Modification and Control of Fermentation by Exogenous Energy Input

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ABSTRACT

Solar radiation influences virtually all biological process on earth. Yeasts, the microbial driver of ethanol fermentation, evolved on the surface of vegetation and had to adapt to survive photonic assault. Past research has demonstrated that white light affects yeast metabolism along with the ability to entrain circadian rhythms, although no known genetic mechanism accounts for this. High intensity narrow wavelength light-emitting diodes were employed to illuminate synthetic cultures under fermentation. Multiple colors along the visible spectrum were used, corresponding to the peak absorbance wavelengths of Saccharomyces sp. yeast. Impacts in primary metabolite evolution were found, dependent on wavelength. Longer wavelengths produced higher amounts of acetic acid and glycerol; shorter wavelengths produced more ethanol. Because past research showed light timing had pronounced effects, illumination schemes on the scale of milliseconds to hours were tested for ethanol production. Light schemes on the scale of enzymatic reactions, yeast generation times, and circadian rhythms produced the most ethanol. Discrete blocks and duration of illumination were used to elucidate where light had the most influence over yeast metabolism and fermentation. Late lag phase and mid log phase illumination impacted ethanol fermentation more than any other period of time. Light effects were tested on apple juice to see if they extended from synthetic media to natural products. Significant impacts on ethanol production were discovered and flavor/aroma impacts were noted. Light, color, intensity, and timing have all been shown to control and affect fermentation with both positive and negative effects established.

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GENERAL AUDIENCE ABSTRACT

Sun light influences virtually all biological process on earth. Yeasts, the microbial drivers of ethanol fermentation, evolved on the surface of vegetation and had to adapt to survive destructive effects of the sun. Past research has demonstrated that white light affects yeast metabolism along with the ability to develop growth cycles similar to day / night patterns, although it is currently not believed this possible due to the biology of yeast. High intensity single color light-emitting diodes were employed to illuminate laboratory formulated cultures under fermentation. Multiple colors along the visible spectrum were used, corresponding to the peak absorbance wavelengths of *Saccharomyces* sp. yeast. Green/yellow/red wavelengths produced higher amounts of acetic acid (vinegar) and glycerol; blue and ultraviolet wavelengths produced more ethanol. Because past research showed light timing could change how yeast grow and consumed carbohydrates, light timing on the scale of milliseconds to hours were tested for ethanol production. Light timing on the scale of milliseconds, hours, and daylight cycles produces the most ethanol. Discrete blocks and duration of illumination were used to find where during fermentation light had the most impact. It was found that from immediately after the beginning of fermentation to the middle of fermentation is where yeast responded the most strongly. Light effects were tested on apple juice to see if they extended laboratory cultures to natural products. Significant changes in the amount of ethanol produced were discovered and changes in the taste and smell of fermented apple

juice were noted. Light, color, intensity, and timing have all been shown to control and affect fermentation with both positive and negative effects established.

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Dedication

For Eldon K. Hurley Sr., William G. Mays, Benjamin W. Mays, S.R. Hurley, and C.C. Hurley

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Chapter 1

Overview

Fermentation modification and control and are essential for consistent quantity and quality production of end products. These products range from food to fuels, medicines to polymers, to even just clean water. The most common interventions are microbial selection, pitching rates, temperature, feedstock type and quantity, and micronutrients. In the last 50 years researchers have occasionally focused on the application of light to the fermentation process. The specific focus has been on the fermentation of carbohydrates to alcohol by the yeast *Saccharomyces cerevisiae*. Interestingly, this yeast is not known to contain any chromophores specifically associated with harvesting light for growth or metabolism (M. Merrow & Raven, 2010). In contrast to past research, detailed herein is the use of specific wavelengths of light under tightly controlled timing regimens to control the production of ethanol and other metabolites. The specific objectives were:

Objective I. Characterize the effect of different wavelengths of light on ethanol fermentations under consistent energy flux.

Objective 2. Identify the wavelength(s) and intensity of light that maximize ethanol production.

Objective 3. Identify color schemes that may impart changes to flavor and aroma compound evolution during fermentation of synthetic media and apple juice.

Objective 4. Utilize the timing of light application to qualify the mechanism(s) responsible for maximal ethanol production.

The principal consequences of this work are to identify light application parameters that lead to maximal ethanol production for the food and biofuel industries and to provide a foundation for future wavelength / intensity / timing studies for the modification of fermentation on different food products .

Introduction

Fermentation and civilization are inseparable - John Ciardi (1916-1986)

Fermentation is broadly defined as the conversion of carbohydrates to alcohols and/or acids by the metabolism of microorganisms. The anthropological record indicates this process has been implemented for food modification and production for almost nine millennia (P. E. McGovern et al., 2004). Humanity's first foray into the consumption of fermented products likely started with near-rotted fruit or high-moisture honey that had time to spontaneously ferment (P. E. McGovern, Mirzoian, & Hall, 2009). The stark change in sensory perception and desired consequences on body and mind likely directed people to repeat this process on demand (P. E. McGovern, Glusker, Exner, & Voigt, 1996). The base desire for fermented products can be seen in the recent discoveries of ancient fermentation paraphernalia and residues. Open-air Neolithic fermentation jars have been identified in Georgia (Eurasia) dating back to 6000 - 5800 BCE; relatively complex winemaking and storage operations have been identified and excavated in an Armenian cave dating back 6,100 years (Barnard, Dooley, Areshian, Gasparyan, & Faull, 2011).

The quote above, widely referenced in electronic and print histories of fermentation, brings the idea that the desire for fermented products has led to the development of agriculture, and agricultural products have fostered the evolution of fermentation. Together they laid the

foundation for permanent settlements and subsequently towns and cities...what we now call civilization (Standage, 2009). What is commonly overlooked, or at least taken for granted, is that the fermentations that led to civilization were at the very least desirable, and more so beneficial. Uncontrolled fermentations can and have caused instances of mild unpalatability (as an understatement) and, more dramatically, illness and death. Grape fermentation benefits from the naturally low pH and high acidity of grape juice to stave off pathogens and the rapid evolution of ethanol completes the trifecta of food safety. Even the spoilage of fermented grape juice (wine) to acetic acid (vinegar) forms a palatable food antimicrobial. Grain fermentations in the form of beer and bread do not have the acidity and pH levels to fend off non-desirable, possibly dangerous, fermentations. The main preventative controls of pathogens in these staples are by the high reproductive and growth rate of yeast coupled with ethanol tolerance above 2%. The application of a starter culture is likely the first control of fermentation enacted. By introducing a known, favorable, fermented material, these first fermentation specialists directed spontaneous natural fermentation toward a predictable end-product based on previous experience.

Temperature as a fermentation control can be seen in the archaeological record by the use of caves, pits, and very large earthenware jars as fermentation vessels (Henderson, Joyce, Hall, Hurst, & McGovern, 2007; P. E. McGovern et al., 1996; P. E. McGovern et al., 2004; P. E. G. D. L. McGovern, 1999). Saccharomyces cerevisiae generally has an optimum growth at 32°C with some strains viable at 45°C (Perego, Cabral de S. Dias, Koshimizu, de Melo Cruz, Borzani, & Vairo, 1985). Fermentations at these temperatures tend to be high in esters and harsh aromatics, presenting an almost solvent-like profile (Šmogrovičová & Dömény, 1999). The use of caves for fermentations, which may be as cold as 12°C, indicates a realization of temperature effects.

Large clay fermenters in hot arid environs would benefit from evaporative cooling, along with their base thermal mass, leading to more desirable end products.

The focus on temperature as a prime fermentation control can be seen in the patent by Merrow et al (M. W. Merrow, 2010). Whether the microorganism is eukaryote or prokaryote, the patent focuses on isothermal and circadian temperature changes to effect the desired outcome of fermentation. The ability to strictly control temperatures is a modern phenomenon, but still has its roots in the Copper-age Armenian wine cave.

Pasteur's identification of living yeast as the driver of fermentation in 1857 completely changed the understanding of fermentation science. Knowing that fermentation is the metabolism of a living, breathing (or not), reproducing, microorganism allowed fermenters to now comprehend the effect environmental modifications had, along with an understanding of organism selection and, at a higher level, breeding.

Microscopically, *Saccharomyces* reproduction can be seen in process as buds are formed and released. Under ideal conditions this process can be as fast as 90 minutes (Sherman, 2002). Understanding this process leads to defined pitching rates as a fermentation control. In the initial and exponential phase of fermentation, pitching twice the number of cells yields a shorter fermentation, but only by ~90 minutes to two hours. Pitching rate guidelines of 2.5 billion to 40 billion cells per barrel have been developed for beers depending on sugar content and yeast strain, targeting end products with desired characteristics for defined beer styles (Unknown, 2018).

Saccharomyces sp. are ubiquitous in nature; being found on the surface of plant material, they have seemed to evolve without any specific light-sensing apparatus (M. Merrow et al., 2010). Circadian clock mechanisms are known throughout all branches of life, but none of these

genes or their analogues have been identified in yeasts. The extent of known light utilization by this specific strain of yeast is in the form of the light-powered DNA repair enzyme photolyase (Weber, 2005).

Growth Factors

The fermentation controls listed above range from the oldest (microorganism and temperature) to the newest (cell number and sugar type and content). It has only been in the last century that micronutrients essential for fermentation have been taken into consideration. Use of yeast extracts along with a carbohydrate source has been used as a propagation medium since pure cultures were first identified (Davidson, 1940; Harden & Young, 1906). Why yeast grew better on dead yeast wasn't identified until the 1950's when nicotinamide adenine dinucleotide (NAD) and B vitamins were identified as a growth factors (Hopkins & Pennington, 1950). Yeast produce these growth factors intrinsically, but addition at the start of fermentation boosts growth rates. As fermentation has become more complex a science, the addition of macro- and micro-nutrients has flourished. Recognition of nitrogen and sulfur compounds, along with vitamins and minerals, is the most recent addition to the arsenal of fermentation controls to produce optimal desired outcomes (Broach, 2012). As an example, approximately 25% wine samples submitted to the VT Enology Analytical Services Laboratory in 2006 had sulfur-like off-odors attributed to a nitrogen deficiency in the fermentation medium. Since the institution of nitrogen testing in the Virginia wine industry, the rate of sulfur-like off-odors as dropped to approximately 4% of samples ((Hurley Jr., 2008)). This type of testing is only effective when proper amelioration protocols and products are used; too early in the growth phase and they may be toxic, too late and they go unused.

Yeast themselves affect the growth medium by the production of quorum sensing molecules (Avbelj, Zupan, Kranjc, & Raspor, 2015; Chen & Fink, 2006; Sentheshanmuganathan & Elsden, 1958). The known quorum molecules in *Saccharomyces* are all aromatic alcohols - 2-phenylethanol, tryptophol, and tyrosol (Avbelj et al., 2015). These molecules signal high cell densities, which in turn change growth rates and cell morphology. Ethanol itself may be seen as a growth factor or a growth-affecting factor, but because of its role in metabolism and use by the cell, it generally does not meet criteria outlined for quorum sensing molecules.

Biochemistry

Discussions of *Saccharomyces cerevisiae* metabolism typically list the organism as a facultative anaerobe, the definition of which is commonly simplified as "...able to grow with or without free oxygen." In complex organisms such as yeast this transition isn't simply just the other lane on a two-way highway; the metabolic pathways, cellular machinery, end products, and net energy balance are almost entirely different.

Both anaerobic and aerobic yeast metabolism start with glucose (preferentially) or fructose and proceed in the cytoplasm to pyruvate. In the presence of free oxygen, the pyruvate is taken up by the mitochondria and oxidized to acetic acid which in turn feeds into the tricarboxylic acid cycle. Coupled to the electron transport chain, yeast can generate 28 adenosine triphosphate (ATP) molecules per molecule of hexose. In contrast, the anaerobic pathway converts pyruvate to ethanol in two steps, but only generating two molecules of ATP per molecule of hexose.

The redox chemistry of the electron transport chain involves numerous proteins and cofactors. Of specific interest to this dissertation are the cytochromes (proteins) and flavins. Cytochromes, so named because they are pigmented molecules, are the main drivers of redox

chemistry in the electron transport chain. The individual types of cytochromes are identified and named based on the ultraviolet-visible absorption bands (Slater, 2003).

The main cofactors in the electron transport chain are nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). Depending on redox state both these molecules may attain pigmented or nonpigmented states, with FAD having nine redox states comprising four different color states, those depending on redox state and local pH, and NAD having only two redox and color states.

The above proteins and cofactors though are not limited to aerobic respiration. There are numerous non-respiratory roles for different cytochromes in cellular growth, maintenance, and repair (Rosenfeld & Beauvoit, 2003). NAD plays a significant role in anaerobic fermentation, being the main redox cofactor involved in the conversion of pyruvate to ethanol. FAD is a prominent redox carrier in many cellular processes independent of aerobic/anaerobic metabolism and part of flavoproteins involved in cell growth, maintenance, and repair.

Energy inputs affecting fermentation

As mentioned above, temperature control is one of the oldest but still most used controls in yeast fermentations. Zymological processes for beer and wine can range from 7°C to 30°C with biofuel production temperatures as high as 35°C (Eelderink-Chen, Mazzotta, Sturre, Bosman, Roenneberg, & Merrow, 2010; Lloyd, 1998; Roenneberg & Merrow, 2001). Temperature effects exist as basic as growth, reproduction, and death rates or as complex as flavor and aroma compound formation. Strictly controlled temperature flux has been shown to be effective in targeting metabolite production as mentioned above, but this process has also been shown able to develop a sustainable circadian growth pattern (Eelderink-Chen et al., 2010; M.

Merrow et al., 2010). Temperatures at the extremes of cold and heat can induce sluggish fermentation and, of course, cell death.

The effect of light on microorganisms has its roots in the study of the Anthrax pathogen (Ward, 1893). In his research to determine why the ubiquitous bacteria was not causing more infections, Ward found that light, specifically ultraviolet light, was a lethal agent. The mechanism of light-driven cell death was discovered due to the recognition of the UV absorbance of DNA, the molecule essential for life (Rastogi, Richa, Kumar, Tyagi, & Sinha, 2010). Later studies of ultraviolet light on yeast found that irradiated cells release growth promoting factors (Davidson, 1940). The growth promoting factors, likely NAD and other adenine complexes, were cell exudates, and cell lysis was not detected or required for production of growth enhancing factors. This indicates that this is a specific cellular light-driven process, not just a consequence of increased nutrient bioavailability due to cell destruction.

