contained 150 mM NaCl (4.3 g), 4 mM $MgCl_2 \cdot 6\ H_2O$ (0.41 g), 20 mM MES (1.95 g), in deionized water was added to make 0.5 liter and buffered to pH 6.0. Triton buffer contained 50 mM MES (4.9 g), 5mM $MgCl_2$ (0.51 g), 15 mM NaCl (0.43 g), in deionized water was added to make 0.5 L and buffered to pH

6.0. SMN buffer contained 400 mM sucrose (68.5 g), 50 mM MES (4.9 g), and 15 mM NaCl (0.43 g), in deionized water was added to make 0.5 L and buffered to a pH of 6.0. Triton X-100 stock was prepared by adding 10 g Triton X-100 to 33 mL Triton buffer for a total of 40 mL, and was stirred overnight in the refrigerator to ensure adequate mixing of the thick Triton X-100 with the buffer. All buffers were refrigerated after preparation.

A large amount of fresh spinach leaves were prepared for the procedure by washing in ice water. All leaves with tears and blemishes were discarded, as well as the major fibrous vein in each leaf. All solutions and tubes were chilled on ice before use. Grind buffer and spinach leaves were added to a blender with 1 mL of grind buffer per gram of spinach. Leaves were ground using short bursts to chop thoroughly, but not puree. The mixture was poured into a large funnel prepared with layers of cheesecloth large enough to wrap around spinach. Most of the liquid was squeezed out with care being taken not to warm the spinach with hands. As much pure green liquid as possible was extracted, avoiding the fibrous pulp. The extract was poured into GSA tubes and centrifuged only up to 1500 rpms at 4° C. From here on, the spinach preparations were kept on ice at all times they were not in the refrigerated centrifuge. The supernatant was poured into clean GSA tubes. These tubes were centrifuged at 6500 rpm for 10 minutes at 4° C. The supernatant was then discarded and the pellet saved on ice. A volume of 5 mL of chilled wash buffer was added to each tube and the pellets resuspended using a paintbrush. After pouring this liquid into homogenizer tubes, 5 mL wash buffer was again added to each tube, resuspending the pellet. This was added to the homogenizer tubes as well. The mixture was homogenized completely, poured into SS34 tubes and centrifuged for 10 minutes as 8000 rpm at 4° C. The supernatant was poured off. 2 to 2.5 mL Triton buffer was added to each tube and resuspended with a paintbrush. Using only 2.5 mL more total Triton buffer, the

remaining material was resuspended and poured into a homogenizer tube. This step was repeated to get all of the material out of the tubes. The mixture was homogenized completely. The volume was measured and the mixture was poured into a 125 mL Erhlenmeyer covered in dark electrical tape on ice.

The chlorophyll concentration was measured using UV-VIS spectroscopy. A volume of 10 μL of the well-mixed suspension was removed and added to a 1.5 mL Eppendorf tube with 1 mL of 80% acetone solution. Tubes were vortexed to dissolve all green particulate and then microcentrifuged for 30 seconds to isolate a small white starch pellet. The sample was further diluted to allow for proper absorption of light using the UV-VIS spectrometer. The absorbance was measured at 663.2 nm and 646.8 nm and the chlorophyll$_{a+b}$ concentration was calculated using the equation $(7.15\ A_{663} + 18.71\ A_{646})$=μg Chl/ mL, making sure to account for previous dilutions. The concentration of chlorophyll in the next step must be 2 mg/ mL. The concentration measurement from the UV-VIS allowed for calculation of total volume needed to reach this concentration. Also, there needed to be 25 mg Triton per mg of chlorophyll. Since a 25% Triton solution was used, the amount of Triton X-100 stock needed could be calculated. This amount was subtracted from the total volume, along with the current volume of the sample. The amount remaining indicated the amount of Triton buffer that needed to be added.

