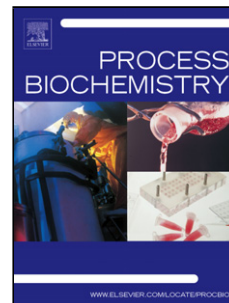# Accepted Manuscript

Title: The effect of hydrogen peroxide produced during ultraviolet disinfection of CHO cell culture media

Authors: Emma V. Dare, Michelle Gabriel, Afroza Begum, Michael Sasges, Marc G. Aucoin

Please cite this article as: Dare Emma V, Gabriel Michelle, Begum Afroza, Sasges Michael, Aucoin Marc G.The effect of hydrogen peroxide produced during ultraviolet disinfection of CHO cell culture media.*Process Biochemistry* http://dx.doi.org/10.1016/j.procbio.2017.06.025

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The effect of hydrogen peroxide produced during ultraviolet disinfection of CHO cell culture media

Emma V. Dare[a], Michelle Gabriel[b], Afroza Begum[c], Michael Sasges[d], and Marc G. Aucoin*[e]

[a]Department of Chemical Engineering, University of Waterloo, 200 University Ave West, Waterloo, ON, Canada, N2L 3G1, edare@uwaterloo.ca

[b] Trojan Technologies, London, Ontario, Canada

[c] Trojan Technologies, London, Ontario, Canada

[d] Trojan Technologies, London, Ontario, Canada

[e] Department of Chemical Engineering, University of Waterloo, 200 University Ave West, Waterloo, ON, Canada, N2L 3G1, maucoin@uwaterloo.ca

*corresponding author

Highlights:

- Moderate UV can disinfect cell culture media without damage to the media
- H_2O_2 generated by high UV was investigated as an initiator of deleterious reactions
- UV causes changes beyond what are caused by H_2O_2 formation
- H_2O_2 in media only caused changes in pyruvate, acetate, sarcosine and formate
- Catalase addition prevented changes in pyruvate, acetate, sarcosine and formate

Graphical

abstract
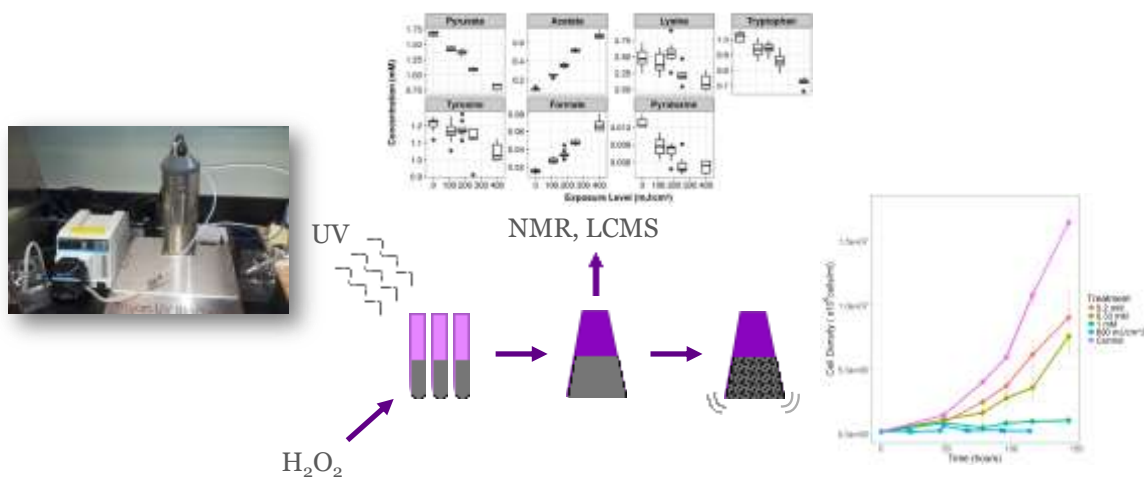
Abstract

Ultraviolet (UV) irradiation is being considered for protection against viral contamination in cell culture media. Hydrogen peroxide (H_2O_2) produced by UV irradiation has been suggested as the cause for poor cell growth in irradiated media, but this hypothesis has not been carefully evaluated. The impact of H_2O_2 on Chinese Hamster Ovary (CHO) cell culture medium was compared with the impact of UV irradiation. Media composition was analyzed via nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography mass spectrometry (LCMS), and cell growth in treated media was also evaluated. Although addition of H_2O_2 to medium caused significant changes in pyruvate, formate, acetate, and sarcosine concentrations, there was less effect on CHO cell growth compared with irradiation. UV irradiation caused other changes in composition that did not occur as a result of H_2O_2 addition. Catalase inhibited the effects of adding H_2O_2 to the media, but catalase added before irradiation did not affect most irradiation-induced changes, even though catalase retained activity. In conclusion we found that

while H$_2$O$_2$, which can be generated as a result of UV-irradiation, may be the cause of some changes in medium composition, it does not directly account for impaired CHO cell growth after high UV doses.

Chemical compounds studied in this article:

Hydrogen Peroxide (PubChem CID: 784); Pyruvate (PubChem CID: 107735); Sarcosine (PubChem CID: 1088); Riboflavin (PubChem CID: 493570); Lumichrome (PubChem CID: 5326566); Thiamine (PubChem CID: 1130); Pyridoxine (PubChem CID: 1054)

Introduction

During the culture of cells in the biopharmaceutical industry, the risk of viral contamination remains of major concern [1,2]. Filtration of cell culture media is used for the removal of contamination; however, several organisms, including minute mouse virus (MMV), leptospira, and mycoplasma, are too small to be removed by filtration and continue to be a risk in biopharmaceutical industry cell culture systems [3-5]. Other techniques that have been used for viral inactivation or clearance from biological materials include gamma irradiation, high-temperature short-time treatment (HTST) and nanofiltration [6,7]. Gamma irradiation of serum used in cell culture has been shown to reduce CHO cell growth efficiency at doses below what are necessary to clear MMV completely [8,9]. HTST has also been shown to be an ineffective treatment for low levels of MMV contamination [10]. Nanofiltration is effective at removing very small organisms [11]; however, the operating costs associated with their use can be prohibitive [12]. Ultraviolet (UV) irradiation is known to be an effective method for disinfection of biological solutions [13,14]. UV-C irradiation has been used in the biopharmaceutical industry for the treatment of blood products such as purified plasma proteins and blood clotting agents [15,16], and has shown great potential in the inactivation of many pathogens, including non-enveloped viruses such

3

Hepatitis A and parvovirus [17,18]. Furthermore, we previously reported on a UV-C continuous flow disinfection system that has the potential to achieve industry required standards for sterility of cell culture media [19]. Given the complexities and diversity of cell culture media however, there is a need for a deeper understanding of the potential deleterious effects that UV-related reactions can have on essential cell culture media components.

Some studies have shown that UV treatment can cause photo-damage to amino acids through photolysis and oxygen radical oxidation [20]. These results raise concern about potential UV-induced changes in biological activity as well as toxicity. UV irradiation of cell culture media can result in formation of hydrogen peroxide (H_2O_2), which is a reactive oxygen species (ROS) that can start free radical reactions causing oxidation of fatty acids and amino acids, and enzyme deactivation, leading to diminished growth and cell death [21]. Previously, we have shown that although high doses of UV-irradiation can lead to decreased cell growth and viability [19], moderate doses result in damage more akin to daylight exposure [13]. It has been speculated by others that the generation of H_2O_2 during UV irradiation plays a major role in the destruction of several media components, via reactions with tryptophan and riboflavin, leading to poor cell growth [22]. Riboflavin is likely a major player in the damage to amino acids. Reaction of riboflavin with UV in the presence of O_2 yields intermediate forms such as lumichrome and lumiflavin [23] and subsequent formation of singlet oxygen molecules via H_2O_2 [24], consistent with our demonstrated improvement in an oxygen-free UV-C irradiation [19]. The objective of this work was to determine if H_2O_2 generated during irradiation is responsible for poor growth of cells in the medium thereafter, and if damage by UV irradiation can be prevented by the addition, prior to irradiation, of catalase, which is an enzyme that catalyzes the decomposition of hydrogen peroxide to oxygen and water [25].

Materials & Methods

Cell culture media and H_2O_2

4

CD-CHO growth medium, supplemented with GlutaMAX^{TM}, and hypoxanthine and thiamine (HT) (Life Technologies Inc., Burlington, ON, Canada), was used for irradiation and culture experiments. A 0.1 M H_2O_2 was prepared in double distilled H_2O from a 8.8 M H_2O_2 stock solution (Sigma-Aldrich, Co., Oakville, ON, Canada) and filter sterilized.