In studying the destructive capabilities of light on microorganisms, light-driven rescue and repair mechanisms were discovered accidentally. After irradiating microbes with ultraviolet light, the transparent vessels were placed in a glass-fronted water bath. The glass allowed illumination by daylight but limited additional UV irradiation (Kelner, 1949). This allowed the enzyme DNA photolyase to repair the UV induced damage, the enzyme itself being powered by blue light. In much the same way, light effects on the respiration of yeasts were discovered.

Light and Fermentation

Studying respiration adaptation in yeasts Sulkowski et al. (Sulkowski, Guerin, Defaye, & Slonimski, 1964) used a new piece of equipment that happened to be made of plexiglass. Experiments that had previously been reproducible and consistent had become erratic. Investigation of this phenomenon led to the first understanding that not only does light impart

changes involving cell death but it can significantly change anaerobic and aerobic metabolism. Cells grown in the dark anaerobically could not switch back to aerobic respiration in the presence of light. This work was later followed by more detailed analysis of the conditions that inhibit recovery of oxygen respiration (Guerin & Sulkowski, 1966).

The key findings were:

- The response to illumination was dose dependent.
- Light induced changes were cumulative to the point of being irreversible after sustained illumination, but reversible with smaller doses or periods of dark recovery.
- Fermentation, separate from oxygen respiration, could be inhibited by very large doses of light.
- Stationary-phase yeast are less sensitive to light than yeast at other growth phases.

Ultraviolet light research programs discussed above used either natural sunlight or high intensity mercury vapor lights, and visible light experiments used fluorescent white lights. Although mercury vapor lights have a very high intensity specific output at 365 nm, they produce a relatively broad-spectrum white light, much like the other light sources used. Specific identification of inhibitory wavelengths of light were not attempted in these studies and, therefore, the effect of multiple different wavelength-based changes on metabolism was not considered.

Follow up work did focus on elucidating the wavelengths of light that had the most biological activity with the goal of determining the mechanism(s) involved in changes to cell growth. Using colored light filters, four specific inhibitory wavelengths (500 nm, 540 nm, 575 nm, and 630 nm) were identified (Guerin & Jacques, 1968). Additionally, light below 450 nm was found to be increasingly inhibitory, especially in the range of 404 nm. These can be

described generally as blue-green, green, yellow, red, and violet, respectively. The 500 nm - 630 nm wavelengths described correspond very well to the absorbance of porphyrin molecules, specifically the cytochromes. The broad-spectrum action in the 404 nm range corresponds well to the absorbance spectrum of porphyrins as well as flavin molecules. This work, as well as previous referenced work, found that cytochrome protein synthesis was inhibited by light. Later developments showed that not only was light inhibitory to protein synthesis but it could deactivate the cytochrome molecule by photooxidation of the heme group (Ninnemann, Butler, & Epel, 1970).

Blue light, centered around 425 nm, had the ability to destroy the electron transport chain cytochromes in starved (non-Crabtree effect) aerobic cultures and stimulate the anaerobic metabolism pathways. The destructive effects were only noticed when oxygen was present. Anaerobic cultures or aerobic cultures treated with cyanide and azide and cytochrome protectants did not experience any cytochrome destruction. Although not extensively studied, this oxygen- and light-mediated destruction may be related to the ability to induce electronic transitions and drive chemical reactions in cytochromes by the application of light of the proper color, intensity, and duration (Amesz, Pulles, Visser, & Sibbing, 1972; Ben-Hayyim, 1974; Knaff, 1973; Knaff, Buchanan, & Malkin, 1973; Naik & Nicholas, 1986).

In addition to direct effects listed above, light was found to inhibit membrane transport of amino acids into the cell (J. R. Woodward, V. P. Cirillo, & L. N. Edmunds, Jr., 1978b). This has the effect of limiting amino acid availability for growth but also blocks the production of other compounds that use amino acids as building blocks, thereby having an extremely broad impact on metabolism. Light also has the effect of making the yeast membrane “leaky” towards sugars, affecting anaerobic fermentation, which is not dependent upon cytochromes. These membrane

transport and integrity deficiencies are more pronounced at the blue end of the spectrum than the red.

The effects of light on yeast cells are more pronounced at colder temperatures (Ulaszewski et al., 1979). At 20°C, amino acid transport decreased by ~10% in the presence of high intensity light. Temperature decreases of 4°C and 8°C led to 50% and ~90% decreases in amino acid transport, respectively. The temperature effects are postulated to be related to the formation of free radicals, augmented by the increased solubility of oxygen at lower temperatures and the decreased activity of protective catalases and dismutases at the lower temperatures.

Research above details specific effects light has on the fermenting cell, focusing on specific mechanisms and molecules. These reactions are carried out in a living cell and have holistic effects on the organism. Although cell death, or even decreased growth rates, may be seen as holistic effects, more complex behavior patterns have been discovered. A yeast in the same family as *Saccharomyces*, *Aspergillus nidulans,* has been shown to require 680 nm light for conidiation (Mooney & Yager, 1990). Whether or not this extends to *Saccharomyces* has yet to be demonstrated. Under conditions of constant darkness or constant illumination (both under constant temperature) cells grew consistently throughout the exponential growth and division phase, albeit slower under light tension. What is interesting is that when a light-dark cycle was introduced, this changed (L. N. Edmunds, Apter, Rosenthal, Shen, & Woodward, 1979).

Utilizing fluorescent white light, yeast growth and cell division could be induced to follow a 10-hour light / 14-hour dark pattern, this entrained pattern being sustained after 7 cycles followed by culture in constant darkness (all at constant temperature). The temperature and light intensity used was sufficient to cause significant cell retardation, with the cell growth specifically

matching the light entraining pattern. The oscillatory pattern did stretch from 24 hours to ~27 hours in the absence of the imposed light regimen but remained relatively consistent thereafter. Cell division, as monitored by cell counts per milliliter of culture, was almost perfectly constant and near zero during the light phases. When the culture was returned to darkness cell division began again with little-to-no lag.

It would be contraindicated to qualify circadian patterning in the stationary phase by monitoring cell division. Going back to the metabolic process of amino acid uptake, it was found that the entrained circadian pattern was sustained in darkness throughout the early-to-mid stationary phase with the peak of amino acid uptake occurring at the transition between light and dark. Cytochrome deficient mutants were shown to be minimally affected by white light (Ulaszewski et al., 1979).

All the work referenced above was focused exclusively on the light effects on the yeast themselves. Around approximately 1980, there was an abrupt shift away from the research of light effects on *Saccharomyces* with most work looking at cell oscillations and clock mechanisms in other organisms (L. N. Edmunds, Jr., 1983, 1984). It wasn't for another 30 years that research shifted back toward *Saccharomyces* with a new focus on the output of fermentation, namely ethanol (Shu, Huang, & Tsai, 2009).

Fluorescent lights can provide a significant amount of illumination, but the light is spread out over a large spectrum. Due to their dependence on fluorophores, wavelength intensity may not be consistent between manufacturers and the output of a specific wavelength may be very weak. The advent of light-emitting diodes (LEDs), specifically high intensity LEDs over the last 60 years has allowed the very specific and focused input of energy on systems (Dupuis & Krames, 2008).

Shu et al., using high intensity red and blue LEDs, reported that they could increase fermentation rate and efficiency using specific wavelengths, timing, and intensities. Results of their experiments demonstrated a 25% decrease in the time a batch fermentation took to complete coupled with a near-theoretically perfect conversion of glucose to ethanol. The process developed depended on red light (~632 nm) stimulated aerobic fermentation, followed by anaerobic fermentation under red light and then blue light (~472 nm). As it applies to this dissertation, it may seem that the work of Shu et al. answered some of the prime research questions. In comparing this work to all the historic research, though, almost every result reported is in complete opposition to what has been shown (L. N. Edmunds et al., 1979; Ninnemann et al., 1970; Sulkowski et al., 1964; Ulaszewski et al., 1979; J. R. Woodward, V. P. Cirillo, & L. N. Edmunds, 1978a), specifically their demonstration of faster fermentation times with greater amounts of biomass produced.

Time-based Elucidation of Chemical & Biological Reactions

When discussing the numerous reactions listed above, one way to differentiate the type of reaction is by examining the rate of reaction (Douglass & Miller, 2013). Direct light-driven reactions take place on the scale of nanoseconds, enzymatic reaction rates in the microsecond to minute range, yeast protein turnover in yeast in approximately 0.5 hours, but ranges from minutes to 24 hours, generation times on the order of 2 hours, and lifetime in the realm of weeks. A comparison of these different rates is shown in Figure 1.

All the previous referenced literature used continuous illumination or circadian illumination for their studies. In the life span of a yeast, these illumination schemes affect cellular processes, from fluorescent energy transfer up to cell generation, growth, and death times. Most of the limited body of information on intermittent illumination again found that

cytochromes were the molecules most affected (Amesz et al., 1972; Ben-Hayyim, 1974; Knaff et al., 1973; Sybesma & Kok, 1969). Cytochrome reaction times as fast as 30 ms and as slow as 3 minutes were recorded. The findings correspond well with the chart of timescales already presented: The 30 ms flashes were dependent on free oxygen and were a direct oxidation event on the cytochrome; the minutes-long reactions corresponded to enzymatic reactions carried out by the cytochromes. The light acted as a direct "pump" to drive chemical reactions in place of the normal electron transfer chain.

The known light-driven reaction in *Saccharomyces,* photorepair, is modulated by the absorbance and electron transfer reactions of tryptophan (Weber, 2005). Light-mediated tyrosine reactions have been studied in vivo and in vitro (Kim, Ayala, Steenhuis, Gonzalez, & Barry, 1998; Reece, Seyedsayamdost, Stubbe, & Nocera, 2007). When considering that two of the three known quorum sensing molecules in yeast are tryptophan and tyrosine derivatives, it is interesting to consider what effect light may have on quorum sensing molecules. Tryptophan photoproducts have been shown to directly impact cytochrome function (Diani-Moore, Labitzke, Brown, Garvin, Wong, & Rifkind, 2006); light-modified quorum sensing molecules may directly impact the cytochromes.

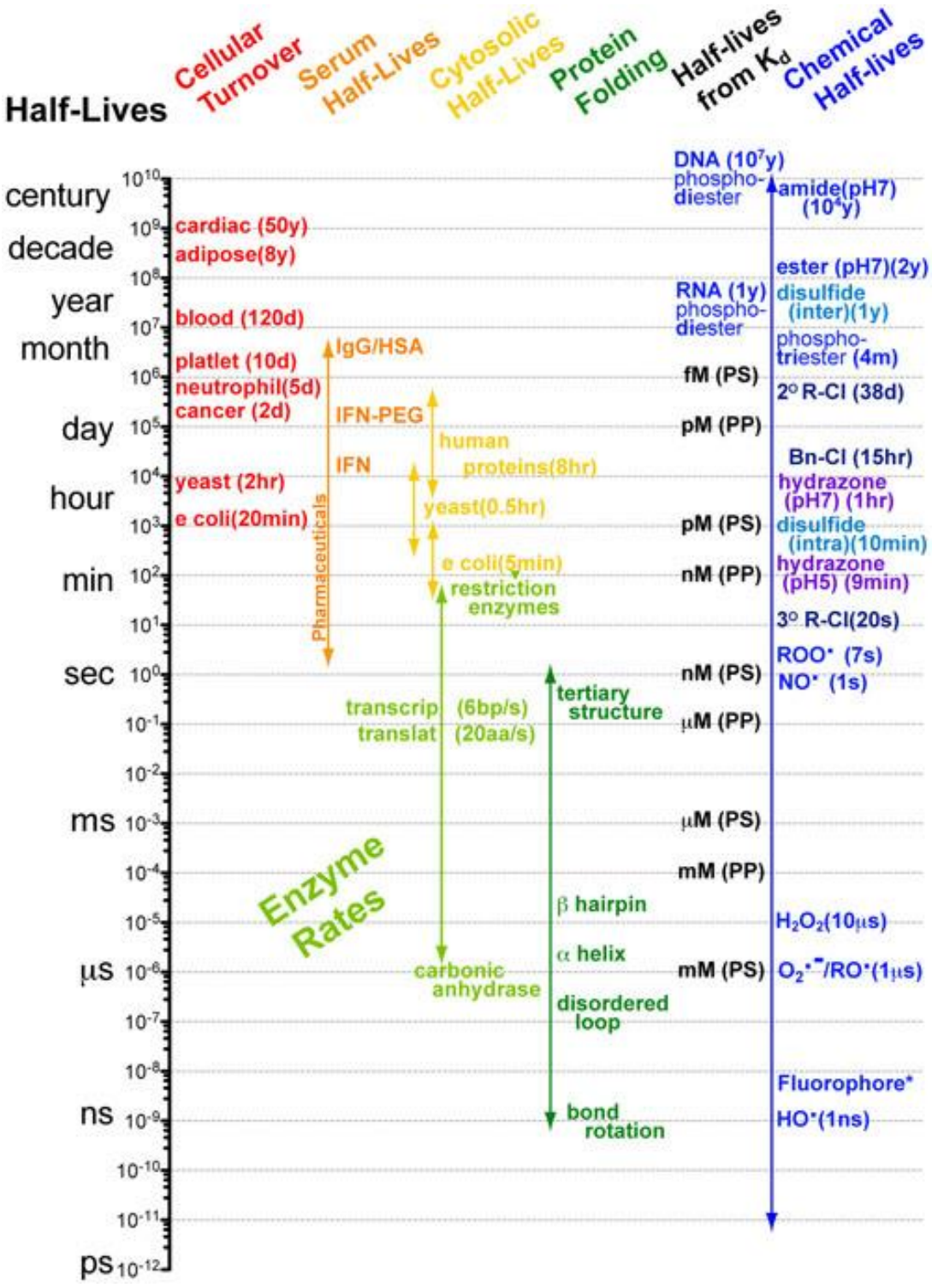

Figure 1. Half-lives of chemical and biological reactions from Douglass & Miller (Douglass et al., 2013). This work by Eugene Douglass and Chad Miller is licensed under a Creative Commons Attribution-NonCommerical-ShareAlike 3.0 Unported (CC BY-NC-SA 3.0) agreement.