The Triton buffer was used to rinse out the graduated cylinder and homogenizer tube. Everything but the Triton X-100 stock was added to the Erlenmeyer flask. After this step, everything was carried out in the dark, continuing to keep everything on ice. The Triton X-100 stock was added dropwise over 5 minutes into the flask, while shaking it continuously on a shaker table. A timer was started about halfway through the addition of the Triton X-100 stock and the mixture was incubated for 30 minutes. Since PSII is isolated during this step, it was very

important to ensure proper cooling, darkness and timing. After incubation, the sample was put into SS34 tubes and immediately centrifuged for 30 minutes at 20,000 rpm at 4° C. The supernatant was discarded and 5 mL of SMN buffer was added to each tube. The pellet was resuspended, taking care not to disturb the white starch pellet, but to obtain all chlorophyll. This mixture was added to a homogenizer tube. The SS34 tubes were cleaned again, using a total volume of SMN buffer equal to the total volume used in the Triton step. This mixture was homogenized again and centrifuged at 20,000 rpm for 30 minutes at 4° C. The supernatant was discarded and the pellet was resuspended in SMN buffer. This was the final suspension, so the amount of SMN buffer was chosen to reach the desired concentration of mg Chl/ mL. This mixture was homogenized for the final time and poured promptly into small tubes. The samples were placed in a -80 ° C freezer. A sample was retained for determination of the final concentration of mg Chl/ mL using the same UV-VIS procedure as before.

*Oxygen Evolution Assay*

An oxygen assay was performed using the Oxygraph oxygen electrode from Hansatech Instruments. The instrument was calibrated following software prompts using sodium dithionite and the membrane was prepared with 50% KCl solution. For the assay, electron acceptors and donors were needed. DCBQ (2,6-Dichloro-1,4-benzoquinone) was recrystallized from warm methanol. 0.5 mL of 100mM DCBQ in ethanol solution was prepared using 0.00885 g DCBQ. 0.5 mL of 100 mM Potassium Ferricyanide solution in SMN buffer was prepared using 0.0165 g $K_3Fe(CN)_6$. Along with a flea stir bar, 15 μL of Potassium Ferricyanide solution, 8 μL DCBQ, and a sample containing 100 mM of PSII BBY prep sample was added (about 10 μL at a concentration of 2.78 mg Chl/ mL), along with enough SMN buffer to equal 1500 μL. A very bright red tinted light with an IR filter was shone on the sample. The computer software was

used to record data and oxygen evolution rates were calculated with the units μ moles $O_2$/ mg Chl · hr.

*GC Method Development*

A method was developed for the separation and identification of 2-propanol and acetone. Separation of these compounds using gas chromatography proved to be difficult. Initial method development using a GC-MS and non-polar column was ineffective. Acetone has a boiling point of 56 ° C, while 2-propanol has a boiling point of 82 ° C. However, the difference in boiling points was counteracted by their interactions with the non-polar column. Headspace analysis allowed for the detection of only volatile organics, but 2-propanol and acetone both eluted at approximately the same time (1.42-1.48 minutes), even at a constant 40 ° C run. Their peaks and mass spectra were indistinguishable.

Thus, a method was developed for derivatization of acetone with the strong reducing agent hydroxylamine to create acetone oxime (McDonald et al. 1975). Acetone oxime has a boiling point of 135 ° C and a similar polarity to 2-propanol, allowing for adequate separation on a non-polar column. After oxidation using PSII, a few crystals of hydroxylamine were added to the solution and shaken. The difficulty of being unable to inject this sample containing large PSII proteins into the GC-MS was overcome by heating the solution in the presence of an SPME fiber made of Carboxen/ PDMS. The fiber adsorbed volatile compounds in the closed chamber over time and was easily inserted in the GC-MS for analysis. This method was run at 50 ° C, with acetone oxime eluting at 2.05 minutes. At higher concentrations, acetone oxime displayed a large, irregular peak.

Method development continued with use of a GC with a flame ionization detector. Using a polar column, samples were run at 40 ° C to show the elution of acetone at 1.479 minutes and

2-propanol at 2.062 minutes. Headspace analysis of these compounds allows for ease of analysis of these volatile organics, even when mixed with PSII.

The method for separation of propionaldehyde and *n*-propanol was on the polar column of the GC-MS. The temperature began at 40 °C and ramped up slowly to 45 ° C in 2.50 minutes. Propionaldehyde eluted at 1.45 minutes and *n*-propanol clearly eluted later at 1.60 to 1.70 or more, depending upon the concentration of *n*-propanol. The *n*-propanol did not interfere with the propionaldehyde peak.