Cell Culture

Cell culture was performed with CHO^{BRI} cells (National Research Council, QC, Canada) adapted to grow in serum-free growth media supplemented with HT supplement and GlutaMax™. Pluronic F-68 non-ionic surfactant (10 mL per 1 L of medium), and anti-clumping agent (10 mL per 1 L of medium) (Life Technologies, Inc., ON, Canada) were added post-irradiation for culture purposes. The parental culture was incubated at 37°C, 5% CO_2, and atmospheric O_2, with agitation at 100 rpm. Erlenmeyer flasks (125 ml non-pyrogenic polycarbonate, Corning Inc., NY, USA) with a vented cap were used for the parental and experimental cultures, with a working volume of 30 mL. After reaching a viable cell density of 2 to 3 × 10^6 cells/mL in the parental flask, the experimental cultures were inoculated with a seeding density of 0.2 × 10^6 viable cells/mL. Experimental cultures for each treatment group were inoculated and cultured in triplicate in 30 mL batch cultures grown identically to the parental cultures. Cell density and viability were monitored daily for up to eight days using a haemocytometer by Trypan blue exclusion.

H_2O_2 Decay in Cell Culture Medium

To quantify H_2O_2, a Hydrogen Peroxide Assay Kit (Abcam Inc., Toronto, ON, Canada) based on binding H_2O_2 to a fluorescent OxiRed probe in the presence of Horse Radish Peroxidase (HRP), was used. The protocol was followed according to the manufacturer's instructions. Briefly, for the fluorometric standard curve, the reaction buffer consisted of 1 µL OxiRed, 1 µL HRP, and assay buffer up to a total volume of 50 µL added to each well followed by a 10 min incubation at room temperature. The fluorescence was measured at Ex 535/Em 587 nm. A standard curve of 0 to 0.5 pmol/well was used.

5

To determine the decay of H_2O_2 in the medium, 10 mL of media was spiked with 0.1 M stock H_2O_2 in ddH$_2$O to final concentrations of 200, 500, and 1000 µM. These samples were incubated in the dark at room temperature and H_2O_2 was measured at 30 min intervals up to 120 min, using appropriate dilutions to fall within the standard curve for the fluorometric assay.

To measure H_2O_2 generated during UV-irradiation, the assay was performed on samples taken immediately upon exiting the reactor (time to read was approximately 20 min). Samples were diluted to fall within the standard curve. Measurements were taken at time zero (initial read) and at 60 min.

The decay of H_2O_2 after exiting the tubular reactor was modeled based on first order kinetics using the equation 1:

$$\frac{dC}{dt} = k_2 C$$

Where C is the concentration of H_2O_2 (µmol/L) and k_2 is the decay rate constant (/min). The change in H_2O_2 in the UV reactor was modeled as a plug flow reactor such that the concentration change along the length of the reactor was given by equation 2:

$$\frac{dC}{dV} = \frac{k_1 V - k_2 C}{v_0}$$

Where V is the volume of the tubular reactor (in L), k_1 is the rate of production constant (µmol/(L^2*min)), which is a function of exposure to UV, and v_o is the volumetric flow rate (L/min). The ordinary differential equations were solved in R statistical software using the deSolve package built under R version 3.1.3.

UV Irradiation

Oxygen-saturated medium was utilized to promote the formation of H_2O_2. An ez-Control system (Applikon Biotechnology B.V., Delft, Netherlands) was used to control and monitor the oxygen saturation level in the medium as well as measure pH and temperature [19]. Medium was pumped into the

bioreactor at 170 mL/min where it was saturated with O_2 gas. The high-purity compressed O_2 gas flowed from a regulated compressed gas cylinder (Praxair Canada, Inc., Mississauga, ON, Canada) through the ez-Control system at 20 mL/min into the bioreactor until the dissolved oxygen reached equilibrium at 100% as determine with a dissolved oxygen sensor. Upon equilibration at oxygen saturation, the media was pumped through a continuous-flow UV-reactor system [19] using a Masterflex L/S computerized peristaltic pump with Easy-Load pump head (Cole-Parmer Canada Co., Montreal, QC, Canada). The UV reactor system consisted of Teflon tubing arranged around a central low-pressure mercury UV lamp emitting at 254 nm. The reactor system was designed to achieve good mixing and uniform fluence to the fluid. To achieve the desired 600 mJ/cm^2 fluence, the media was passed through the reactor system at 7 mL/min (~10 min residence time). After discarding a volume of fluid equal to three UV system volumes, samples were collected into sterile 15 mL tubes for analysis. The actual fluence delivered was verified using the procedure described in the UV Fluence section. The pH was monitored throughout O_2 saturation and during pumping through the UV reactor and ranged from 8.0 to 8.4.

UV Fluence

To determine the UV fluence delivered during media irradiation, a viral clearance test with a challenge organism, MS2 phage, was conducted. MS2 (GAP EnviroMicrobial Services, London, ON, Canada) was irradiated at a number of discrete UV fluence values using a collimated beam apparatus according to the protocol of Bolton and Linden [26]. Survival curves were plotted to determine the UV sensitivity of the phage. Supplemented media was inoculated with the characterized MS2 coliphage to a titre of 10^8 pfu/mL, and this media was pumped through the UV reactor at flow rates of 50 and 100 ml/min. The log inactivation of MS2 was determined by GAP EnviroMicrobial Services. From this data, it was determined that the 100 mL/min flow rate of media through the reactor delivered a fluence of 49 mJ/cm^2, and the 50 mL/min flow rate of media through one reactor received 98 mJ/cm^2. It was calculated that the

7

flow rate of 7 mL/min achieved the target fluence of 600 mJ/cm^2. The high fluence condition could not be validated directly, since it would have eliminated all viable phage.

Collimated Beam Irradiation of Catalase

A 20 mg/mL crystalline stock solution of bovine catalase (Sigma–Aldrich) was diluted to 20 µg/mL in 50 mM potassium phosphate buffer pH 7.0, with 0.1% Triton X-100. 5 mL samples of dilute catalase solution in 10 mL beakers were irradiated using a calibrated collimated beam apparatus (Trojan Technologies, London, ON, Canada) incorporating a low-pressure mercury lamp emitting at 254 nm [26]. Beakers were placed in an ice water bath with continuous stirring to avoid thermal degradation during irradiation. Optical absorbance of the fluid was measured with a Cary 100 spectrophotometer (Agilent, USA) equipped with a 6-inch integrating sphere. Samples were continuously stirred and exposed to UV light for doses of 50, 100, 200, 500, and 1000 mJ/cm^2. Control samples were also placed in an ice water bath, but were not exposed to UV light. The catalase enzyme activity was determined using a colorimetric Catalase Assay Kit (Sigma-Aldrich) as per the manufacturer's instructions.

H_2O_2 and Catalase Addition to Cell Culture Medium

To evaluate the effects of H_2O_2 directly on the cell culture medium, H_2O_2 was added from a fresh 0.1 M stock to final concentrations of 0.2, 0.33, 0.5, and 1 mM with or without the addition of bovine liver catalase (Sigma-Aldrich) to a final concentration of 100 U/mL. Samples were incubated at room temperature for 2 hours in the dark, followed by storage at -80°C. Experiments were also performed where 100 U/mL catalase was added to the medium immediately after irradiation with 600 mJ/cm^2 to assess if any further damage to media components due to H_2O_2 occurs post-irradiation. Samples were incubated for 2 hours in the dark, followed by freezing and storage at -80ºC.

H_2O_2 and catalase were also added prior to irradiation for some samples. Catalase was added to

8

a final concentration of 270 U/mL to the oxygen saturated media in the bioreactor and mixed for ~1 min, before being pumped through the UV reactor at the appropriate flow rate. In another experiment, catalase (370 U/ml final concentration) followed by 0.33 mM H_2O_2 were added to the oxygenated media in the bioreactor prior to irradiation. Samples were collected for further analysis.

To assess the possibility of compounds being formed during irradiation that may inhibit H_2O_2-induced damage, H_2O_2 was added immediately after irradiation (0.5 mM H_2O_2 final concentration with or without additional 100 U/ml catalase) to samples irradiated at a 600 mJ/cm^2 fluence. As additional controls, fresh, non-irradiated media with 0.5 mM H_2O_2, with and without 100 U/mL catalase, were also evaluated. The media were incubated in the dark for 2 hours, followed by freezing at -80ºC.

To determine whether H_2O_2 or other compounds generated from UV-irradiation of media can react further with fresh media, fresh medium was added immediately post-irradiation to 600 mJ/cm^2-irradiated media in a 1:1 ratio. Samples were incubated in the dark at room temperature for 2 hours followed by freezing at -80ºC.