Chapter 2

Materials and Methods

Materials

Yeast Strains

ATCC 9804 - *Saccharomyces cerevisiae*, type strain of *Saccharomyces chevalieri*, was purchased from ATCC. The strain was isolated in 1914 from a palm wine fermentation on the Ivory Coast of Africa. This strain was chosen due to its use in prior work by Shu et al. 2009.

Lalvin W15 - *Saccharomyces cerevisiae* was a generous gift from Gordon Sprecht, North American Area Manager for Lallemand Inc. (Montreal, Canada). The strain was isolated in 1991 from a Müller-Thurgau (white grape) must at the Viticulture Research Station in Wädenswil, Switzerland. This strain was chosen because of its high alcohol tolerance (16%) and utility in all fermentation mediums, according to the supplier.

DistilaMax DS - *Saccharomyces cerevisiae* was a generous gift from John Little of Smooth Ambler Spirits, Maxwelton, WV. This strain is a commercial strain of distiller's yeast; the lineage is not published. This strain was chosen due to its broad usage profile in regard to temperature, alcohol, starting sugar content, and acid tolerance, as well as its utility in beverage and fuel fermentations.

Culture Media

Synthetic Media - MPYD was prepared from 3 g/L Malt Extract, 5 g/L Peptone, 3 g/L Yeast Extract, and 2g/L Glucose. MPYHG was prepared the same as MPYD, except that the glucose level was raised to 200 g/L. MPYDS was prepared the same as MPYD but containing 30% glycerol.

Apple Juice - Store brand frozen apple juice concentrate was obtained from Kroger (Cincinnati, OH). Frozen concentrate was chosen due to the absence of preservatives and consistency between containers of the same lot. The concentrate was diluted to 14.0° brix for experiments.

Grape Juice - Wine Expert brand merlot and viognier grape juice concentrates were obtained from Northern Brewer (Roseville, MN). Concentrates were diluted to 21.0° brix for experiments.

Light Emitting Diodes (LEDs)

Wavelength	**Supplier**	**Part Number**	**Type**
Warm White - 5400K	Super Bright LEDs St. Louis, MO	RL5-WW15030	T1
733 nm (Red-Near IR)	Ledtronics, Inc Torrance, CA	L200CWIR731-30D	T1
630 nm (Red)	Super Bright LEDs St. Louis, MO	WFLS-R300-WHT	Strip
630 nm (Red)	LED Supply Randolph, VT	L2-0-R5TH20-1	T1
590 nm (Yellow-Orange)	Super Bright LEDs St. Louis, MO	WFLS-Y300-WHT	Strip
575 nm (Yellow)	LED Supply Randolph, VT	L1-0-Y5TH20-1	T1
535 nm (Green)	Cree, Inc	C503B-GAS-CB0F0791	T1

	Durham, NC		
505 nm (Cyan)	LED Supply Randolph, VT	L4-0-T5TH30-1	T1
470 nm (Blue)	Super Bright LEDs St. Louis, MO	WFLS-B300-WHT	Strip
470 nm (Blue)	LED Supply Randolph, VT	L1-0-B5TH15-1	T1
405 nm (Purple)	Super Bright LEDs St. Louis, MO	WFLS-UV-300-WHT	Strip
400 nm (Purple)	LED Supply Randolph, VT	L3-0-U5TH15-1	T1
380 nm (UV)	Super Bright LEDs St. Louis, MO	RL5-UV0315-380	T1
351 nm (UV)	LED Supply Randolph, VT	L5-1-U5TH15-1	T1

Table 1. Light emitting diode Wavelengths, Colors, and Sources

Electronic Controls

Microcontrollers - Arduino Uno R3 and Arduino Mega 2560 R3 Microcontrollers were purchased from Amazon (Seattle, WA). Microcontrollers were programmed using the most current version of the Arduino IDE from Arduino (Ivrea, Italy).

Grove System - The Grove Shield, Grove MOSFET, and Grove Real Time Clock (RTC) controllers were obtained from Seeed [sic] Development Limited (Guangdong, People's Republic of China).

High Power MOSFET - A Ciseco (Nottingham, UK) dual-channel high power MOSFET shield was used for controlling LED strip lights.

Miscellaneous Electronics - Miscellaneous wires, resistors, and other electronic components were purchased from Amazon.

Light Meters

Initial light measurements were made using an Extech LT-45 LED light meter (FLIR Commercial Systems Inc, Nashua, NH) tuned to the appropriate color setting. For light energy measurements, a custom-made light meter was fabricated from an AMS Taos TSL237 photodiode (Premstaetten, Austria) interfaced to an Arduino Uno.

Fermenters

Small Scale Fermenters - Supelco (Bellefonte, PA) 5 mL reaction vials were used for small scale experiments. Clear glass marbles were used as caps to block oxygen intake but allow venting of carbon dioxide.

Large Scale Fermenters – Custom-designed 3 L jacketed fermenters (Figure 2) were made by the VT Chemistry Glass Shop (Blacksburg, VA). The internal diameter of the fermentation vessel is 82 mm with a length of 955 mm ending in flanged ends. The bottom was sealed with a flat glass circle and the top covered with a bell-shaped cap fitted with a 24/40 standard taper receiver. A bell-shaped reflux condenser was attached via the standard taper joint to prevent loss of volatiles. The reflux condenser was maintained at less than 5°C.

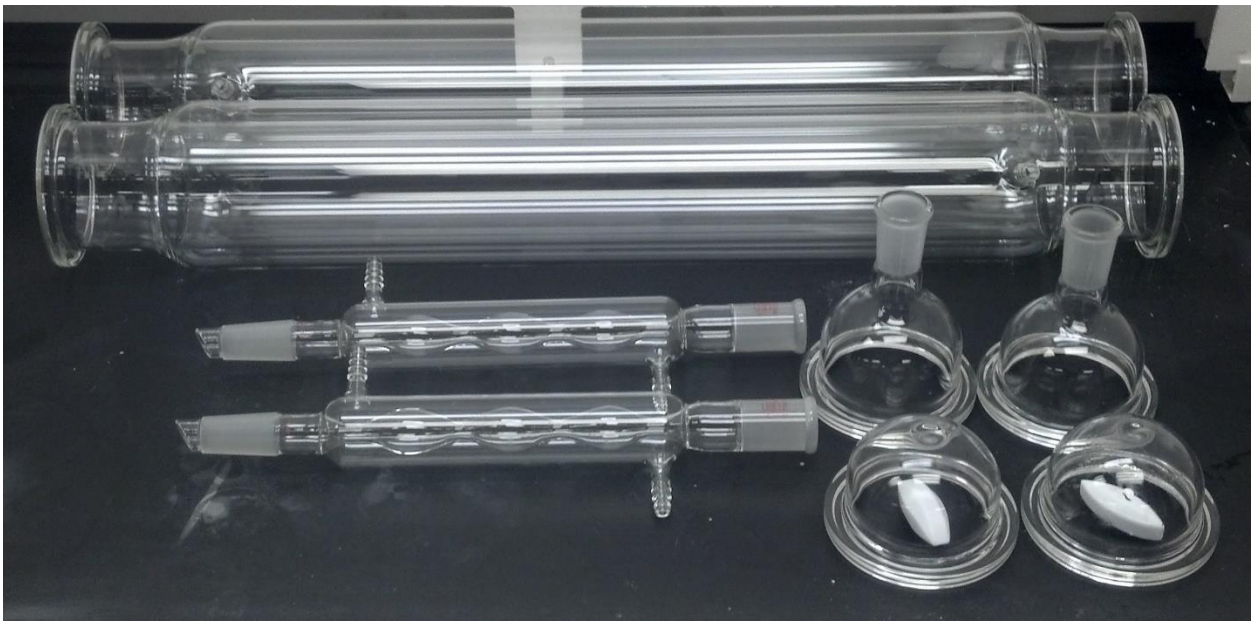

Figure 2. Custom-made 3L Jacketed Glass Fermenters

Temperature Controls

A Thermo Fisher (Pittsburgh, PA) Isotemp 1.3L refrigerated bath was used to maintain low temperatures in the reflux condensers. A custom-made water bath was used to control the temperature of the large-scale fermenters at elevated temperatures for the replication of the Shu et al experiment. Briefly, an Arduino Uno coupled to a temperature probe was used to control a 500W heating element. A Thermo Fisher Isotemp 6.5L refrigerated/heated recirculating bath was used to maintain moderate temperatures (17.5 °C).

Analytical Instrumentation

HPLC-DAD-RID Analysis - The liquid chromatography system was Hewlett Packard/Agilent HP1100 (Santa Clara, CA). HP ChemStation software was used for instrument control and analysis. The column was a Bio-Rad (Hercules, CA) HPX-87H thermostated at 65°C.

GC-FID Analysis - The gas chromatography system was a Hewlett Packard/Agilent 5890 Series II Plus equipped with a 100-sample holder and a Flame Ionization Detector (FID) using HP ChemStation software for control and analysis. An Agilent DBWax-ETR megabore capillary column (0.530 mm ID x 30 m, 1.0 μm film thickness) was mounted in the GC to effect separation. HP ChemStation software was used for instrument control and analysis.

Methods

Yeast Strain Preparation and Storage - Yeast strains were obtained from their respective sources and cultured in 100 mL MPYD medium for 16 hours at 21°C. Cultures were then centrifuged at 3,000 x g for 10 minutes at 10°C. The supernatant was discarded, and cells were resuspended in 100 mL ice cold MPYDS. After a 15-minute incubation on ice, the cells were divided into 1 mL aliquots and snap frozen in a dry ice-isopropanol bath. The cells were stored at -76°C until used. All experiments were performed using fresh aliquots from this initial preparation.

The 1 mL aliquot was thawed on ice then allowed to equilibrate at room temperature. The aliquot was added to 100 mL MYPD medium and incubated 16 hours at 25°C with shaking. The optical density at 600 nm was measured to ensure cultures had not progressed past the exponential phase and 0.01 g (1 mL at an optical density of 1.000 at 600 nm) was added to 200 mL of MPYHG, thoroughly mixed, and divided into small scale fermenters.

Repeat Experiment of Shu et al – The method of Shu et al. 2009 (Shu et al., 2009) was replicated using two large-scale fermenters wrapped in black tape for the control or red (WFLS-R300-WHT) and blue (WFLS-B300-WHT) LED strips for the treatment. Light intensity and color were digitally controlled through high power MOSFETs controlled by an Arduino Uno using custom software. Temperature was maintained at 28 °C using the custom-made water bath. Air

and nitrogen gas flows were switched and maintained manually. All other aspects of the experiment were as described in the publication.

Constant Intensity/Varied Wavelength - LED intensity was controlled by varying the current using a potentiometer. The 535 nm LEDs were set to 2,000 Lux measured using the Extech LT-45 Light Meter. The energy output of the 535 nm LEDs was measured using the custom light meter. The light intensity of all other LED colors was adjusted to the same power output as the 535 nm LEDs using potentiometers. Sensitivity of the TSL237 to different wavelengths of light was considered, based on manufacturers published specifications, when making intensity calculations.

Constant Wavelength/Varied Intensity (CWVI) - Near-UV LEDs with an output wavelength of 405 nm were set to a specific power output by using potentiometers to adjust the current flowing to the LED. The highest intensity level was chosen by finding the maximum output of the LEDs and reducing it by 10%. This was to prevent premature burnout of the LED by operating at maximum intensity. Eight additional energy levels between the maximum intensity and zero light were obtained by use of appropriately sized potentiometers. Intensity measurements were made using the Extech LT-45 Light Meter.

Constant Wavelength/Constant Intensity/Varied Time - Near-UV LEDs with an output wavelength of 405 nm were set to 1510 Lux, 90% of their maximum intensity, using appropriately sized potentiometers.

Three different time scales were used in experiments listed in the table below (Table 2). Timing was managed by the Arduino Uno connected to a Real Time Clock module triggering an interrupt every second. This directly exercised the LEDs at the desired time scales and allowed

precision timing up through the duration of days/weeks. All experiments had constant on and constant off illumination controls.

The first timing pattern based on a periodic times scale, denoted PTS, had the LEDs on for one 9-hour period, distributed over the first 3 days of the 6-day fermentation. Initial testing showed that complete fermentation took 6 days, with the yeast entering stationary phase in the middle of day 3. The sample was held in the dark before and after its illumination period.

The second timing pattern based on illumination from the start but terminating during fermentation, denoted TSS, started with the LEDs on but after a period of time the LEDs would turn off until the end of fermentation. In the table below (Table 2) the number denotes how many hours after inoculation the LED on that sample would be turned off.

The third time scale pattern based on chemical and biological reaction times, denoted RTS, had the LEDs flash on and off at intervals from one millisecond to twelve hours. In all instances, the LEDs were illuminated at the start of the experiment.

Constant Wavelength Varied Intensity (Lux)	LED Timing Patterns		
	PTS	TSS	RTS
6	0-9 Hrs	14 Hrs	1 mS
12	9-18 Hrs	28 Hrs	10 mS
24	18-27 Hrs	42 Hrs	100 mS
47	27-36 Hrs	56 Hrs	1 Second

94	36-45 Hrs	70 Hrs	1 Minute
188	45-54 Hrs	84 Hrs	1 Hour
375	54-63 Hrs	98 Hrs	2 Hour
750	63-72 Hrs	112 Hrs	12 Hour
1500	Const. On	126 Hrs	Const. On
Dark	Const. Off	Const. On	Const. Off
		Const. Off	

Table 2. Intensity and Timing of LEDs

Large Scale Fermentations - The glass fermenters designated as dark controls were wrapped in masking tape and black duct tape to prevent intrusion of light. Any part that was not completely covered by the tape was wrapped with two layers of aluminum foil. Fermenters used for treatments were wrapped with the designated LED lights and secured with duct tape. Uncovered portions of the fermenters were wrapped in two layers of aluminum foil. Additionally, both sets of fermenters were surrounded by card board to shield stray light. Overnight yeast cultures were measured for optical density and to ensure growth had not extended to stationary phase. A constant number of cells was added to bulk fermentation media and allowed to mix with stirring for 5 minutes. The media was then divided between the large-scale fermenters to achieve homogeneous and consistent inoculum between control and treatment fermenters. The fermenters were immediately capped with chilled condensers and the jackets connected to a pre-

equilibrated water bath maintained at 17.5°C. Light regimens and monitoring systems were started immediately after all connections were made.

Analysis

Clarification and Biomass - Pre- and post-fermentation samples were centrifuged at 12,000 x *g* using 0.45 μm Corning Costar CA filters (Corning, NY). Tared filter units were weighed to measure biomass production. Samples were then frozen at -20°C until analyzed.