*Reaction of PSII with 2-propanol*

After this extensive method development, attempted oxidations of 2-propanol with PSII occurred. 400 μL DCBQ solution and 750 μL Potassium Ferricyanide solution as described for the oxygen assay were combined with an aliquot of 2-propanol ranging from 1 to 4 mL in a dark room. This was mixed and 360 μL PSII BBY prep sample was added after being thawed on ice. All solutions were place in a small vial with a rubber septum top and a stir bar. The mixture was stirred constantly under intense red light with an IR filter for 15 minutes and analyzed. The 2-propanol (HPLC grade) and the ethanol (200 proof) were analyzed for purity on the GC-MS to ensure no contamination with acetone. All syringes were rinsed in ether and no acetone was used in the cleaning of any glassware.

*Reaction of PSII with n-propanol*

Before attempted oxidation of *n*-propanol with PSII, the alcohol was dried with potassium carbonate under calcium chloride for 48 hours and then distilled to remove impurities. Protocol paralleled Frasch et al. (1988), which was done on an unknown scale. In a small Erlenmeyer flask, 0.0800 g MES (50 mM) was dissolved in 7.5 mL deionized $H_2O$, which was buffered to a pH of 6.0. A minute amount of KCN, 0.0025 g (500 μM), and 0.0022 g EDTA (1

mM) were then dissolved in the MES solution. A volume of 200 μL of PSII BBY prep was added and then 1.12 mL of *n*-propanol (2 M). After this, 1.11 mL of $H_2O_2$ (1.3 M) was added and the reaction was capped with parafilm and a rubber septum. The flask was stirred with a stir bar in the dark for at least 20 minutes. A sample absorbed to a Carboxen/ PDMS fiber was analyzed with the previously mentioned GC-MS method for *n*-propanol and propionaldehyde.

**Results**

*Spinach PSII preparation and oxygen evolution assay*

The final concentration of chlorophyll from the BBY prep was about 2.75 mg Chl/ mL. Attempts at a higher concentration were thwarted, as the PSII was difficult to resuspend in lower amounts of SMN buffer. The oxygen assay showed oxygen evolution rates of about 280 μ moles $O_2$/ mg Chl · hr.

*Reaction of PSII with 2-propanol*

Reaction of PSII BBY prep with 2-propanol and subsequent derivatization with hydroxylamine showed a small peak at 2.05 minutes (Figure 3).

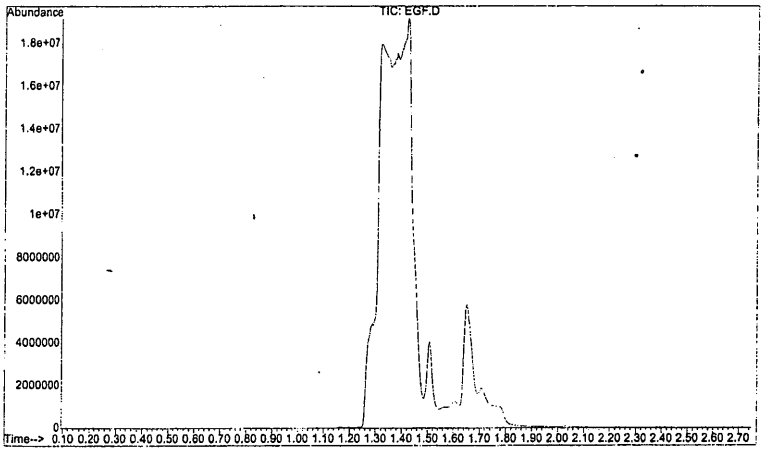


**Figure 3:** GC results of derivatization with hydroxylamine. Note peak under arrow at 2.05 minutes.

Due to the large amount of 2-propanol that eluted and overloaded the filament, the difference in the height of these peaks is exaggerated. The corresponding mass spectrum of this peak at the expected time shows the characteristic fragmentation pattern, including the distinctive combination of 58 and 73 m/z (Figure 4).