Nuclear Magnetic Resonance Spectroscopy and Metabolic Profiling

Samples were randomized prior to NMR scanning to minimize variability and profiling bias. To prepare samples for NMR analysis, 70 µL of internal standard (99.9% D_2O with 5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid and 0.2% w/v sodium azide) were added to 630 µL of media. The prepared samples were mixed in a vortex mixer and pipetted into 5 mm NMR tubes (NE-UL5-7, New Era Enterprises Inc., NJ, US). The NMR spectra were acquired with a 600 MHz Advance spectrometer equipped with a TXI 600 triple resonance probe (Bruker, MA, US) using the first increment of the NOESY pulse sequence with a 1 s pre-saturation pulse, followed by a 4 s acquisition time. Following spectra acquisition, the pH of each sample was measured and recorded and ranged from 8.0 to 8.4. The Chenomx NMR Suite 8.2 (Chenomx Inc., AB, Canada) was used to process the spectra. Baseline, phase, shim, and chemical shape corrections

9

were performed manually using the software. The compounds were identified and quantified by targeted profiling with the built-in library of chemical resonances and the internal standard as a reference compound.

LC-MS Analysis

Calibration curves were developed for biotin, vitamin B12, lumichrome, riboflavin and thiamine, using serial dilutions of vitamin standards between 0.025 and 250 ppb (Sigma Aldrich, Oakville, ON, Canada). Stock solutions were prepared by dissolving in water or ammonium hydroxide, as appropriate, and stored at 4 °C.

Media samples were diluted 10:1 with HPLC grade water, and a solid-phase-extraction (SPE) method, using Supelco Supelclean LC18 SPE cartridges (Sigma Aldrich), was used to extract the vitamins from interfering compounds. Cartridges were conditioned prior to use with 3 mL of HPLC-grade methanol (Fisher Scientific, Ottawa, ON, Canada) and 3 mL of water at a flow rate of 3 mL/min. After extraction, 3 mL of 85% methanol was used as an elution solvent. The methanol was subsequently evaporated using a rotary evaporator, and then reconstituted with water to an appropriate final dilution. Samples were filtered with a Phenex 0.2 µm syringe filter (Phenomenex, Inc., Torrance, CA) prior to analysis.

The LC-MS system comprised an Agilent 1100 HPLC (Santa Clara, CA, US) equipped with an autosampler and thermostated sample chamber, and an Agilent Poroshell 120, 2.7 µm SB-C18, 3.0 × 50 mm analytical column. An optimized gradient elution method was used to achieve good chromatographic separation. The gradient was formed using solvent A, which consisted of a 5 mM ammonium formate in water with 0.1% formic acid, and solvent B, which consisted of 5 mM ammonium formate in 95% acetonitrile in water with 0.1% formic acid. The chromatographic run was carried out over 14 min with 100% A up to 5 min, then 25% of B at 5.10 min, 43% of B at 6.60 min, followed by 98% B from 6.70 to 8 min, and back to 100% A from 8.10 to 14 min.

10

The mass spectrometer was a SCIEX 6500 triple quadrupole mass analyzer (SCIEX, Concord, ON, Canada). Multiple Reaction Monitoring (MRM) was used as the mass spectrometer's detection mode, with unique ion precursor and fragment ions monitored for each vitamin [19]. Statistical data analysis was conducted using Analyst® 1.6.2 software (SCIEX, Concord, ON, Canada) using the following parameters for processing calibration curve data: Gaussian smooth width – 3 points, noise percentage – 50%, peak splitting factor – 2 points, and a weighting of $1/x^2$.

Results

Decay of H_2O_2 in Culture Media

H_2O_2 is known to be generated upon UV irradiation of fluids containing sensitizers such as riboflavin [27] and has been reported to be the cause of toxicity in irradiated tissue-culture medium [22]. To determine if H_2O_2 was the principal cause of any negative effect of UV-irradiating media in our flow-through reactor system, and to see if the effect could be mitigated, we undertook an in-depth investigation of the generation and impact of H_2O_2 in cell culture media. Although a dose of 600 mJ/cm^2 is more than 30 times that required to eliminate 10 log of mycoplasma [28], and would probably never be used in any practical application, this dose, and fully oxygen-saturated conditions, were chosen to provide the best possible conditions for H_2O_2 generation, and detection. H_2O_2 concentration measured at the outlet of the reactor after receiving a dose of 600 mJ/cm^2 was approximately 30.2 µM. The same sample after a 60-minute incubation at room temperature in the dark, had concentrations of H_2O_2 reduced to baseline.

To establish the rate of H_2O_2 decay, media was spiked and monitored over time (Figure 1). By 2 hours post-spiking, H_2O_2 is no longer detectable in the media regardless of the starting concentration. The decay of H_2O_2 in the medium follows, approximately, first order kinetics with respect to H_2O_2 when starting

in the same media composition (Figure 1A). There is some evidence, however, that the rate of decay decreases with higher initial H_2O_2 (Figure 1B), consistent with higher order reaction mechanisms in which a secondary species participating in the reaction is ultimately influencing the rate of decay.

Based on the determined first-order kinetics of H_2O_2 decay (k = 0.08 min^{-1}), and extrapolating backward from time of H_2O_2 measurement, it is calculated that the concentration of H_2O_2 at the outlet of the UV reactor used to irradiate the cell culture medium in the experiment described above was approximately 150 µM (Figure 1C). The decay profile of the H_2O_2 once leaving the outlet of the reactor was modeled as shown in Figure 1D, assuming first order generation based on exposure to UV light (k_{UV} = 550 umol/(L^2*min)).

Influence of H_2O_2 in Media on Cell Culture

CHO cells were cultured in medium spiked with H_2O_2 prior to seeding. The results indicated that some significant negative effects on growth occurred; however, the viable cell density observed in H_2O_2-spiked medium (0.2 or 0.33 mM) was not as poor as that observed in medium spiked with 1 mM H_2O_2 or medium irradiated at 600 mJ/cm^2 (Figure 2).

UV Exposure and Effect on the Composition of Cell Culture Medium

NMR profiling was used to determine effects on media components, and all the compounds that had significant changes in response to UV dose from 0 up to 600 mJ/cm^2 are listed in Table 1. Samples were run in triplicate from two sets of experiments. Compounds that do change significantly in response to UV include: acetate, arginine, cystine, ethanolamine, formate, histidine, hypoxanthine, methionine, pyridoxine, pyruvate, sarcosine, tryptophan, tyrosine, and trans-4-hydroxy-L-proline. All significant changes were decreases, except for increases in acetate, formate, and sarcosine, which represent degradation products. These results are similar to, and follow the same trends as, those reported

previously [19].

Effect of Adding H_2O_2 to Cell Culture Medium

To evaluate the effect of H_2O_2 on fresh medium, NMR analysis was performed after a two-hour incubation with H_2O_2 (Figure 3). Significant changes were only found to occur to acetate, formate, pyruvate, and sarcosine, compared to the many changes that occur as a result of UV-irradiation (Table 1). This indicates that the changes to these 4 compounds are a direct result of the H_2O_2, and that the other changes associated with irradiation likely occur secondary to these reactions with H_2O_2 or because of other reaction mechanisms. The simultaneous addition of 100 U/ml catalase completely prevented any changes to these compounds as a result of H_2O_2 addition (Figure 3). Furthermore, equivalent changes in pyruvate and acetate resulting from a 600 mJ/cm^2 UV exposure would have required the generation of 0.33 mM of H_2O_2 (Figure 6), which is much higher than the anticipated production based on outlet measurements (Figure 2).

To measure specific vitamins, LCMS was used. Decreases in vitamin B12, riboflavin (with a corresponding increase in lumichrome), and thiamine were measured after irradiation at 600 mJ/cm^2 (Figure 4). By contrast, the addition of H_2O_2 up to 1 mM did not lead to any changes in the vitamin concentrations compared to the control (Figure 4).

Catalase Activity After Irradiation

To investigate the effect of UV irradiation on catalase and to see if it could be added to prevent damage to the composition of the medium, a catalase solution was irradiated using a collimated beam apparatus [26] and the enzyme activity was measured. The catalase activity decreased with increasing UV dose (Figure 5A). For 711 U/mL (20 mg/ml) catalase, a dose of 600 mJ/cm^2 resulted in an approximate 40% loss of enzyme activity. Therefore, even though there is loss of enzyme activity, it is expected that

13

enough activity remained to counter the production of H_2O_2 during the irradiation.