GC Analysis - Samples were analyzed for alcohol and fusel oils according to the Department of Alcohol Tobacco Tax and Trade Bureau (TTB), Scientific Services Division (SSD), Method TM200 revision 4 (Unknown, 2011), modified according to AOAC Method 983.13 (International, 1988), and using propylene glycol as an internal standard.

HPLC Analysis - Samples were analyzed for carbohydrates, organic acids, and alcohols according to the method of McFeeters (McFeeters, 1993). Samples were loaded on to the column and eluted isocratically with 0.005 M sulfuric acid. Elution was monitored by refractive index and UV absorption at 210 nm.

Statistical Analysis - Chromatographic results were analyzed using JMP (SAS, Cary, NC) and graphically using Igor Pro (WaveMetrics, Tigard, OR).

Chapter 3 - Results

Repeat Experiment of Shu et al.

Both the light sample and dark control were analyzed for cell mass, optical density, alcohol, and glucose. The overall conversion ratio (% ethanol / % glucose used) for the control was 0.583, for the light sample it was 0.587. Although not detailed in the protocol, it was noted that at the time of the switch from aerobic conditions with red light to anaerobic conditions with blue light, there was a marked change in aroma throughout the lab. The aroma was distinctly reminiscent of fermentation, specifically alcohol and ester characteristics. As noted in Table 3, the alcohol level produced between the control and the treatment only differ by 0.07% ethanol. The only result with a large variation is the OD, which had a 6.6% increase between treatment and control. Fermentations were essentially finished at the end of 60 hours post inoculation.

Time (hours)	Optical Density @ 600 nm (A.U.)	Cell Mass (g/mL)	Glucose (g/L)	Alcohol (% v/v)
0	0.067 / 0.058	0.006 / 0.006	200 / 200	0.0 / 0.0
6	0.093 / 0.095	0.006 / 0.006		
12	0.739 / 0.839	0.010 / 0.011		
18	5.74 / 5.60	0.021 / 0.020		
24	9.99 / 9.61	0.022 / 0.020		
30	10.34 / 10.36	0.023 / 0.025	87.4 / 82.8	6.48 / 6.87

36	12.94 / 13.28	0.023 / 0.023	64.6 / 59.0	7.88 / 8.34
48	13.32 / 13.74	0.025 / 0.026	23.1 / 17.3	10.38 / 10.84
60	12.34 / 13.16	0.025 / 0.025	2.8 / 3.0	11.49 / 11.56

Table 3. Repeat of experiment by Shu et al. Underlined values are for the dark control.

Constant Intensity / Varied Wavelength

Fermentation in the presence of LED light produced consistent results where wavelengths at the blue end of the spectrum tended to produce more ethanol, where longer wavelengths tended to produce the same or slightly less ethanol than the dark control. Because variations in starting carbohydrate content could strongly skew the results for ethanol production, the results of each triplicate run were analyzed by the ratio of ethanol produced compared to the dark control sample. One-way analysis of variance (ANOVA) demonstrates that the differences in ethanol production are statistically significant ($p < 0.0001$, Figure 3, Table 1). Tukey's range test was applied to these results as well, demonstrating that UV treatment was significantly different from the dark control and all light treatments above 500 nm. Also demonstrated was that wavelengths in the range of 375 nm to 470 nm were significantly different from all light treatments above 500 nm. It should be noted that analysis of one of the 630 nm replicate fermentations was flawed due to equipment error and was not included in the analysis of ethanol, acetic acid, or glycerol.

Ethanol Production – Constant Intensity / Varied Wavelength

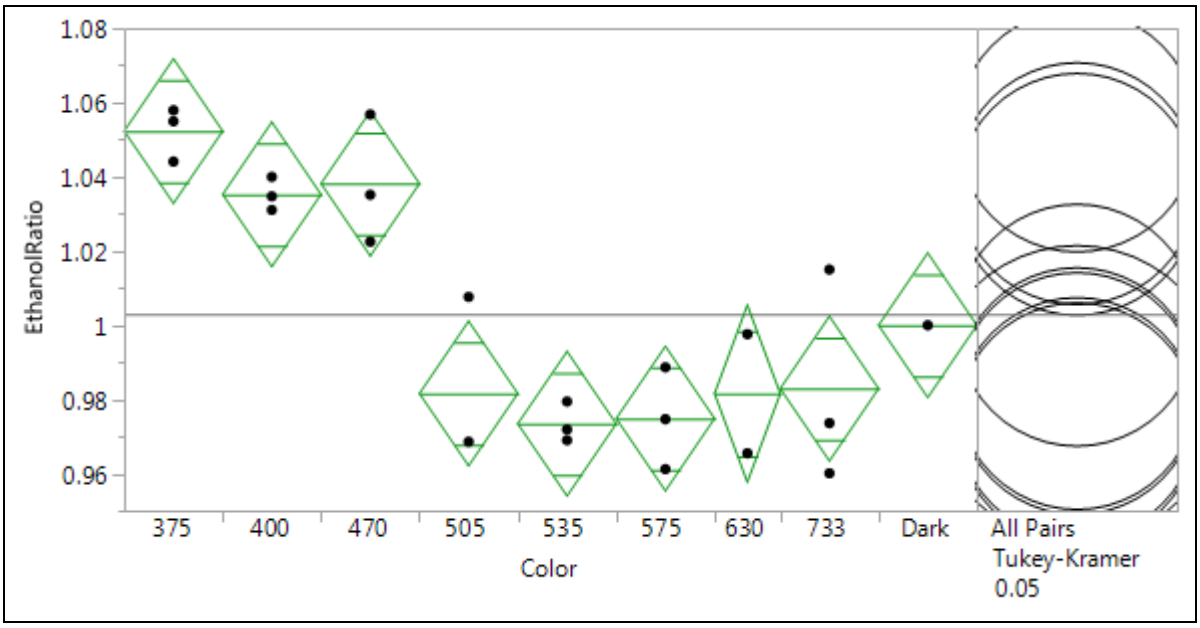

Figure 3. Graph of means, ANOVA, and Tukey's HSD analysis of ethanol production under Constant Intensity / Varied Wavelength treatments.

Color	Wavelength				Mean of Ethanol:Dark Ratio
UV	375 nm	A			1.05
UV-Purple	400 nm	A	B		1.04
Blue	470 nm	A	B		1.04
	Dark Control		B	C	1.00
Teal	505 nm			C	0.98
Green	535 nm			C	0.98
Yellow	575 nm			C	0.98
Red	630 nm			C	0.97
Cherry Red	733 nm			C	0.97
Levels not connected by same letter are significantly different.					

Table 4. Tukey's HSD Analysis of Means of Constant Intensity / Varied Wavelength treatments.

Acetic Acid Production – Constant Intensity / Varied Wavelength

Like the ethanol analysis, the production of acetic acid was also measured and ratio to dark control was analyzed. ANOVA demonstrates that although not significantly different from dark controls, 375 nm and 400 nm treatments are significantly different from 505 nm and 535nm treatments ($p < 0.0020$, Figure 4, Table 5).

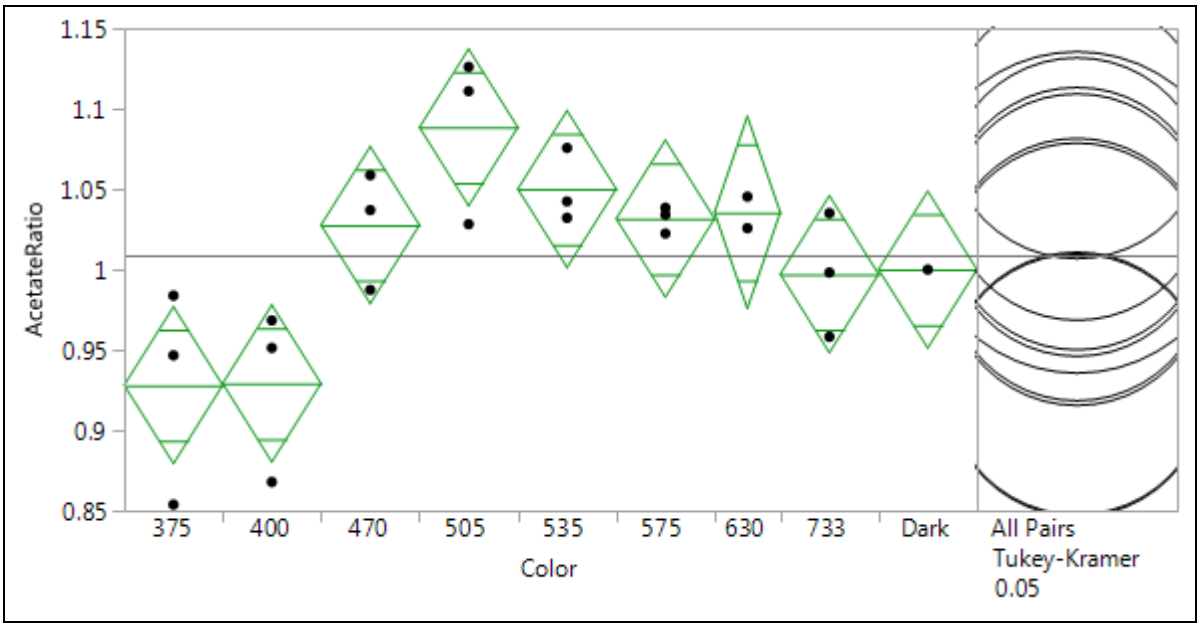

Figure 4. Graph OF Means, ANOVA, and Tukey's HSD analysis of acetic acid production under Constant Intensity / Varied wavelength treatments.

Color			Mean of Acetic Acid:Dark Ratio
505 nm	A		1.09
535 nm	A		1.05
630 nm	A	B	1.04
575 nm	A	B	1.03
470 nm	A	B	1.03
Dark Control	A	B	1.00

733 nm	A	B	1.00
400 nm		B	0.93
375 nm		B	0.93
Levels not connected by same letter are significantly different.			

Table 5. Tukey's HSD Analysis of Means of acetic acid production under Constant Intensity / Varied Wavelength treatments.

Glycerol Production – Constant Intensity / Varied Wavelength

Analysis of production of glycerol showed some variation, but the variation was not found to be statistically significant ($p = 0.0879$, Figure 5).

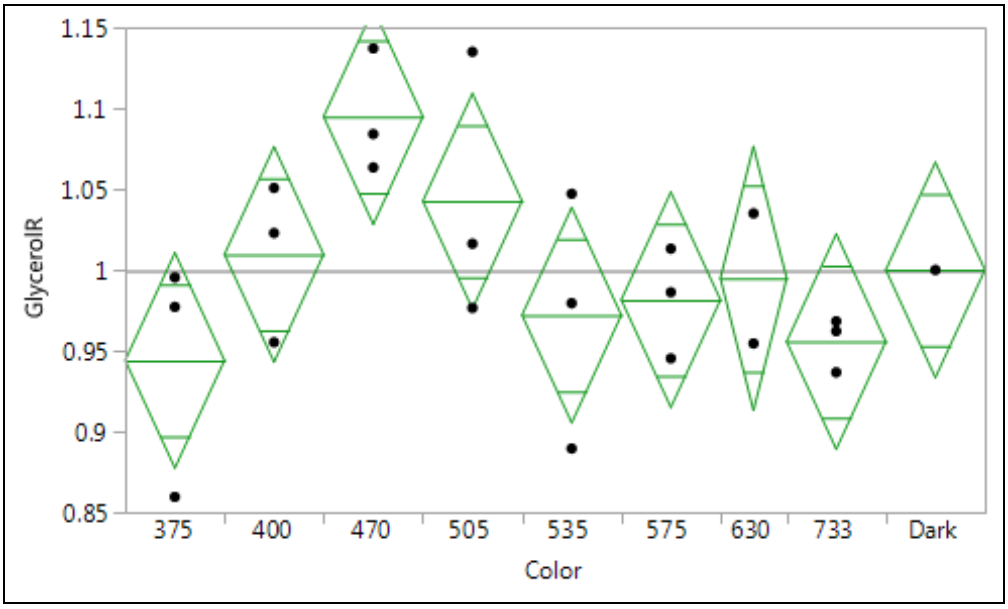

Figure 5. Graph of Means and ANOVA of glycerol production under Constant Intensity / Varied / Wavelength treatments.

Discriminant Analysis of Ethanol, Acetic acid, and Glycerol

Discriminant analysis of the covariates ethanol, acetic acid, and glycerol by LED color (Figure 6) demonstrates that there is a difference between blue-end treatments and >500 nm treatments, with near-UV and UV treatments significantly different from dark controls. Blue-end treatments favor the production ethanol and glycerol, while green-red treatments favor acetic

acid production. Although acetic acid production in all cases is not significantly more than the Dark control, the UV treatments do produce significantly less acetic acid. The difference in these light treatments indicates that there may be a significant effect on sensory perception of fermented products based on light treatment.

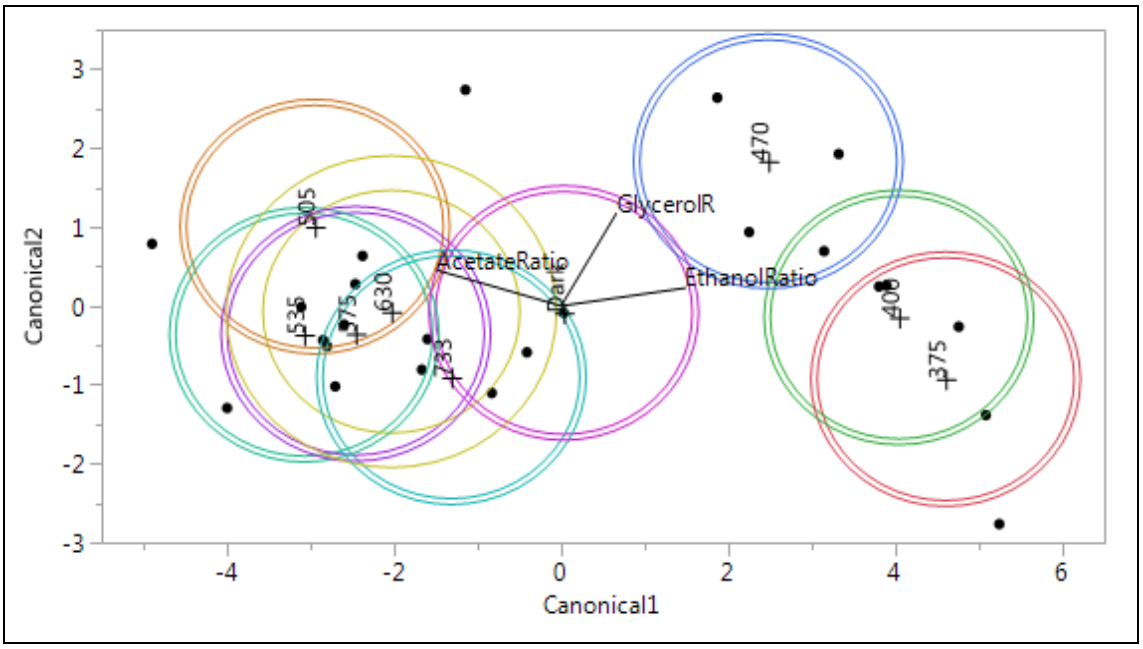

Figure 6. Discriminant Analysis of the covariates ethanol, acetic acid, and glycerol.