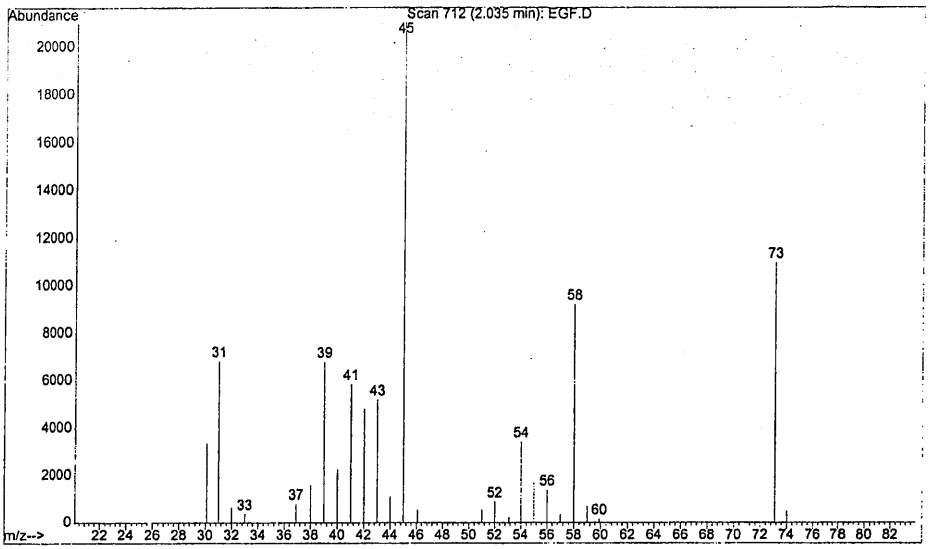


**Figure 4:** Mass spectrum of derivatization with hydroxylamine at 2.035 minutes. The odd m/z are indicative of a compound containing nitrogen, i.e. hydroxylamine.

Contamination from the large 2-propanol peak appears with the fragment of 45 m/z. The PSII and 2-propanol reaction was also analyzed using the GC with a flame ionization detector. While small, a peak is distinguishable at 1.479 minutes, the exact elution time of acetone. (Figure 5.)

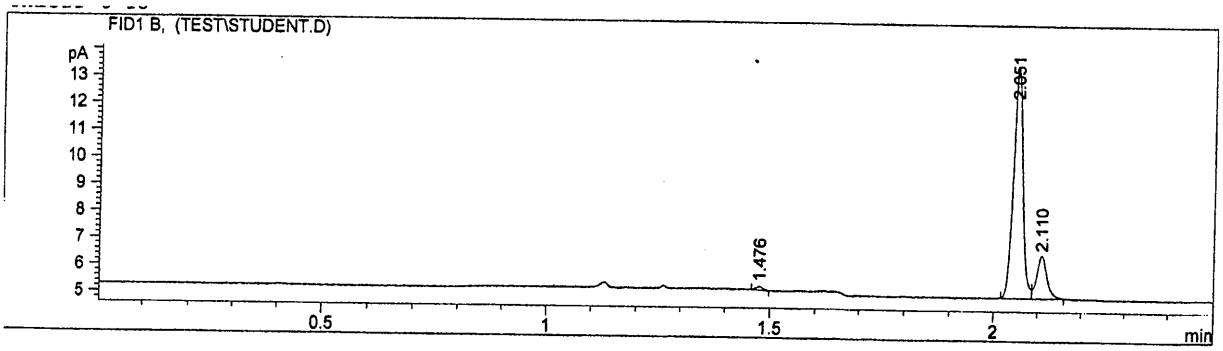


**Figure 5:** GC results showing acetone peak at 1.476 minutes. At 2.051 minutes is the reactant, 2-propanol, and the solvent, ethanol, is at 2.110 minutes.

The peaks at 2.051 and 2.110 minutes respectively correspond to 2-propanol and the ethanol used to dissolve the DCBQ.

*Reaction of PSII with n-propanol*

Oxidation of *n*-propanol with PSII showed a very small peak of propionaldehyde when analyzed with the GC-MS, especially when compared to the large peak of the reactant, *n*-propanol (Figure 6).