Preventing H_2O_2 Damage during Irradiation Using Catalase

To determine which changes during irradiation were due to H_2O_2 reactions directly, catalase was added to the media prior to passing the media through the UV reactor. Two different concentrations of catalase were evaluated, 100 U/mL and 270 U/mL. In both cases, the added catalase reduced, but did not eliminate, the impact of irradiation on acetate, pyruvate, formate and sarcosine (Figure 5C). Other impacts of UV irradiation were not affected by the added catalase, such as those listed in Table 1. Increasing the catalase concentration from 100 U/ml to 270 U/ml showed no significant change. Based on the study of irradiating catalase, there should still be approximately 50 and 135 U/mL of catalase activity after irradiation, similar to the levels that prevented metabolite changes without exposure to UV (Figure 3). This indicates that changes to these compounds are being caused partially by H_2O_2 and partially by other mechanisms. We also performed an experiment where 100 U/ml of fresh catalase was added immediately following UV irradiation of CD-CHO at 600 mJ/cm^2 (data not shown). We found no additional changes to irradiated media due to catalase addition compared to unsupplemented irradiated media, indicating that no significant peroxide-induced reactions occurred after irradiation. In addition, no improvement on CHO cell culture was observed when catalase was added prior to irradiation (Figure 5B).

Effect of UV and H_2O_2 on medium composition

It is also possible that the presence of H_2O_2 in the cell culture media may cause other secondary reactions to take place in the presence of UV in addition to the changes observed with acetate, pyruvate, formate and sarcosine. To assess this, 0.333 mM H_2O_2 was added to media immediately prior to being subjected to a UV dose of 600 mJ/cm^2. The results (Figure 6) indicated that adding H_2O_2 to the media caused the expected changes to occur (to acetate, pyruvate, formate and sarcosine); however, no additional significant changes to other compounds were observed. This indicates that there are no

14

detectable additional reactions occurring when H_2O_2 is in the presence of UV and no compounding effect on changes to the affected media components. It is known that 254 nm irradiation of hydrogen H_2O_2 can produce hydroxyl ($^{\cdot}$OH) radicals resulting in oxidation reactions [29]. The present results indicate that this is only a minor pathway in the photochemistry of UV-C irradiated media.

When H_2O_2 was added immediately after irradiation (Figure 7), additional changes to acetate, pyruvate, formate and sarcosine were seen as in Figure 3 – the same compounds for which changes were seen when H_2O_2 was added to fresh media. When catalase was added immediately after irradiation, no further prevention of changes occurred. Adding fresh media immediately after irradiation did not lead to any additional changes (data not shown). These results imply that reactions with H_2O_2 appear to only take place within the reactor.

Discussion

We have shown through this current and previous work [19] that high-dose UV-irradiation of cell culture media leads to the destruction of many important nutrients and formation of new compounds that can lead to poor cell growth. Our previous work [19] indicates that in the presence of oxygen, the effects of UV-irradiation on the culture media are more pronounced. Therefore, it is likely that ROS are involved in the destructive reactions with media components. The current results indicated that H_2O_2 addition, and formation in the presence of UV, only led to changes in acetate, pyruvate, formate and sarcosine concentrations in the media. It is not clear why only these specific compounds are affected, but the reduction of UV-induced changes to only these compounds by the addition of catalase prior to irradiation, provides further evidence of this conclusion. It has also been reported that H_2O_2 addition to wastewater is a poor method for disinfection [30]. Therefore, it is not likely responsible for the majority of the viral clearance that takes place with UV irradiation.

15

Several mechanisms can be proposed as to the specific reactions taking place to cause the observed changes. Formation of sarcosine may be a result of reactions with serine, threonine, glycine or other upstream compounds in glycolysis in the presence of excess H_2O_2. During cell growth, sarcosine – which is a metabolite and by-product of glycine synthesis – is oxidized to form H_2O_2 and glycine through the actions of the enzyme sarcosine oxidase [31], which can then go on to form (and can also be formed from) serine or threonine.

The destruction of pyruvate and formation of acetate appear to be tightly linked in the presence of H_2O_2. During normal cell metabolism, pyruvate and acetate are both involved in the formation of acetyl co-enzyme A, which is required for many pathways in metabolism. Pyruvate is a precursor to acetate during the citric acid cycle in the cell, but can also be converted to acetate in the presence of H_2O_2 [32]. Schone and Herrmann [32] reported a second order rate constant of 0.75 $M^{-1}s^{-1}$. The second order rate constant for the reaction of catalase with H_2O_2 was reported as 2 $M^{-1}s^{-1}$. This likely explains the ability of catalase to prevent the majority of changes to pyruvate when in the presence of H_2O_2 as the reaction occurs more quickly than the reaction of pyruvate with H_2O_2. Formate is formed normally in both the metabolism of pyruvate and sarcosine, which is likely why it is produced when pyruvate is degraded in the presence of H_2O_2.

Meunier et al [19] previously demonstrated that after disinfection of media with the UV reactor also used in this study, CHO cells grow poorly under certain oxygenated conditions and above certain UV doses. Our current results show that H_2O_2 addition to culture media (tested only up to 1 mM) led to a decrease in viable cell density. We attribute this decrease to an imbalance within the citric acid cycle of the cell and altered glucose metabolism caused by a decrease in pyruvate and an increase in acetate in the culture medium. It is important to note that the expected concentration of H_2O_2 produced at effective UV doses is much smaller than 1 mM. In fact, we determined that a fluence of 600 mJ/cm^2 of UV would

16

be equivalent to ~0.33 mM given the results determined by NMR of spiked H_2O_2 in media (Figure 3) and the 600 mJ/cm^2 UV-irradiated media (Table 1).

It can be noted that when catalase was added to media prior to irradiation, the impact of irradiation on pyruvate, acetate, formate and sarcosine was decreased; however, blocking these reactions was not complete regardless of the quantity of catalase added. Even though we found that there was a 40% loss in catalase activity with irradiation at 600 mJ/cm^2, the remaining amount of catalase would still likely be sufficient to block reactions related to H_2O_2, since we saw this to be the case when both catalase and H_2O_2 were added to non-irradiated media. Catalase is only responsible for decomposing H_2O_2 to water and oxygen [33]. It is very likely that other reactions are occurring affecting pyruvate, acetate, formate, and sarcosine, independent of H_2O_2.

Other ROS can be produced by UV irradiation. Reduction of O_2 produces superoxide ($^\cdot O_2^-$), which is reactive and is also the precursor to most ROS including H_2O_2 and $^\cdot OH$ [34]. The generation of superoxide occurs when UV photons interact with atomic oxygen to promote its formation [35]. It is also possible that singlet oxygen may have been produced, which is very reactive, high energy form of oxygen. It is produced after the O_2 molecule reacts with a photosensitizer molecule that has absorbed light or UV [36]. It is expected that irradiation of oxygen-saturated media will produce many ROS, and that these are likely responsible for the other chemical changes not attributable directly to H_2O_2.

Our results indicate that the damage observed to the media after exposure to 600 mJ/cm^2 UV irradiation (a fluence much higher than would be used for medium disinfection) is only partly caused by reactions with H_2O_2, which is formed by UV. In addition, we have shown previously that UV treatment of nitrogen-saturated media (0% dissolved oxygen) also led to the damage of media components [19]. Therefore, oxygen-related damage to media components in the presence of UV is only partly responsible for the changes that occur and lead to poor cell growth.

In conclusion, while we have provided evidence that while H_2O_2 causes damage to cell culture media components, it is not likely the ROS responsible for the negative effects on culture media that result from high doses of UV irradiation. We have provided evidence that H_2O_2 is indeed formed and is responsible for some damage to the media based on the effect of blocking damage with catalase addition. In order to determine the best possible method to mitigate damage to cell culture media during UV irradiation, it will be necessary to continue to search for the compounds that are involved in these destructive reactions. Our results indicate that UV disinfection of cell culture media would be best suited for clearance of organisms that are the most sensitive to UV, such as MMV or mycoplasma [14], as very low production of H_2O_2 and other toxic compounds would be produced at these doses as well as minimal destruction of important molecules. It will be interesting to investigate the effects of UV-C for disinfection of other cell culture-related solutions – such as buffers, serum, enzymes, and other biopharma-related products to determine its further applicability to the biopharma industry.

Acknowledgements

18

References

[1] J. Petricciani, R. Sheets, E. Griffiths, I. Knezevic, Adventitious agents in viral vaccines: lessons learned from 4 case studies, Biologicals. 42 (2014) 223-236.

[2] G. Dublin, J.-F. Toussaint, J.-P. Cassart, B. Howe, D. Boyce, L. Friedland, R. Abu-Elyazeed, S. Poncelet, H.H. Han, S. Debrus, Investigation of a regulatory agency enquiry into potential porcine circovirus type 1 contamination of the human rotavirus vaccine, Rotarix™, Hum. Vaccin. Immunother. 9 (2013) 2398-2408.

,

[3] J. Chen, J. Bergevin, R. Kiss, G. Walker, T. Battistoni, P. Lufburrow, H. Lam, A. Vinter, Case study: A novel bacterial contamination in cell culture production–Leptospira licerasiae, P.D.A. J. Pharm. Sci. Technol. 66 (2012) 580–591.