Constant Wavelength / Varied Titer

The effect of starting titer of microbes in conjunction with light treatment was analyzed for ethanol production. It was again found that 400 nm light significantly increased ethanol production in comparison to dark controls ($p < 0.0006$), but a higher titer of cells under blue light treatment produced more ethanol as well (Figure 7). The effect of titer on 400 nm light in comparison to 575 nm light and dark controls is consistent with previous results where ethanol production is greatly enhanced ($p < .00014$) with 400 nm light and yellow (575 nm) light is not significantly different. Though the average for 575 nm light is lower than dark controls (Figure 8), it is not proportionally lower than previous experiments.

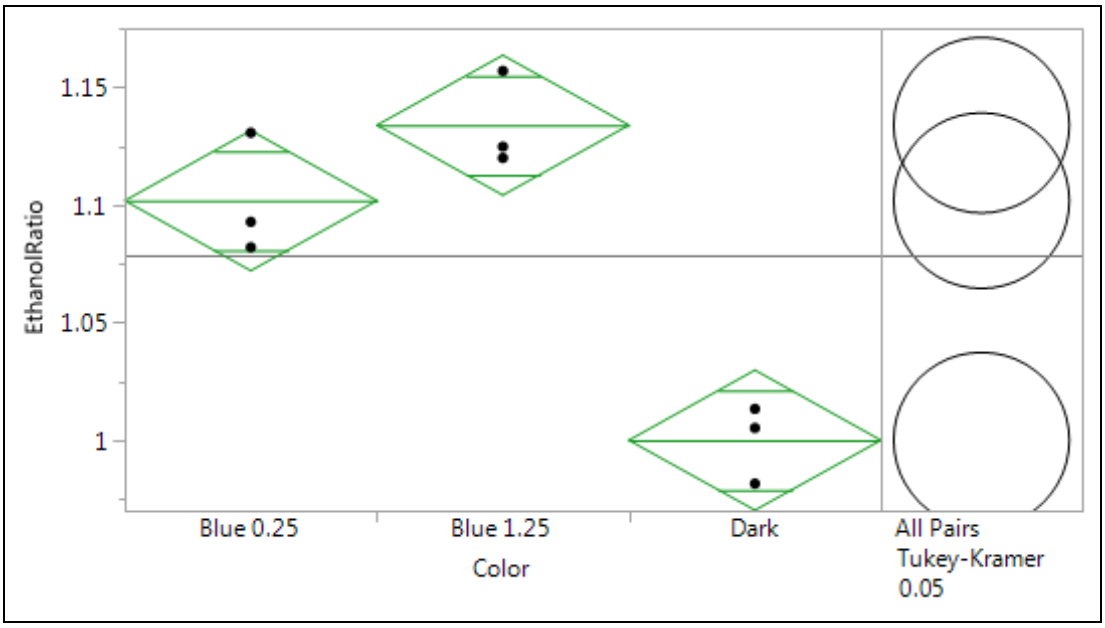

Figure 7. Effect of Varying Starting Microbial Titer on Ethanol Production Under Light Treatment.

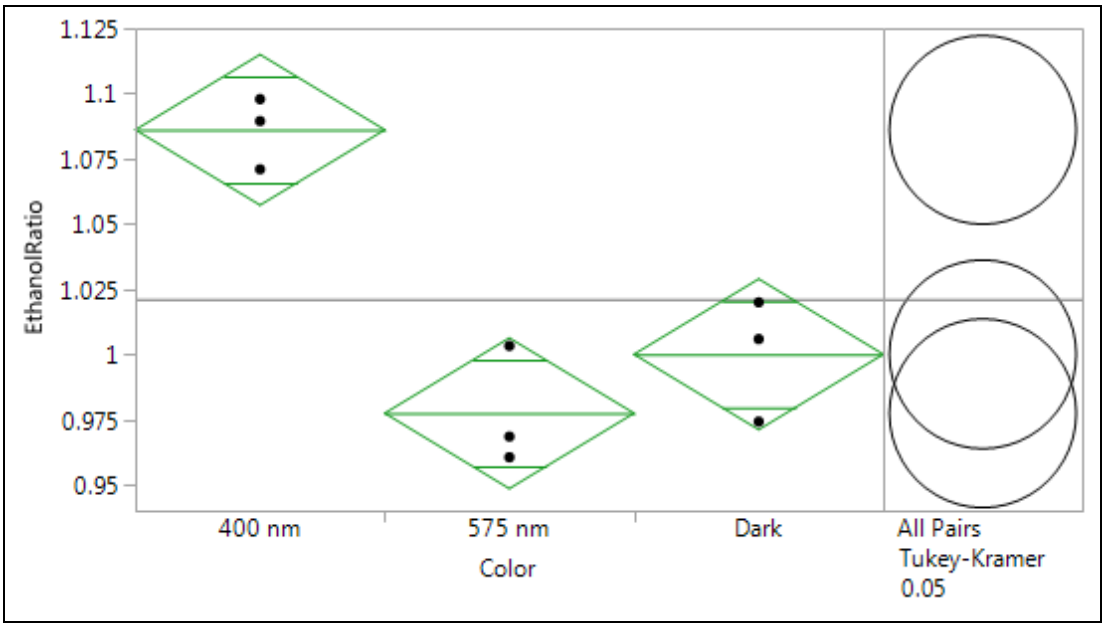

Figure 8. Effect of varying starting microbial titer on ethanol production under varying light treatment.

Modified Shu et al. 2009

Using the data obtained from the experiments above, a modified version of the protocol by Shu et al. was performed. Fermentation vessels were not agitated by introduction of gasses; additionally, condensers were placed at the top of the fermenters to reflux any ethanol or other volatiles. Yellow (575 nm) and Blue/Violet (400 nm) LEDs were used. The illuminated culture finished fermentation at 10.35% ethanol, which is higher than the 9.53% ethanol produced by the dark control. Differences in alcohol and other metabolites can be easily seen in the difference GC-FID chromatogram (Figure 9), where compounds that are either only present or present in higher concentration in the illuminated culture are represented as positive peaks and compounds in excess in the dark control are represented as negative peaks.

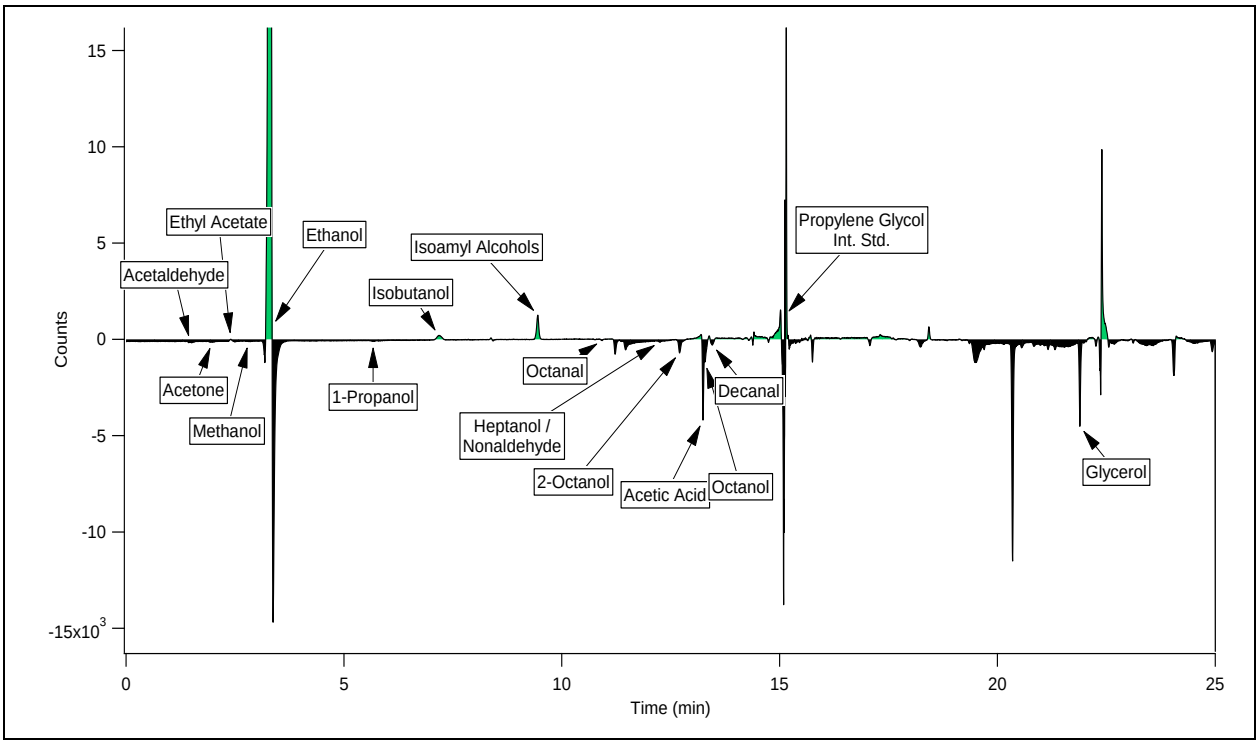

Figure 9. Difference GC-FID chromatogram of fermentation in yellow (590 nm)/blue (470 nm) illumination (positive values) vs dark control (negative values).

Constant Wavelength / Varied Intensity

Pulse-Width Modulation Dimming

Light emitting diodes dimmed using pulsed width modulation (PWM) are essentially on at full brightness, but for varying portions of time (Table 6). The microcontroller's frequency for these experiments was approximately 500Hz, which corresponds to 2 milliseconds per on/off cycle. It should be noted that most commercial LED dimmers utilize PWM dimming methods and the operational frequency is ~550 Hz.

PWM Setting	1	2	4	8	16	32	64	128	255
Duration (µS):									
Light	8	16	32	63	125	250	500	1000	2000
Dark	1992	1984	1968	1937	1875	1750	1500	1000	0
Lux	6	12	24	47	94	188	375	750	1500

Table 6. Light duration (microseconds) as a function of PWM timing and effect on light output.

Using this method of light dimming, the amount of ethanol produced under 400 nm light treatment was quantified. When the continuum of light treatments (Table 6) are analyzed together the results are not statistically significant (p=0.0596, Figure 10). Using a subset of the data, reviewing the minimum light output (PWM=1), half duration (PWM=128), and light full on/constant (PWM=255), it can be seen that light does have an effect throughout the continuum, but only constant light is statistically significant (p=0.0249, Figure 11, Table 1).

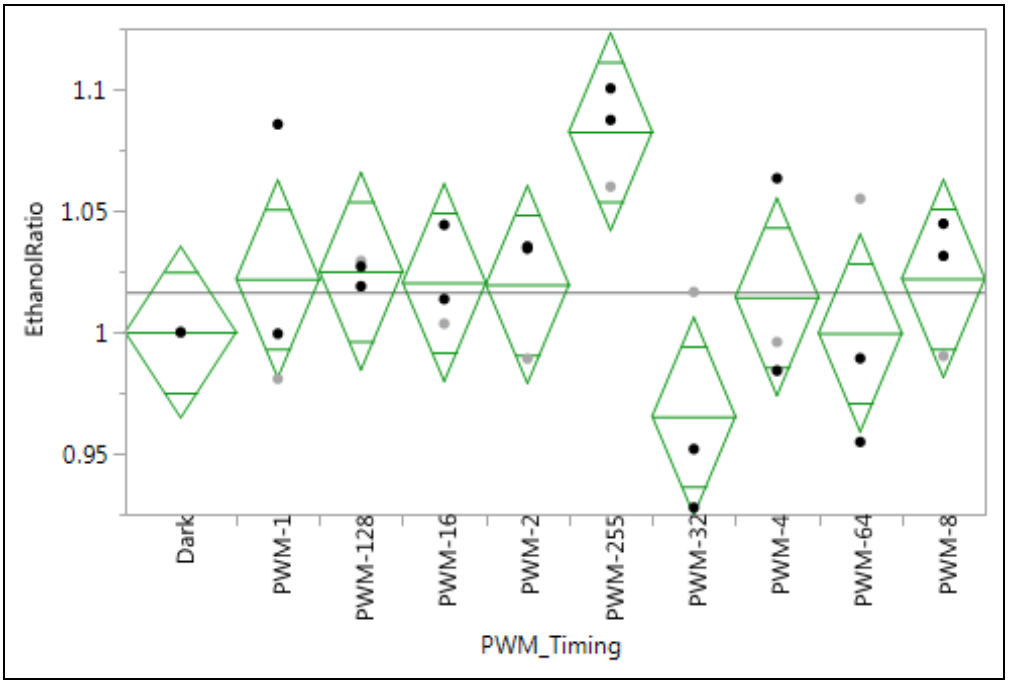

Figure 10. Average ethanol production ratio under a continuum of light intensity affected by Pulse Width Modulation.