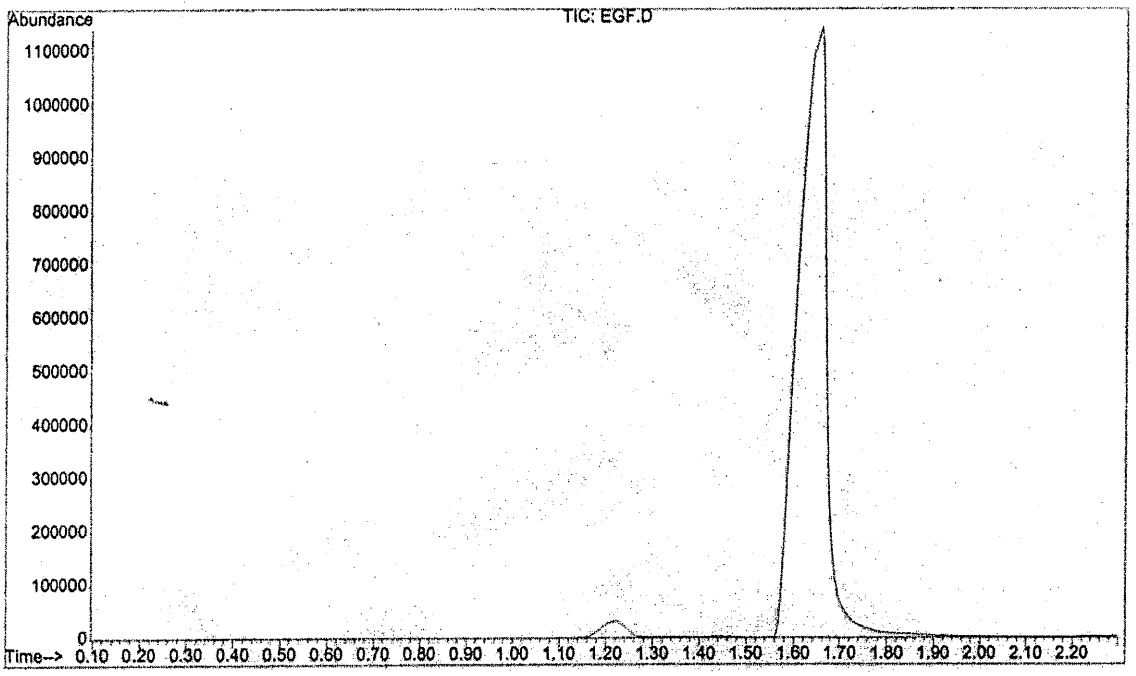


**Figure 6:** GC-MS showing probable propionaldehyde peak at 1.45 minutes. The peak at 1.20 represents an unknown, while *n*-propanol is the large peak at 1.58 to 1.88 minutes

The mass spectrum of the propionaldehyde peak shows the characteristic molecular weight fragment of 58 m/z, along with the loss of $H^{\circ}$ for a fragment weight at one third the height of 57 m/z (Figure 7). Because of the low abundance analyzed, many other spurious fragments appear, but presence of the 57 and 58 m/z peaks confirm the presence of propionaldehyde.

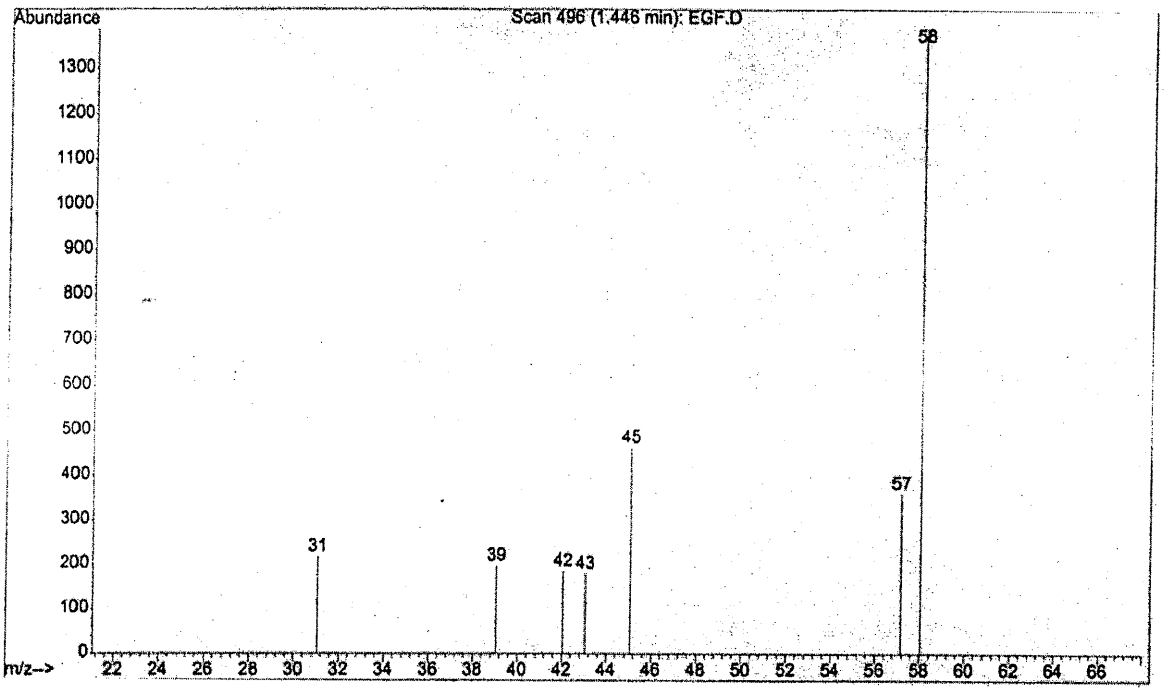


**Figure 7:** Mass spectrum of peak at 1.45 minutes in Figure 6. Note characteristic peaks of 58 and 57, distinctive to the desired product, propionaldehyde.