[4] H.G. Drexler, C.C. Uphoff, Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention, Cytotechnology. 39 (2002) 75–90.

[5] M. Moody, W. Alves, J. Varghese, F. Khan, Mouse minute virus (MMV) contamination–A case study: Detection, root cause determination, and corrective actions, P.D.A. J. Pharm. Sci. Technol. 65 (2011) 580–588.

[6] S. Shah, Pharmacy considerations for the use of IGIV therapy, Am. J. Health Syst. Pharm. 62 (2005) S5-11.

[7] R. Cameron, K. Smith, Virus clearance methods applied in bioprocessing operations: an overview of selected inactivation and removal methods, Pharm. Bioprocess. 2 (2014) 75-83.

[8] Z.M. Plavsic, J.P. Daley, D.J. Danner, D.J. Weppner, Gamma irradiation of bovine sera, Dev. Biol. Stand. 99 (1999) 95-105.

[9] J.D. Keathley, D.E. Wyatt, C.M. Williams, R. Festern, C. Maben, Is there life after irradiation? Part 2: gamma-irradiated FBS in cell culture, BioPharm. 22 (1993) 46-52.

[10] M. Murphy, G.M. Quesada, D. Chen, Effectiveness of mouse minute virus inactivation by high temperature short time treatment technology: a statistical assessment, Biologicals. 39 (2011) 438-443.

[11] T. Barnouf, M. Radosevich, Nanofiltration of plasma-derived biopharmaceutical products, Haemophilia. 9 (2003) 24-37.

[12] M. Taheran, S.K. Brar, M. Verma, R.Y. Surampalli, T.C. Zhang, J.R. Valero, Membrane processes for removal of pharmaceutically active compounds (PhACs) from water and wastewaters, Sci. Total Environ. 547 (2016) 60-77.

[13] S. Yen, S. Sokolenko, B. Manocha, E.J. Blondeel, M.G. Aucoin, A. Patras, F. Daynouri-Pancino, M. Sasges, Treating cell culture media with UV irradiation against adventitious agents: Minimal impact on CHO performance, Biotechnol. Prog. 30 (2014) 1190–1195.

[14] S.M. Meunier, M.G. Aucoin, M. Sasges, Evaluating ultraviolet sensitivity of adventitious agents in biopharmaceutical manufacturing, J. Ind. Microbiol. Biotechnol. 44 (2017) 893-909.

[15] P. Pohler, M. Miller, C. Winkler, D. Schaudien, K. Sewald, T.H. Müller, A. Seltsam, Pathogen reduction by ultraviolet C light effectively inactivates human white blood cells in platelet products, Transfusion. 55 (2015) 337-347.

[16] H.-L. Chan, P.R. Gaffney, M.D. Waterfield, H. Anderle, H.P. Matthiessen, H.-P. Schwarz, P.L. Turecek, J.F. Timms, Proteomic analysis of UVC irradiation-induced damage of plasma protien Serum amyloid P component as a major target of photolysis, F.E.B.S. Letter. 580 (2006) 3229-3236.

[17] S. Chin, B. Williams, P. Gottlieb, H. Margolis-Nunno, E. Ben-Hur, J. Hamman, R. Jin, E. Dubovi, B. Horowitz, Virucidal short wavelength ultraviolet light treatment of plasma and factor VIII concentrate: protection of proteins by antioxidants, Blood. 86 (1995) 4331-4336.

[18] S. Chin, R. Jin, X.-L. Wang, J. Hamman, G. Marx, X. Mou, I. Anderson, L.-O. Lindquist, B. Horowitz, Virucidal treatment of blood protein products with UVC radiation, Photochem. Photobiol. 65 (1997) 432-435.

[19] S.M. Meunier, B. Todorovic, E.V. Dare, A. Begum, S. Guillemette, A. Wenger, P. Saxena, J.L. Campbell, M. Sasges, M.G. Aucoin, Impact of dissolved oxygen during UV-irradiation on the chemical composition and function of CHO cell culture media, P.L.o.S. One. 11 (2016) e0150957.

[20] M.J. Davies, Reactive species formed on proteins exposed to singlet oxygen, Photochem. Photobiol. Sci. 3 (2004) 17-25.

[21] S.G. Rhee, H.A. Woo, I.S. Kil, S.H. Bae, Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides, J. Biol. Chem. 287 (2012) 4403–4410.

[22] R.J. Wang, T. Nixon, Identification of hydrogen peroxide as a photoproduct toxic to human cells in tissue-culture medium irradiated with "daylight" fluorescent light, In Vitro. 14 (1978) 715-722.

[23] I. Ahmad, Q. Fasihullah, A. Noor, I.A. Ansari, Q.N. Ali, Photolysis of riboflavin in aqueous solution: a kinetic study, Int. J. Pharm. 280 (2004) 199-208.

[24] S. Sel, N. Nass, S. Pötzsch, S. Trau, A. Simm, T. Kalinski, G.I.W. Duncker, F.E. Kruse, G.U. Auffarth, H.-J. Brömme, UVA irradiation of riboflavin generates oxygen-dependent hydroxyl radicals, Redox. Report. 19 (2014) 72-79.

[25] M. Zamocky, P.G. Furtmuller, C. Obinger, Evolution of catalases from bacteria to humans, Antioxid. Redox Signal. 10 (2008) 1527-1548.

[26] J.R. JBolton, K.G. Linden, Standardization of methods for fluence (UV dose) determination in bench-scale UV experiments, J. Environ. Eng. 129 (2003) 209–215.

[27] K. Sato, H. Taguchi, T. Maeda, H. Minami, Y. Asada, Y. Watanabe, K. Yoshikawa, The primary cytotoxicity in ultraviolet-a-irradiated riboflavin solution derived from hydrogen peroxide, J. Invest. Dermatol. 105 (1995) 608-612.

[28] G. Furness, Analysis of the Growth Cycle of Mycoplasma orale by Synchronized Division and by Ultraviolet Irradiation, J. Infect. Dis. 118 (1968) 436-442.

[29] F.L. Rosario-Ortiz, E.C. Wert, S.A. Snyder, Evaluation of UV/H2O2 treatment for the oxidation of pharmaceuticals in wastewater, Water Res. 44 (2010) 1440-1448.

[30] M. Wagner, D. Brumelis, R. Gehr, Disinfection of wastewater by hydrogen peroxide or peracetic acid: development of procedures for measurement of residual disinfectant and application to a physicochemically treated municipal effluent, Water Environ. Res. 74 (2002) 33-50.

[31] Y. Nishiya, T. Kishimoto, Alteration of L-proline oxidase activity of sarcosine oxidase and a structural interpretation. J. Anal. Bio-Sci. 33 (2010) 161-166.

[32] L. Schöne, H. Herrmann, Kinetic measurements of the reactivity of hydrogen peroxide and ozone towards small atmospherically relevant aldehydes, ketones and organic acids in aqueous solutions. Atmos. Chem. Phys. 14 (2014) 4503–4514.

[33] P. Chelikani, I. Fita, P.C. Loewen, Diversity of structures and properties among catalases, Cell. Mol. Life Sci. 61 (2004) 192–208.

[34] J.F. Turrens, Mitochondrial formation of reactive oxygen species, J. Physiol. 552 (2003) 335–344.

[35] J. D'Orazio, S. Jarrett, A. Amaro-Ortiz, T. Scott, UV radiation and the skin, Int. J. Mol. Sci. 14 (2013) 12222-12248.

[36] C. Schweitzer, R. Schmidt, Physical mechanisms of generation and deactivation of singlet oxygen, Chem. Rev. 103 (2003) 1685–1757.

Table 1: List of compounds that change in response to UV dose for irradiated media. P-value on the fit of the regression line from 0 to 600 mJ/cm^2 was used to assess the significance of changes in response to UV. SE – standard error for the concentration change per 100 mJ/cm^2.

Figure 1: A) Decay curve of H_2O_2 in media over time. B) Change in rate constant for different initial H_2O_2 concentrations. C) and D) Modeling the decay of H_2O_2 in media.

Figure 2: The viable cell density for 600 mJ/cm^2 UV-irradiated samples and H_2O_2-spiked samples (0, 0.2, 0.33, 1 mM). Error bars represent standard deviations to the means.

Figure 3: Average concentrations determined by NMR profiling at concentration of H_2O_2 for acetate (A), pyruvate (B), formate (C), and sarcosine (D), which were shown to exhibit changes as a result of H_2O_2 addition (blue bars). Red bars indicate changes as a result of catalase and H_2O_2 addition. Error bars represent standard deviations to the means.