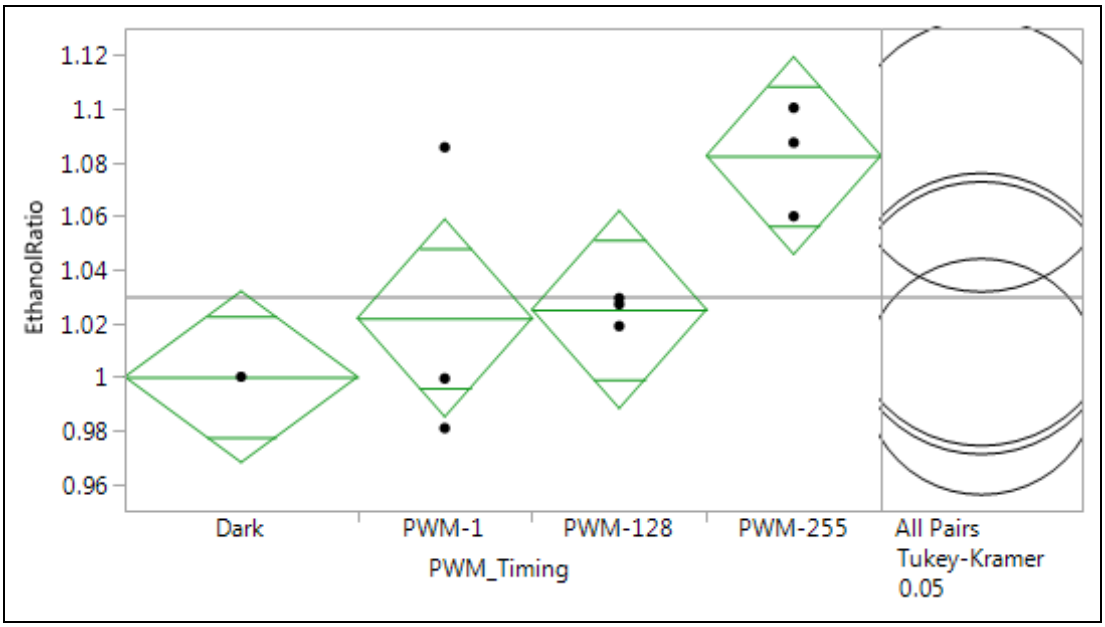

Figure 11. Average ethanol production ratio under a continuum of light intensity affected by Pulse Width Modulation.

PWM Timing			Mean of Ethanol:Dark Ratio
PWM-255	A		1.08
PWM-128	A	B	1.03
PWM-1	A	B	1.02
Dark		B	1.00
Levels not connected by same letter are significantly different.			

Table 7. Tukey's HSD Analysis of Means of average ethanol production ratio under a continuum of light intensity affected by Pulse Width Modulation.

Dimming by Resistance

In contrast to PWM intensity control, actual dimming of the LEDs can be affected by limiting current flow through the device using resistors. This has the effect of truly decreasing the amount of light emitted. Fermentations conducted under light conditions *measured* to be of the same intensity as the PWM light experiments show statistically significant results (p=0.0002,

Figure 12, Table 8) as far as ethanol production as a function of light treatment. The data labels are based on intensity correlating to the PWM frequency experiments (i.e., Int-128 has the same intensity as PWM-128).

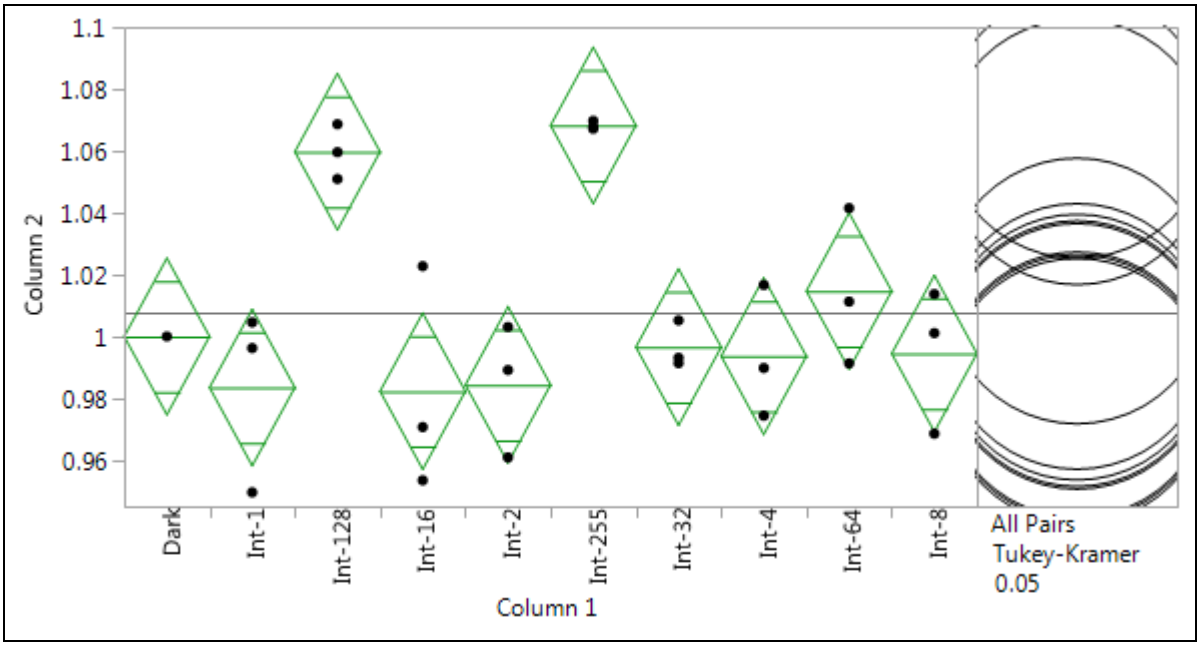

Figure 12. Average ethanol production ratio under a continuum of light intensity affected by Current Limiting (true dimming).

Resistance Dimming				Mean of Ethanol:Dark Ratio
Int-255	A			1.07
Int-128	A	B		1.06
Int-64	A	B	C	1.01
Dark		B	C	1.00
Int-32			C	1.00
Int-8			C	0.99
Int-4			C	0.99
Int-2			C	0.98
Int-1			C	0.98

Int-16			C	0.98
Levels not connected by same letter are significantly different.				

Table 8. Tukey's HSD Analysis of Means of average ethanol production ratio under a continuum of light intensity affected by Current Limiting (true dimming).

When the data for these two sets of experiments are pooled together and analyzed it can be seen that resistance dimming is different from PWM type dimming (p=0.0004, Figure 13). The highest level of illumination is well above the dark controls. Fermentation under half-maximal intensity still produces higher amounts of ethanol when the dimming is done by current limiting in place of pulse light (p<0.0001, Figure 14, Table 9. Tukey's HSD Analysis of Means of full and half maximal intensity light effects on fermentation and variation by dimming mode.).

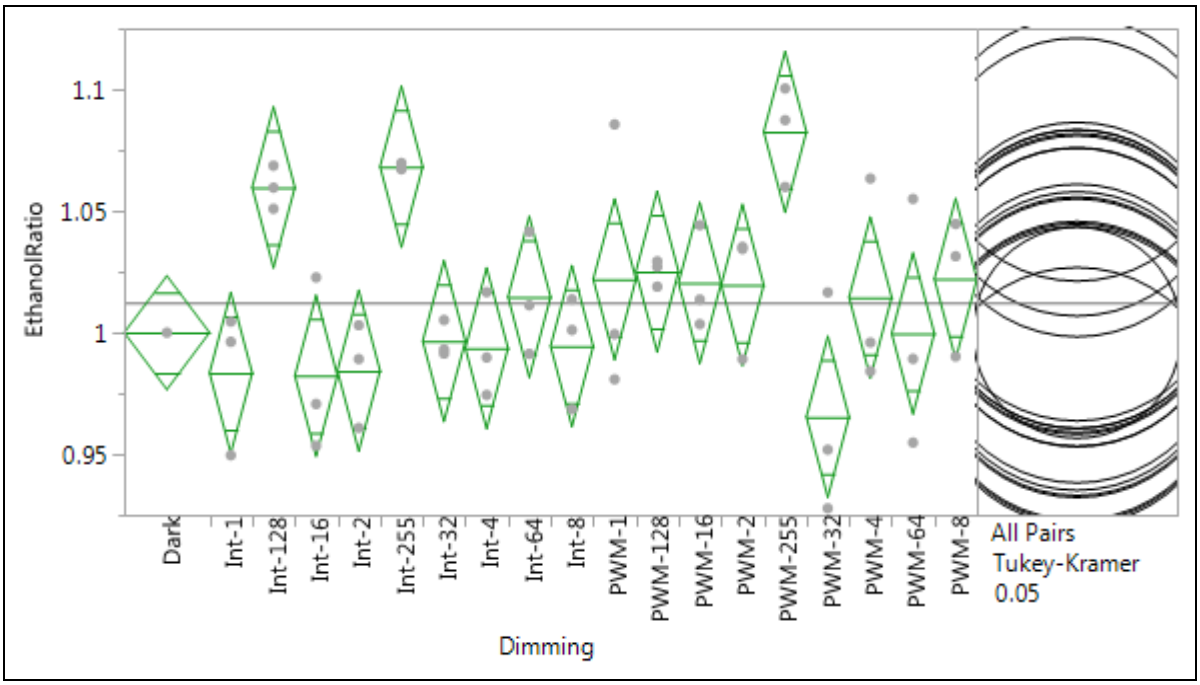

Figure 13. Pooled data from light intensity experiments.

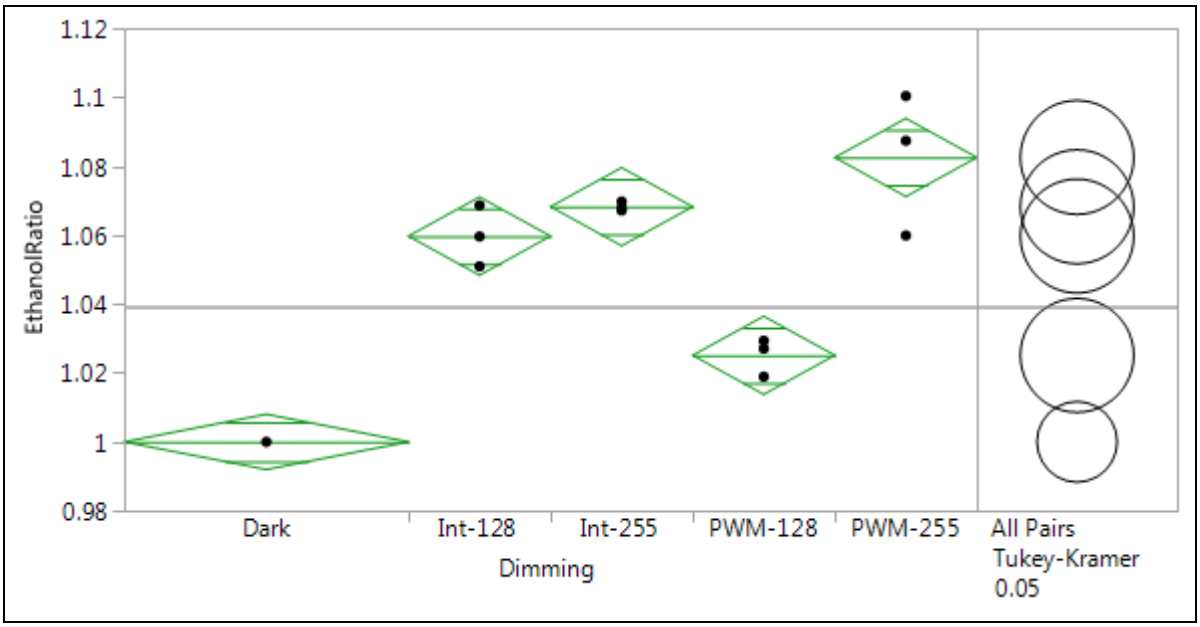

Figure 14. Full and half maximal intensity light effects on fermentation and variation by dimming mode.

Dimming Mode & Intensity				Mean of Ethanol:Dark Ratio
PWM-255	A			1.08
Int-255	A			1.07
Int-128	A			1.06
PWM-128		B		1.03
Dark			C	1.00
Levels not connected by same letter are significantly different.				

Table 9. Tukey's HSD Analysis of Means of full and half maximal intensity light effects on fermentation and variation by dimming mode.

Constant Wavelength / Constant Intensity / Varied Time – Periodic Timing

Discrete Block of Illumination

Light emitting diodes were dimmed using resistors to the equivalent of 1500 lux. The LEDs were then illuminated for discrete portions of time during fermentation (Table 10). At all

other times the fermentation vessels were held in the dark. As with data from previous treatments, the data is “noisy” which may be due to differing fermentation rates between vessels and small variation in cell growth (Figure 15). Significant differences can be seen in comparing the constant illumination and light treatment at the very beginning of fermentation (p=0.0215, Figure 16, Table 11). Illuminating cultures during the first few hours, the lag phase, yields an average of 9.2% less ethanol compared to constant illumination and a 2.6% decrease in ethanol compared to cultures fermented in the dark.

0-9	9-18	18-27	27-36	36-45	45-54	54-63	63-72	Constant On	Constant Off

Table 10. Timing of illumination (hours of fermentation).

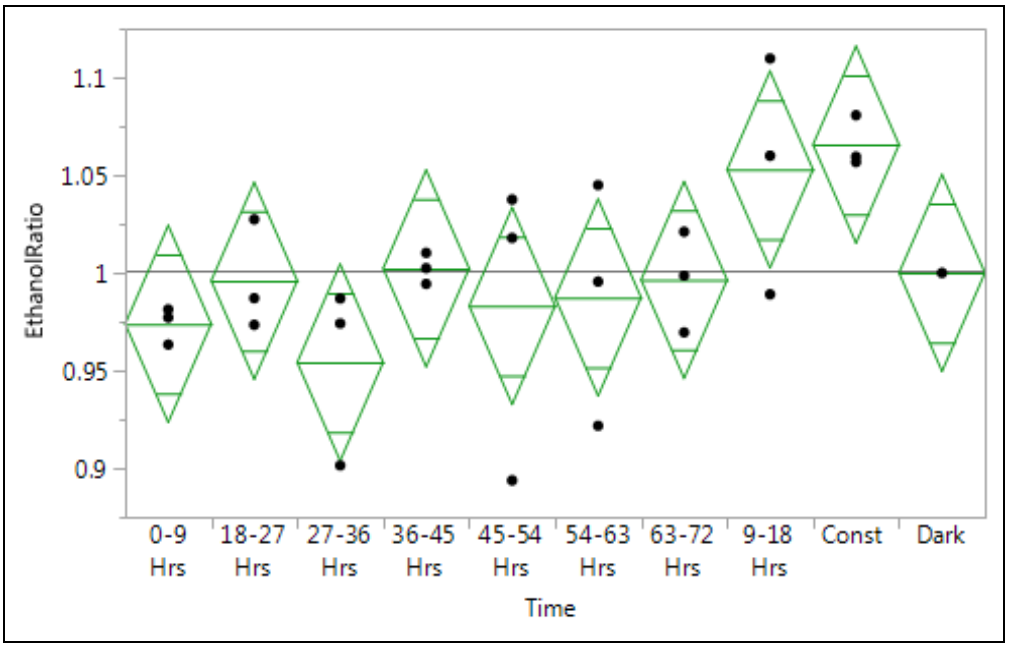

Figure 15. Graph of ethanol production over discrete blocks of time.