## Discussion

*Spinach PSII preparation and oxygen evolution assay*

Oxygen evolution rates from the assay were much lower than expected results. While the measured rate of 280 μ moles $O_2$/ mg Chl · hr does show the PSII to be active, it is lower than desired. Rates of at least 600 μ moles $O_2$/ mg Chl · hr are adequate, but it is possible to attain a rate of 800 to 1000 μ moles $O_2$/ mg Chl · hr with the BBY preparation used. This lower oxygen evolution rate may be due to the inexperience of those preparing the BBY prep from spinach as well as other procedural factors. Because of this low rate, it is possible that the success of all alcohol experiments was inhibited. Further, the oxygen evolution rates appear to be lower after the PSII BBY prep aliquots are thawed for use, and especially after they are thawed, refrozen several times.

*Reaction of PSII with 2-propanol*

Past research shows 2-propanol, as well as *n*-propanol, to be effective inhibitors of oxygen evolution activity with high potential for binding at or near the OEC active site (Force et al. 1998). The peaks eluted from the acetone oxime derivative analyzed on the GC-MS do show oxidation of 2-propanol to acetone (Figures 3 and 4). Testing of all reactants used in the procedure for the presence of acetone on the GC-MS showed all to be pure. The 2-propanol used was HPLC grade and 99.9% pure. Thus, it can be assumed that this acetone resulted from the oxidation of 2-propanol at the OEC of PSII. Analysis of another experiment run with the same parameters on the GC with the flame ionization detector also shows a small peak where the standard acetone eluted (Figure 5). This again supports the successful oxidation of 2-propanol using PSII.

Still, the peaks of the oxidized product, acetone, are very small. The abundance of the peak with the acetone oxime/ hydroxylamine derivate is moderately sized, but still very slight in comparison to the abundance of the reactant, 2-propanol. The same is true for the GC analysis for acetone of the other sample. Both show very minimal peaks of product compared to reactant, but since none of the reactants contained acetone, it is apparent that some oxidation of the alcohol, 2-propanol, did occur with PSII.

*Reaction of PSII with n-propanol*

Many attempts were made to oxidize *n*-propanol using a light activated method. None of these attempts oxidized *n*-propanol to propionaldehyde, so the reaction was attempted using the parameters outlined by Frasch et al. (1988). This method does not capitalize on the light innovatively used to power the other oxidation, but instead used hydrogen peroxide as an exogenous oxidant. While the success of this method has been documented in oxidizing other

alcohols, such as ethanol, glycerol and propargyl alcohol, it is unclear on what scale it was done. The decision was made to do it at a practical scale based upon passed experiments of about 7.5 mL. It is also unclear why a deadly poison, KCN, potassium cyanide, is necessary for a procedure meant to capitalize on the abilities of a living cell. Addition of EDTA can be rationalized as chelating excess metal ions that may interfere with the oxidation. None the less, oxidation of *n*-propanol with this dark method does appear to produce propionaldehyde (Figure 6). The mass spectrum of the small peak at the desired elution time shows fragments indicative of propionaldehyde (Figure 7). All reagents were tested to ensure they were propionaldehyde free and the *n*-propanol was distilled before use. Still, *n*-propanol oxidizes easily over time to propionaldehyde and other oxidation products. Despite this, it appears that *n*-propanol was oxidized to propionaldehyde with PSII.

Of constant annoyance is the peak at about 1.20 minutes on the GC-MS (Figure 6). Consisting of peaks with m/z of 32, 40, and 44, and others appearing variably, this compound has not been identified. It may be a residue left by the SPME fiber or something inherent to *n*-propanol. It shows up even when a blank is run on the GC-MS and in standards, reactions with and without PSII and reactions with or without *n*-propanol.