Figure 4: Vitamin concentrations of biotin (a), vitamin B12 (b), lumichrome (c), riboflavin (d), and thiamine (e). They were measured with LCMS for control (non-irradiated medium), control + 0.2 mM H_2O_2, control + 0.333 mM H_2O_2, control + 1 mM H_2O_2 and medium irradiated at 600 mJ/cm^2 (red bars).

Figure 5: A) The percent enzyme activity of 71 and 711 U/mL catalase solutions after UV irradiation. Error bars represent standard deviation to the means. B) CHO cell viability data of cells cultured in control and irradiated media with and without catalase addition prior to irradiation.

C) Histograms indicating average concentrations detected by NMR for acetate, pyruvate, formate, and sarcosine for control media with and without the addition of catalase as well as 600 mJ/cm² UV-irradiated medium after the addition of catalase.

Figure 6: Average concentrations as determined by NMR profiling for acetate, pyruvate, formate, and sarcosine for medium treated with and without H_2O_2 and/or catalase as well as 600 mJ/cm² UV-irradiated medium with and without pre-treatment with H_2O_2 and/or catalase.

Figure 7: Impact of UV irradiation and H_2O_2 addition on four compounds: Acetate, pyruvate, formate and sarcosine.

Table 1:

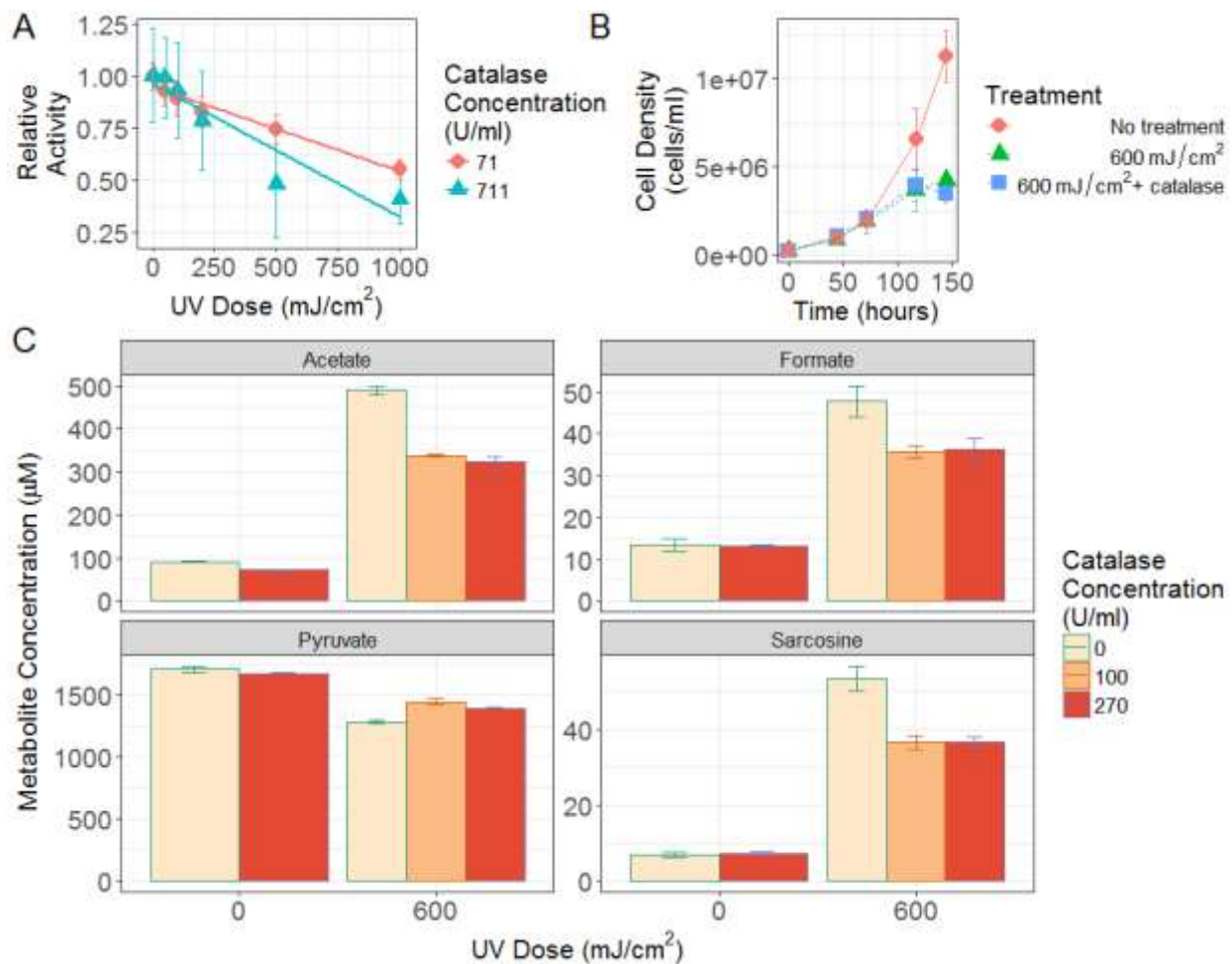| Compound	Concentration change per 100 mJ/cm², µM	SE	p-value
Acetate	74.6	10.1	5.59E-06
Arginine	-47.0	8.80	1.35E-04
Cystine	-16.2	1.99	1.77E-06
Ethanolamine	-3.75	0.82	5.18E-04
Formate	10.5	0.59	1.69E-10
Histidine	-49.5	11.2	6.97E-04
Hypoxanthine	-6.47	1.13	6.94E-05
Methionine	-22.1	6.50	4.80E-03
Pyridoxine	-2.27	0.28	2.19E-06
Pyruvate	-134	11.9	4.50E-08
Sarcosine	10.7	1.08	1.95E-07
Tryptophan	-68.7	7.24	3.32E-07
Tyrosine	-32.9	8.59	2.08E-03
Trans-4-hydroxy-L-proline	-10.3	3.76	1.65E-02