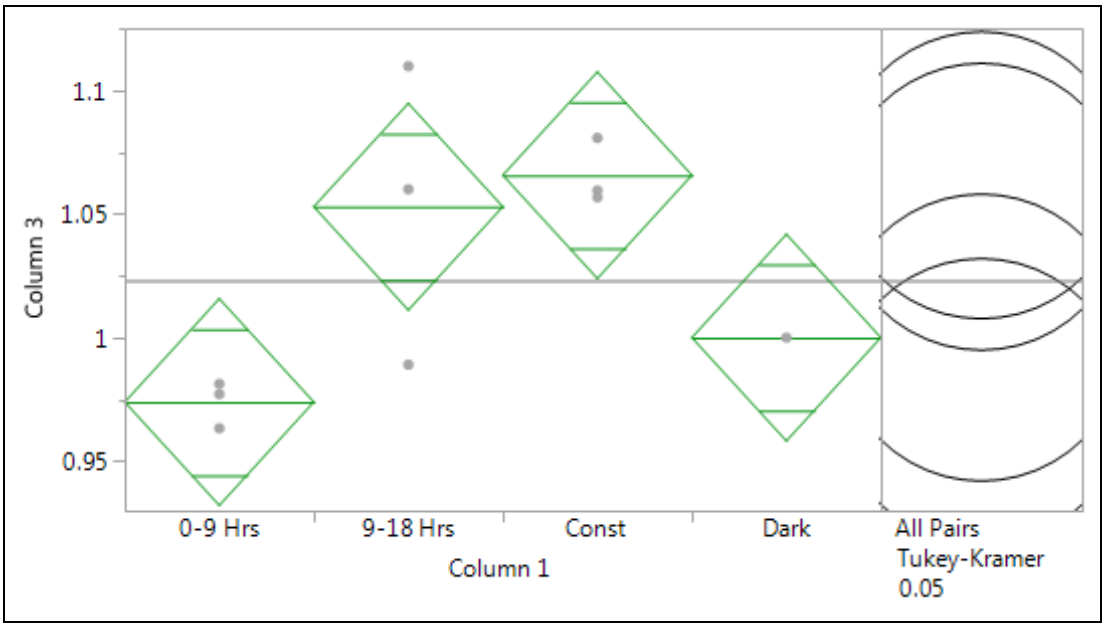

Figure 16. Minimum and maximal ethanol production by block of illumination.

Discrete Illumination			Mean of Ethanol:Dark Ratio
Constant On	A		1.07
9-18 Hrs	A	B	1.05
Dark	A	B	1.00
0-9 Hrs		B	0.97
Levels not connected by same letter are significantly different.			

Table 11. Tukey's HSD Analysis of Means of minimum and maximal ethanol production.

Duration of Illumination

Light emitting diodes were dimmed using resistors to the equivalent of 500 lux. All reaction vessels, save for the dark control, were illuminated at the start of fermentation, and then turned off until the end of fermentation at different times (Table 12). The ratio of ethanol produced, compared to dark controls, from each treatment are displayed in Figure 17. Much like the results from the periodic illumination scheme, there is significant variation in the results

related to timing of light. Comparing the beginning of fermentation to the dark control and the constant on illumination, it can be seen that the increase in ethanol production is significant (p=0.0190, Figure 18, Table 1).

0-14	0-28	0-42	0-56	0-70	0-84	0-98	0-112	0-126	Constant On	Constant Off

Table 12. Duration of illumination (hours).

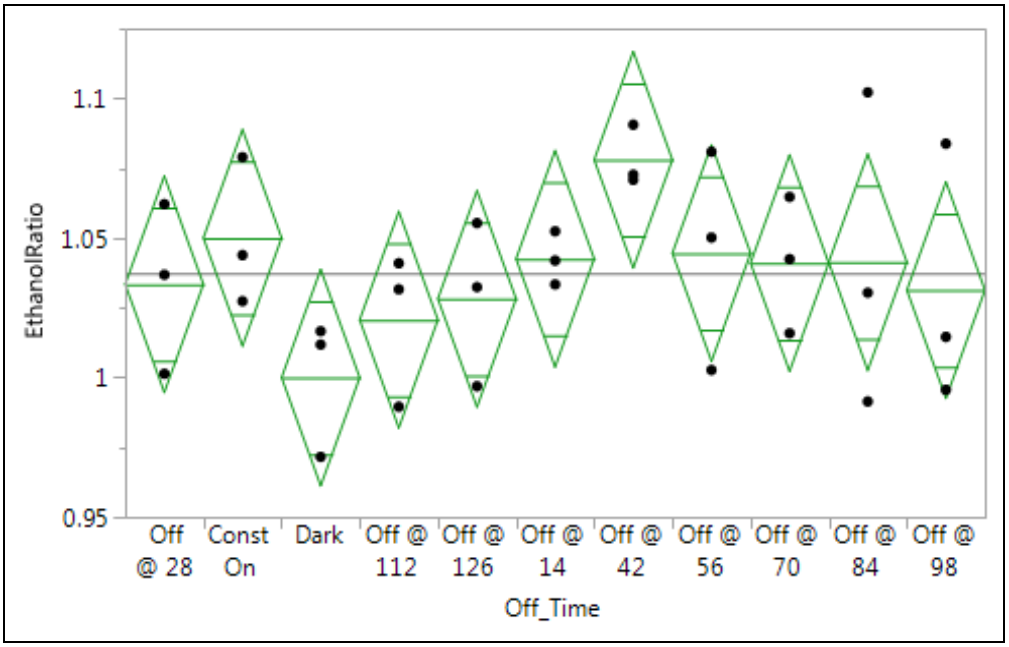

Figure 17. Ethanol produced during fermentations illuminated for different durations from the start of fermentation.

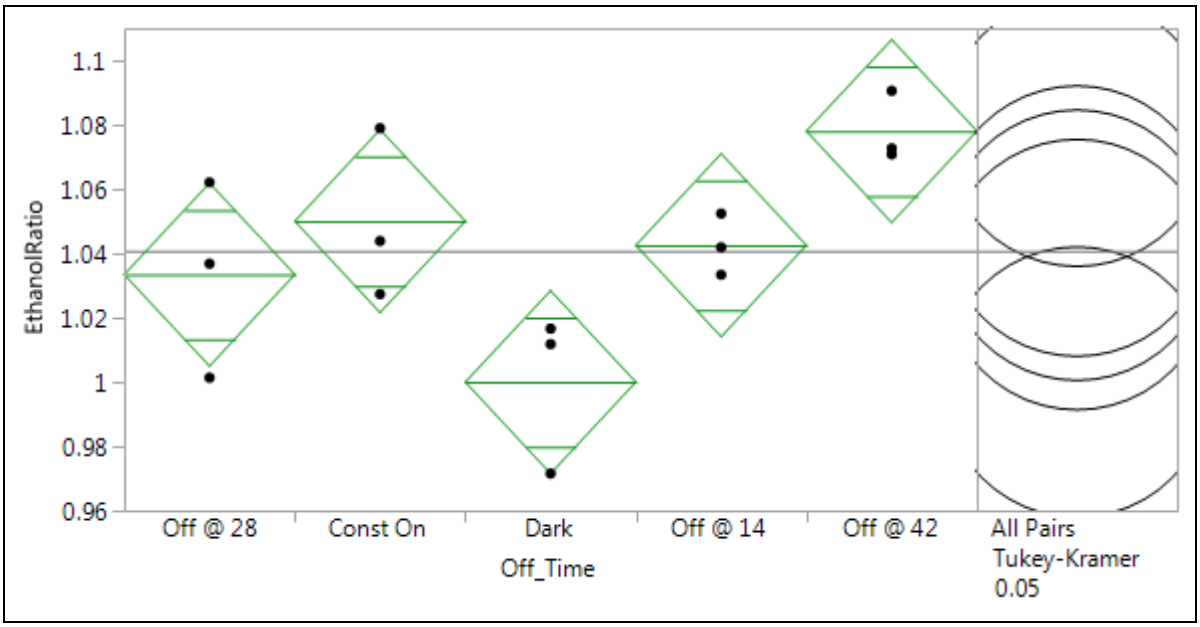

Figure 18. Ethanol production is increased when cultures are illuminated at the beginning of fermentation.

Duration of Illumination			Mean of Ethanol:Dark Ratio
Off @42	A		1.08
Constant	A	B	1.05
Off @ 14	A	B	1.04
Off @ 28	A	B	1.03
Dark		B	1.00
Levels not connected by same letter are significantly different.			

Table 13. Tukey's HSD Analysis of Means of ethanol production when cultures are illuminated at the beginning of fermentation.

Constant Wavelength / Constant Intensity / Varied Time – RTS

Light emitting diodes were dimmed using resistors to the equivalent of 1500 lux. The LEDs were then switch illuminated by pulsing at timescales in the range of milliseconds to hours including constant on and constant off (Table 14). The timing of light pulses on the fermentation

vessels are denoted in the table as duration of each light and each dark period. Ethanol concentrations are scaled to their dark controls. The Constant On samples are consistent with the values reported for the resistance dimmed values, but 100 mS, 2 hour, and 12 hour timing cycles produce more ethanol than all other treatments (Figure 19), with 100 mS and 12 hour timing cycles producing significantly more ethanol than constant illumination and dark controls (p=0.0049, Figure 20, Table 15).

1 mS	10 mS	100 mS	1 Sec	1 Min	1Hr	2 Hrs	12 Hrs	Constant On	Constant Off

Table 14. Reaction time scales.

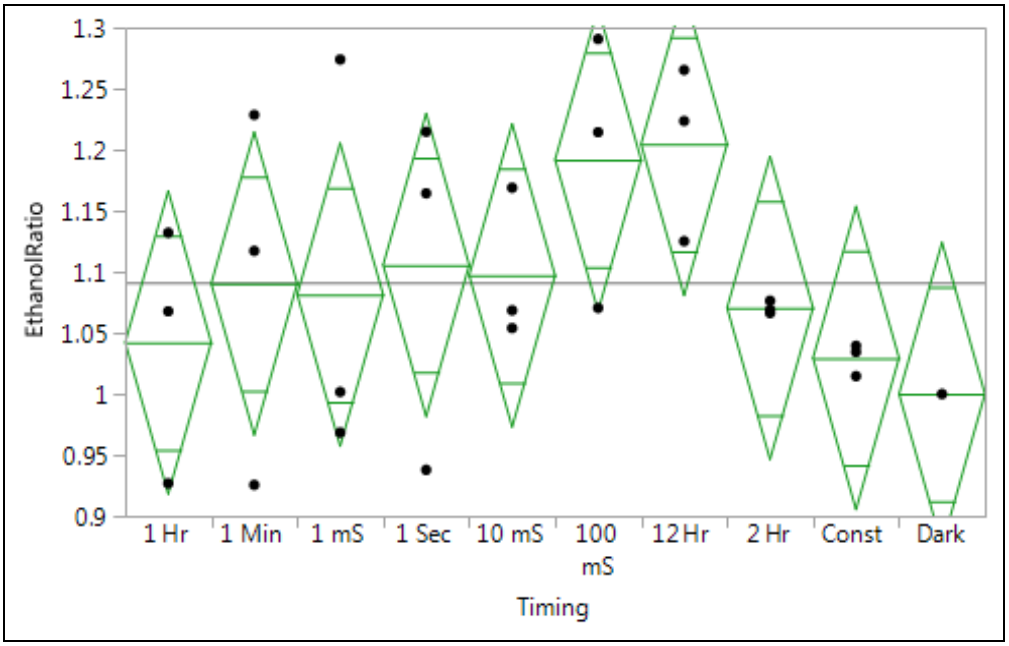

Figure 19. Ratio of ethanol produced compared to dark control using RTS Timing.

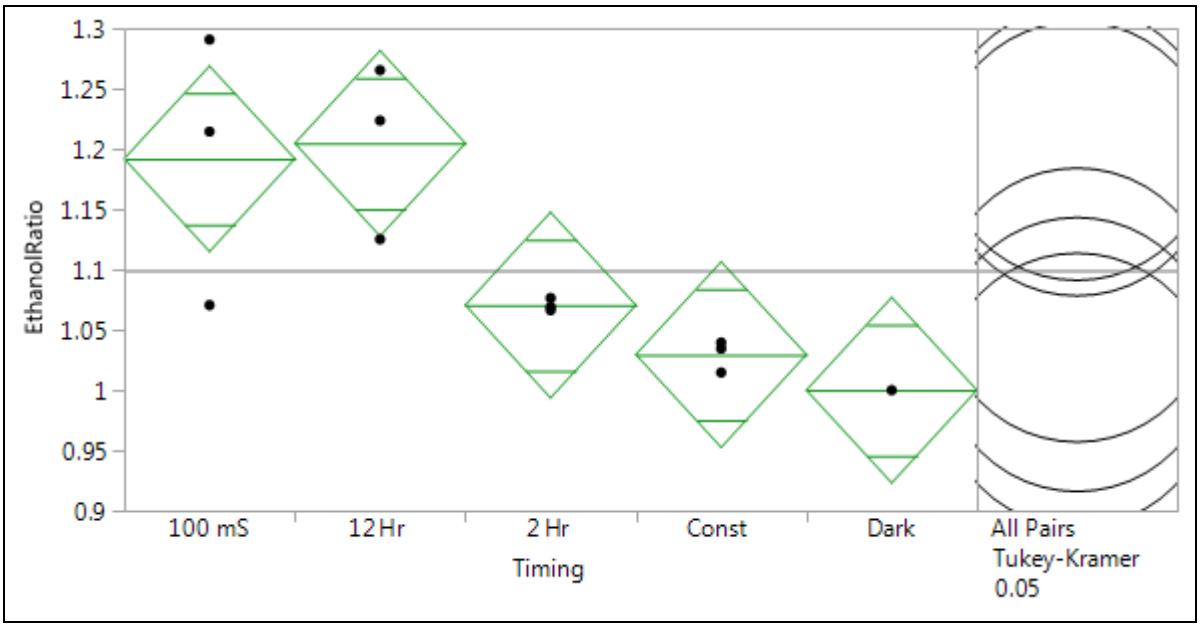

Figure 20. Light Timing Effecting Maximal Ethanol Production.