*Conclusions and future work*

In conclusion, it has been seen that spinach PSII can oxidize the small alcohols 2-propanol and *n*-propanol. The oxidation of these alcohols with PSII was not previously studied. Effective methods using gas chromatography were developed to identify reactants and products. Still, this procedure is not effective for synthesis, as only minute amounts of oxidized product can be seen. The amounts seen in analysis are unquantifiable and definitely unable to be isolated. Thus, the

light and dark oxidation of these alcohols with PSII is an ineffective method of synthesis of acetone and propionaldehyde.

Further research might explore many related topics, such as development of a light activated method to use for the oxidation of *n*-propanol. Also, removal of extrinsic protein subunits from PSII may allow better access for alcohols to the OEC and more oxidation. Attempts to oxidize other alcohols with different structures, such as propargyl alcohol, with a straight chain and triple bond, are worth investigating. Propargyl alcohol is one of the alcohols shown to be oxidized at the highest rate in the dark with $H_2O_2$ (Frasch et al. 1988).

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**References**

Åhrling, Karin A.; Evans, Mike C. W.; Nugent, Jonathan H. A.; Ball, Richard J.; Pace, Ron J. ESEEM Studies of Substrate Water and Small Alcohol Binding to the Oxygen-Evolving Complex of Photosystem II during Functional Turnover. *Biochemistry*, 2006, *45*, 7069-7082.

Berthold, Deborah A.; Babcock, Gerald T.; Yocum, Charles F. A Highly Resolved, Oxygen-Evolving Photosystem II Preparation from Spinach Thylakoid Membranes. *FEBS Letters*, 1981, *134*, 231-234.

Åhrling, Karin A.; Evans, Mike C. W.; Nugent, Jonathan H. A.; Ball, Richard J.; Pace, Ron J. ESEEM Studies of Substrate Water and Small Alcohol Binding to the Oxygen-Evolving Complex of Photosystem II during Functional Turnover. *Biochemistry*, 2006, *45*, 7069-7082.

Evans, M. C. W.; Nugent, J. H. A.; Ball, R. J.; Muhuiddin, I.; Race, R. J. Evidence for a Direct Manganese-Oxygen Ligand in Water Binding to the $S_2$ State of the Photosynthetic Water Oxidation Complex. *Biochemistry*, 2004, *43*, 989-994.

Faber, Kurt. Biotransformations of Non-Natural Compounds: State of the Art and Future Development. *Pure and Appl. Chem.*, 1997, *69*, 1613-1632.

Ferreira, Kristina N.; Iverson, Tina M.; Maghlaoui, Karim; Barber, James; Iwata, So. Architecture of the Photosynthetic Oxygen-Evolving Center. *Science*, 2004, *303*, 1831-1838.

Force, Dee Ann; Randall, David W.; Lorigan, Gary A.; Clemens, Kari L.; Britt, R. David. ESEEM Studies of Alcohol Binding to the Manganese Cluster of the Oxygen Evolving Complex of Photosystem II. *J. Am. Chem. Soc.*, 1998, *120*, 13321-13333.

Frasch, Wayne D.; Mei, Rui; Sanders, Matthew A. Oxidation of Alcohols Catalyzed by the Oxygen-Evolving Complex. *Biochemistry* 1988, *27*, 3715-3719.

Giri, Archana; Dhingra, Vikas; Giri, C. C.; Singh, Ajay; Ward, Owen P.; Narasu, M. Lakshmi. Biotransformations using plant cells, organ cultures and enzyme systems: current trends and future prospects. *Biotechnology Advances* 2001, *19*, 175-199.

Knör, Günther. Bionic Catalyst Design: A Photochemical Approach to Artificial Enzyme Function. *ChemBioChem.* 2001, *7/8*, 593-596.

Markó, István E.; Giles, Paul R.; Tsukazaki, Masao; Brown, Stephen M.; Urch, Chistopher J. Copper-Catalyzed Oxidation of Alcohols to Aldehydes and Ketones: An Efficient Aerobic Alternative. *Science*. 1996, *274*, 2044-2046.

McDonald, I.A.; Hackett, L.P.; Dusci, L.J. The Identification of Acetone and the Detection of Isopropanol in Biological Fluids by Gas Chromatography. *Clinica Chemica Acta.* 1975, *63*, 235-237.

Sheptovitsky, Yelena G.; Brudvig, Gary W. Isolation and Characterization of Spinach Photosystem II Membrane-Associated Catalase and Polyphenol Oxidase. *Biochemistry* 1996, *35*, 16255-16263.