23
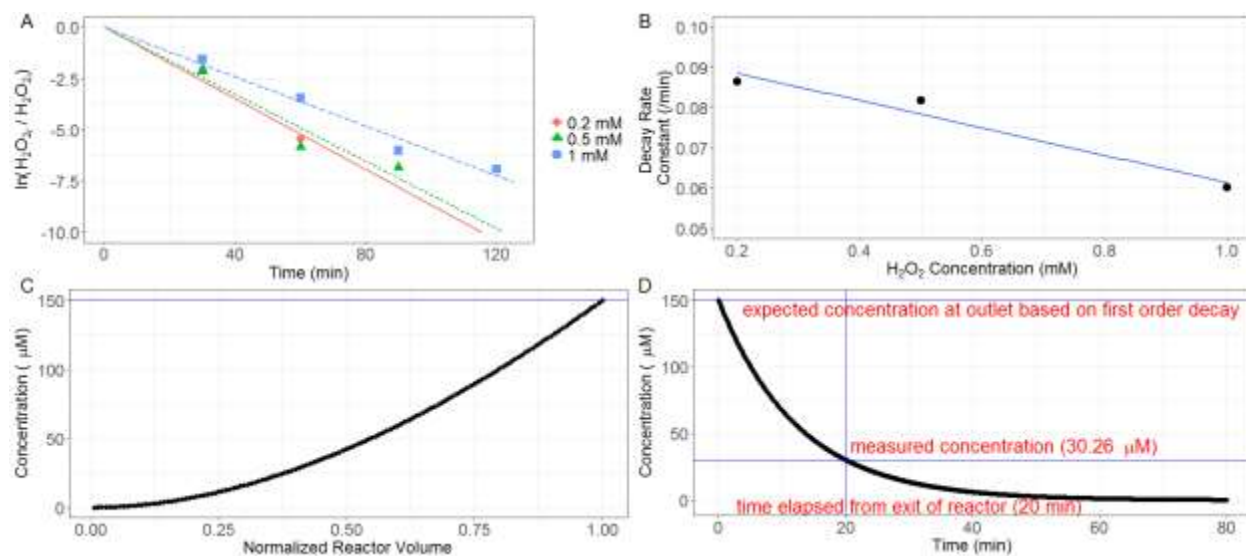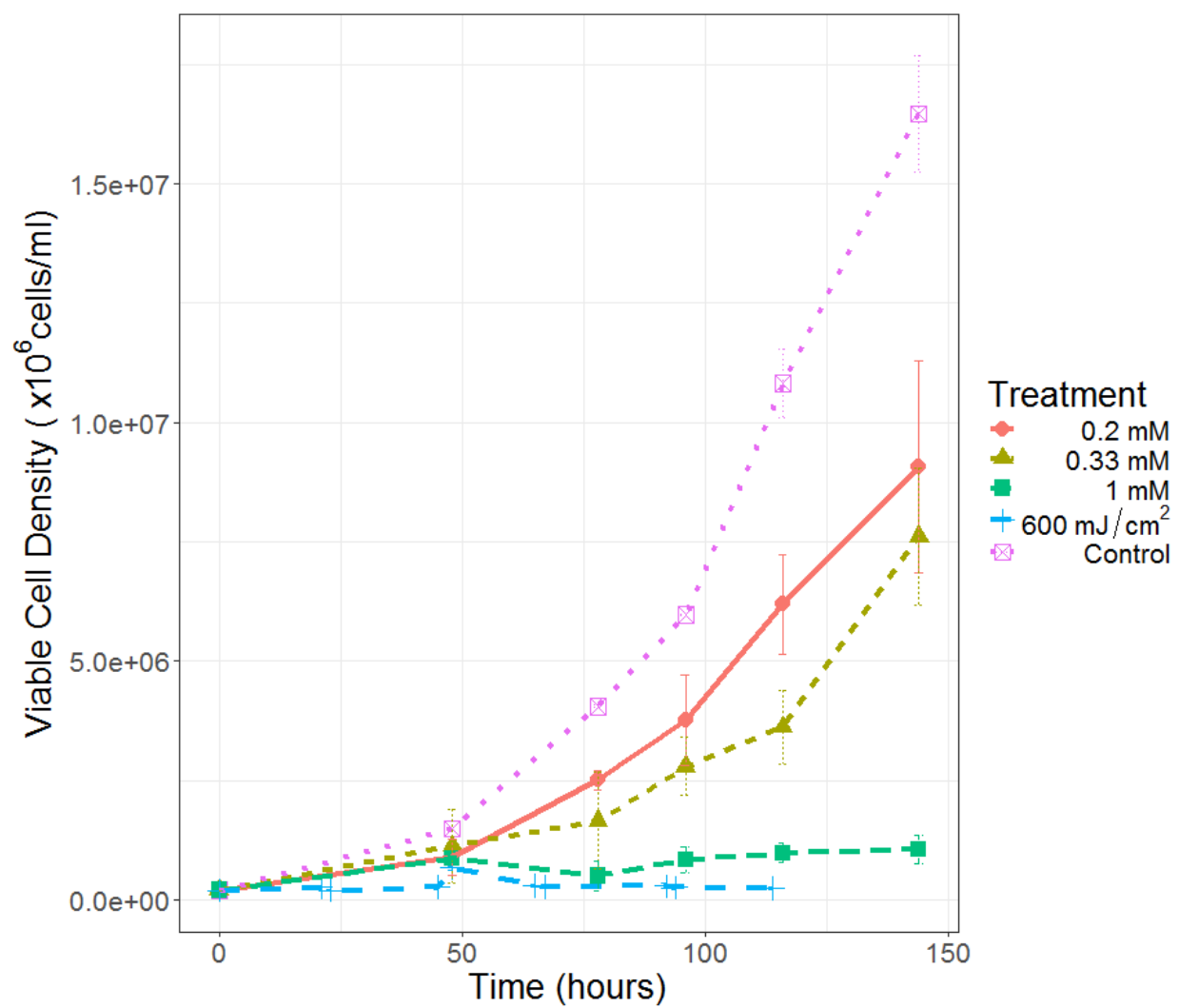
Figure 1:

Figure 2:

Figure 3:
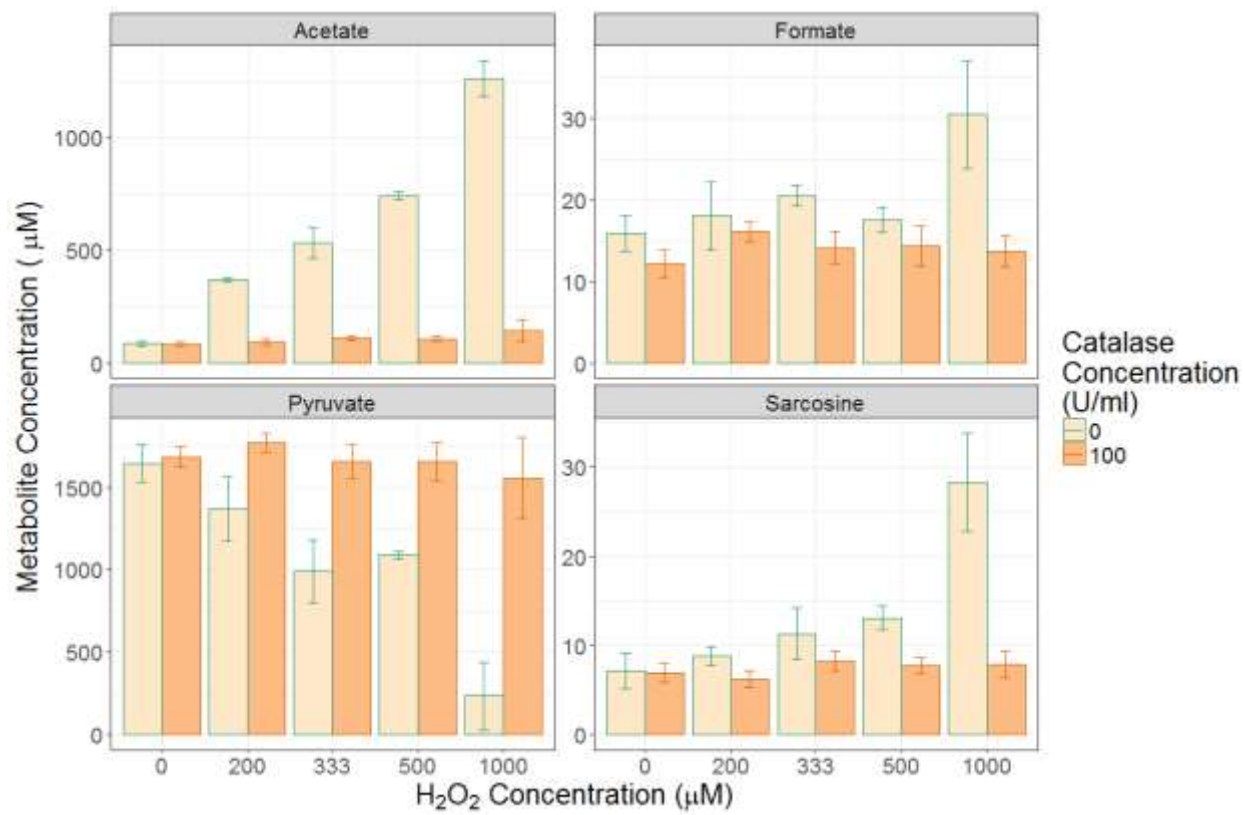
Figure 4:
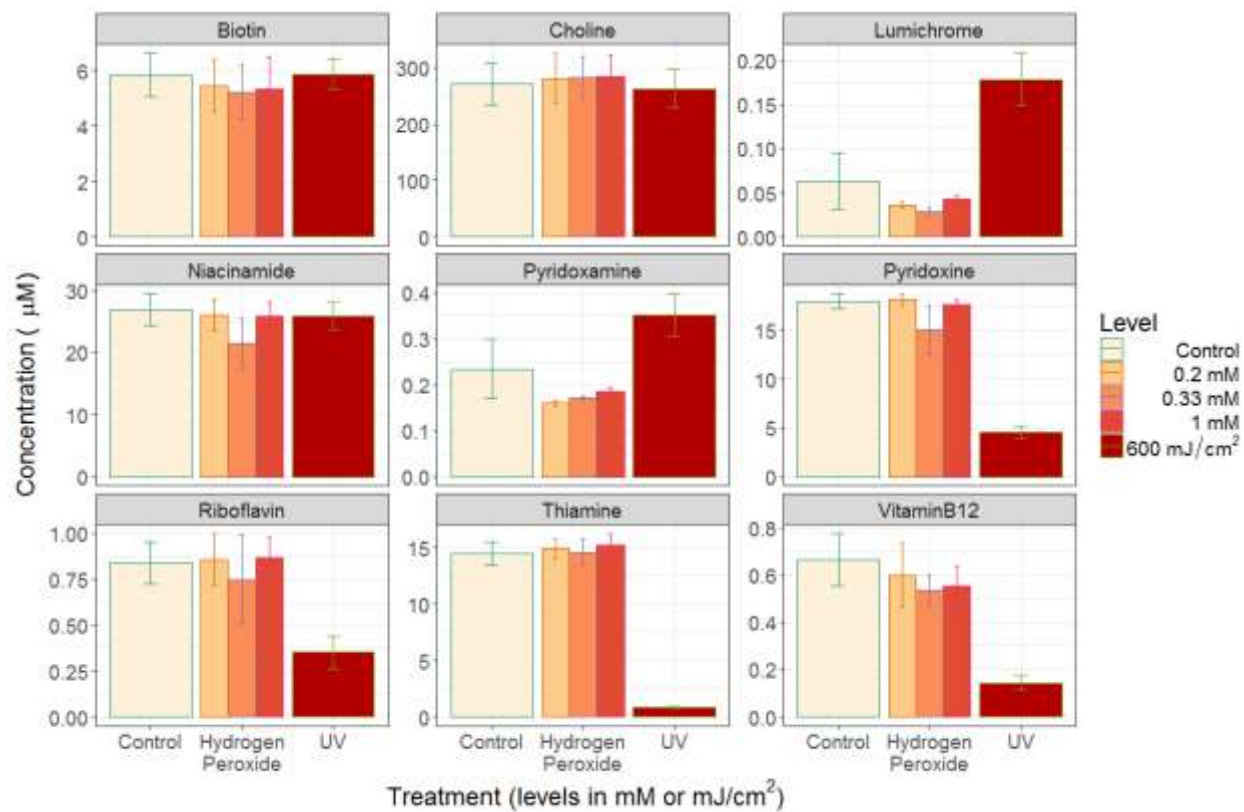
Figure 5:

Figure 6:
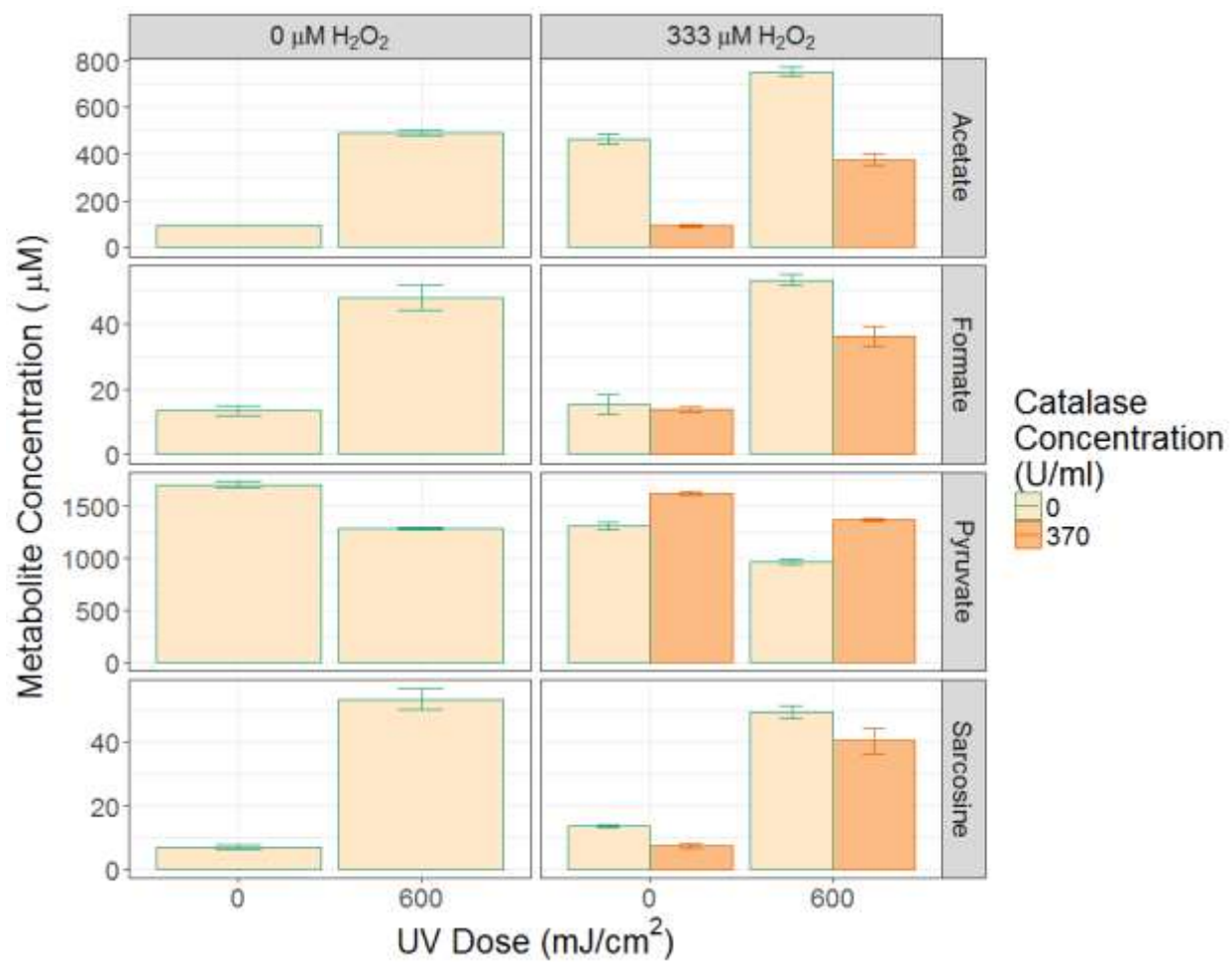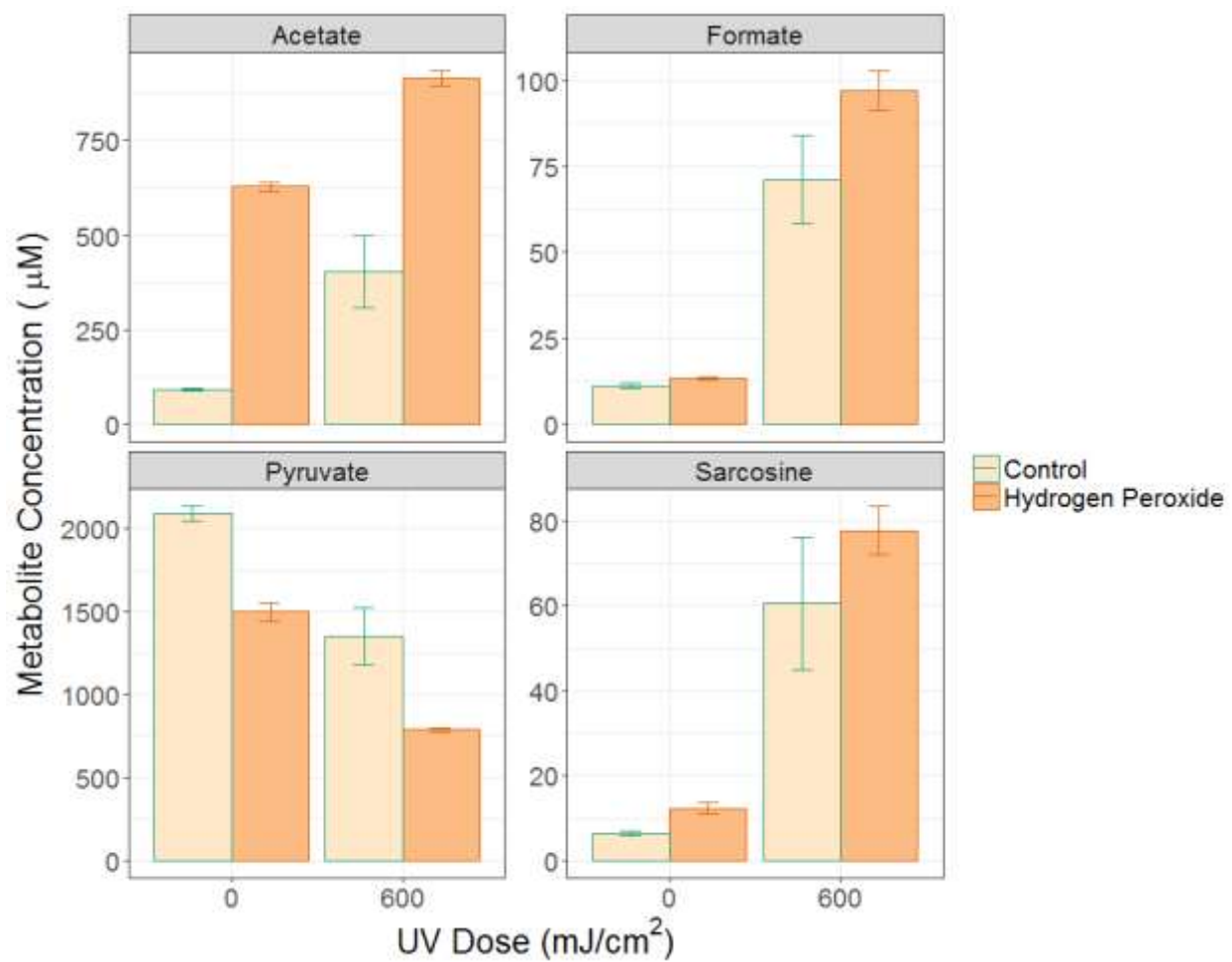
Figure 7:

32

Figure Legends