Pulse Duration			Mean of Ethanol:Dark Ratio
12 Hours	A		1.20
100 mS	A		1.19
2 Hours	A	B	1.07
Constant On		B	1.03
Dark		B	1.00
Levels not connected by same letter are significantly different.			

Table 15. Tukey's HSD Analysis of Means of light timing on ethanol production.

Constant Intensity / Varied Wavelength – Apple Juice

Apple juice fermentations were illuminated using LEDs in the same manner as the Constant Intensity / Varied Wavelength experiments. The fermentations were quantitatively analyzed for ethanol production. No significant increase was seen in the level of ethanol

produced at any wavelength, though surprisingly a significant decrease in ethanol was observed under 375 nm illumination (p=0.0126, Figure 21, Table 16).

Informal sensory analysis by faculty, staff, and a student of the Virginia Tech Food Science and Technology Department (n=5), did note that apple juice fermentations illuminated with 470 nm LEDs had a strong off-putting aroma reminiscent of iso-valeric acid. In contrast, fermentations illuminated by 733 nm and 630 nm LEDs had more fruit characteristics than dark controls.

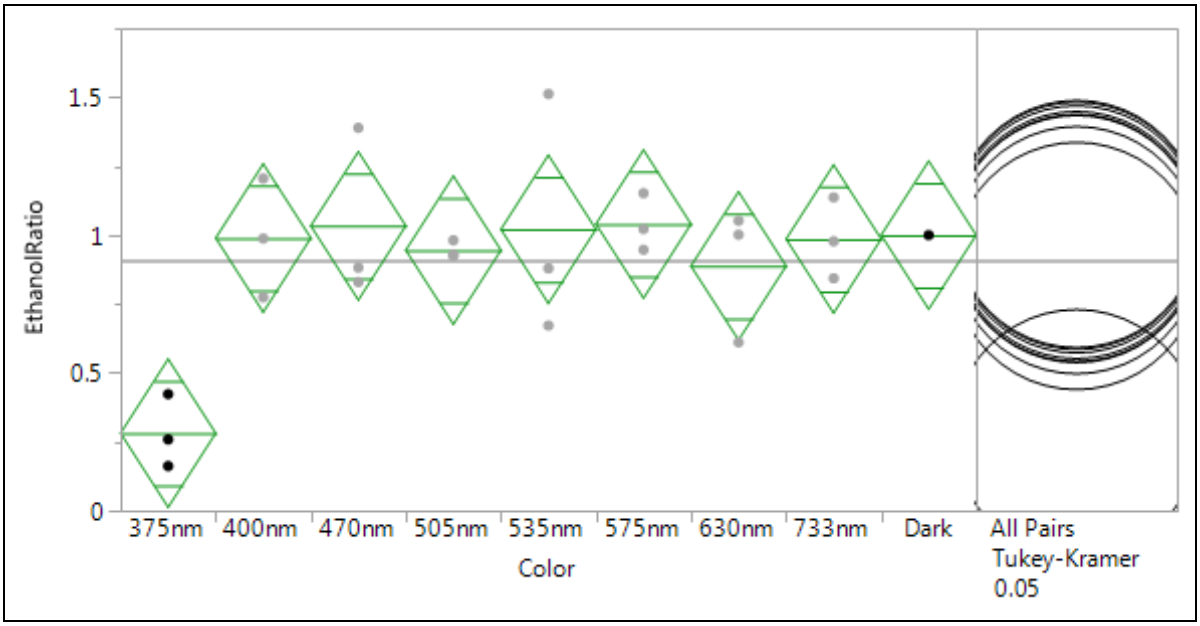

Figure 21. Ethanol produced by the fermentation of apple juice under varying wavelengths of light.

Wavelength – Apple Juice Fermentation			Mean of Ethanol:Dark Ratio
575 nm	A		1.04
470 nm	A		1.03
535 nm	A		1.02
Dark	A		1.00

400 nm	A		0.99
733 nm	A		0.99
505 nm	A		0.94
630 nm	A	B	0.89
375 nm		B	0.28
Levels not connected by same letter are significantly different.			

Table 16. Tukey's HSD Analysis of Means of illuminated apple juice fermentations.

Chapter 4 - Discussion

There are numerous products on the market to change the flavors and aromas of wines. Use of metal catalysts (Clef du Vin) or magnets (Flav-O-Ring) to age wines, aerators to micro-oxygenate (Aervana), and chemicals to treat wine (Drop-It) are all common in the wine industry. The common thread of all these products is that they all are designed for the finished product. The main idea behind this research is that the beginning of the fermentation process can have the largest effect on the outcome. Since the production of alcohol, as well as flavor and aroma compounds, come from yeast metabolic processes, modifying these processes can have the most profound effect.

Previous research has shown that broad spectrum (white) light can affect yeast growth, though those studies focused specifically on cell size, reactor biomass, and nutrient uptake (L. N. Edmunds et al., 1979; Ulaszewski et al., 1979; Woodward et al., 1978a). The effects observed in these studies were correlated to light intensity, and although yeast do not have a known genetic pathway for a light-dependent circadian rhythm, they demonstrated that one could be entrained by the timing of light. The only research found detailing an increase in ethanol due to light treatment during yeast fermentation was the article by Shu et al. Their research found that aerobic fermentation under red light (~630 nm) to the end of exponential growth phase followed by anaerobic fermentation under blue light (470 nm) produced more ethanol than fermentations in the dark. Furthermore, the amount of ethanol produced under the illumination scheme was 96.5% of the maximum theoretical possible.

The experimental design and data brought up two specific questions:

- How do other colors of light effect fermentation?
- Could the method of controlling the light intensity be affecting the fermentation?

Repeat Experiment of Shu et al. 2009

The first step was to repeat the experiment as closely as possible. One key assumption was made—that the LEDs intensity was controlled by Pulse Width Modulation (PWM). PWM is essentially flashing the LEDs at full intensity, but changing the ration of time on : time off to achieve the desired light intensity. The human eye and most common light meters will register the light as constantly on but dimmed even though the lights are at full intensity on and completely off.

The results for the experiment (Table 3) demonstrated very little difference in the light treatment and dark control fermentations with approximately 11.5% ethanol being produced. The maximum theoretical amount of ethanol produced would be 12.96% ethanol, Shu et al. reported 12.51% ethanol due to light treatment and 9.18% for the dark control. One key result from this experiment, not noted in the original paper, was the loss of solution due to evaporation. By then end of the fermentation, ~10% of the volume had been driven off due to the elevated temperature and gasses constantly being bubbled through the reactor. In the current study, the room housing the fermenters (approximately 8,000 ft^3) had a very strong odor of ethanol and volatile esters, indicating loss of these compounds from solution during fermentation.

Considering that replication of the published results was not possible and that not all of the spectrum was represented, research covering light wavelength, intensity, and timing was undertaken to see how ethanol production could be affected.

Constant Intensity / Varied Wavelength

Elucidation of the effects of light on fermentation began with determining which wavelength of light has the most profound effect. This research is based on modifying the metabolism of yeast to modify the fermentation. To that end, the wavelengths most strongly absorbed by yeast (Table 3-2, 400 nm – 630 nm), as described by Guerrin et al. 1966, were

chosen as the wavelengths to use during fermentation. Additionally, 375 nm was chosen due to the strong absorbance of protein bound flavins at this wavelength (Schwinn, Ferré, & Huix-Rotllant, 2020). Also, 730 nm light was shown to be involved in other *Ascomycota* growth (Mooney et al., 1990).

Ethanol production under different colors of light is demonstrably different, with Blue-UV light increasing ethanol production and Green-Red lowering ethanol production (Figure 3). UV light (375 nm) produced significantly more ethanol than dark control, the difference between Blue-UV and Green-Red being significantly different as well. The Blue-UV end of the spectrum has the highest and most distributed absorption due to the high energy of these photons and the number of absorbing species (flavins, porphyrins, cytochromes, etc.). Considering the higher energy per photon at this end of the spectrum it is easy to conceive of more profound effects.

Ethanol is the most significant metabolite during yeast fermentation, but other compounds are produced as well. Acetic acid and glycerol are also produced in significant quantity. The same fermentations referenced above were analyze for acetic acid production. The results were the inverse of ethanol production (Figure 4). The UV end of the spectrum produced less acetic acid, the Red region produced more, with the Green region producing the most. None of these are significantly different from the dark control, though UV and Green illuminated fermentations are significantly different from each other.

Glycerol was measured as a function of illumination wavelength and found not to be significant on its own (Figure 5). When the three metabolites are considered together, demonstrated on a Canonical Plot (Figure 6), it can be plainly seen that the Blue-UV region of the spectrum is separate from the Green-Red end of the spectrum. Fermentations treated with different colors of light will have different flavor and aroma profiles based on these metabolites

and the end value of the fermentation can be increased by applying the appropriate color of light based on the desired end product.

Constant Wavelength / Varied Titer

The results discussed above demonstrate that light treatment of different wavelengths have significant impacts on yeast metabolism. These impacts would be expected to vary the flavor and aroma of fermented foods and beverages, demonstrated in the thesis work of Wright (Wright, 2019). The research focus presented here was shifted to strictly the production of ethanol as a function of light exposure.

As referenced in the materials and methods section, the fermentations were inoculated with 0.01 grams yeast per 200 mL medium. This equates to 1 mL of inoculum at 1 OD_{600nm}. Considered for experimentation was the effect of the starting concentration of yeast on illuminated fermentations. Once diluted, the optical density would be ~0.005, approaching the lower limit of quantitation for most spectrophotometers. Although this is a low level of absorbance, it is not no absorbance. Also, *Saccharomyces* sp. yeast are known to communicate via quorum sensing. A lower or higher concentration of yeast may impact the fermentation. With this in mind, 400 nm illuminated fermentations were prepared using 25% (Blue 0.25) of the previous starting material and 25% increase (Blue 1.25) in the starting material along with a dark control using the standard titer of yeast (Figure 7). The higher titer illuminated fermentation did produce slightly more ethanol than the lower titer fermentation, but they were not significantly different from each other. Of note is both fermentations produced significantly more ethanol than the dark control.

Using this higher titer of yeast, the influence of light wavelength was also measured. All treatments (illuminated and dark controls) were inoculated with the higher titer yeast. The 400

nm illuminated fermentation produced significantly more ethanol than both the dark control and the 575 nm illuminated fermentation (Figure 8). Consistent with previous results, the 575 nm illuminated fermentation produced less ethanol than the dark control, but not significantly different.

The titer of yeast at the start of fermentation does influence the overall ethanol production, with more yeast producing a higher concentration of ethanol. This is possibly related to the shift in yeast metabolism into energy production (ethanol evolution) instead of cell mass and dilution. With a higher concentration of yeast, fermentations will reach stationary phase faster and the shift away from reproduction will begin sooner. Ethanol production is a wholly anaerobic endeavor. A higher yeast titer will also metabolize the dissolved oxygen faster, leading to anaerobic conditions faster. It can be stated with confidence that the wavelength of light is influencing ethanol production and the light effect is independent of microbial concentration. To be sure, though, a very high titer of yeast will begin to decrease the amount of light reaching the center of the fermentation, decreasing its overall effect.

Modified Shu et al.

The results discussed above led modification of the protocol used by Shu et al. The most significant change was that the fermenters were capped with reflux condensers to prevent loss of volatile compounds. Consistent with previous literature, the temperature of the fermenters was lowered to 17.5°C (instead of 28°C) and no oxygenation or gas bubbling was used. Sparging with either air or nitrogen would have the effect of stirring the ferment which would increase speed and efficiency. The bottom of the fermenters was fitted with a flat cap allowing the use of a magnetic stir bar.

A dual illumination scheme was used, with the beginning of fermentation receiving 575 nm light (yellow) and the latter stages of fermentation receiving 400 nm (purple) light. These wavelengths were chosen because they had the most disparate ethanol production while still maintaining roughly similar acetic acid and glycerol production.

The dual-stage illumination fermentation did produce an end product that was different from the dark fermented control (Figure 9). As expected, the amount of ethanol produced was higher, but in processing the two fermentations the aroma was found to be different. Because an aroma profile is difficult to quantify, the two fermentations were compared by gas chromatography-flame ionization detector chromatograms. The difference spectrum generated (Figure 9) is significantly more complex in the negative direction (dark control) compared to the illuminated fermentation (positive peaks). The number and higher intensity of the negative peaks indicates that the dark fermentation will have a higher complexity and overall stronger aroma than the illuminated fermentation. Highlighted in the graph are the higher alcohols and aldehydes, and most notably acetic acid. With the goal of ethanol production in mind, directing the yeast metabolism toward ethanol production in place of higher alcohol and aldehyde production with only the application of light is a significant finding and will be of importance to ethanol industries. For production of purified, high-concentration ethanol, decreasing the amount of glycerol generated is desired to prevent fouling of distillation equipment. This illuminated fermentation does decrease the production of glycerol and further experiments could dictate even more control over the non-desired components.

For all the following described experiments, the blue/near-UV LEDs at 400 nm were used.

Constant Wavelength / Varied Intensity

Illumination of ferments does require control of intensity. For light emitting diodes, there are two electronic methods for controlling the light: PWM dimming and resistor dimming. As mentioned previously, PWM is not "true" dimming. The LEDs are at full intensity but the on period is varied. Most common PWM dimming systems work in the range of ~500 Hz. This means that each second is divided into 500 subsections, 2 microseconds, and those 2 microseconds will be further subdivided into periods of light on and light off. The controllers used in this experimentation have 8-bit resolution, meaning there are 256 possible levels of illumination. The maximum illumination of the LEDs was 1500 lux, which was chosen as the starting point. Light intensity was continually halved to the lowest PWM setting (Table 2). Standard light meters read the pulse width modulated light much like the human eye does, as dimmed. Only high-speed sensors will detect the PWM controlled lights as actually flashing.

Using the measured lux values for the PWM dimmed LEDs, variable resistors were used to dim LEDs to the same intensity as measured for the PWM controlled lights. This is "true" dimming, the LEDs are constantly on and not flashing on any time scale.

Pulse-Width Modulation Dimming

Visual observation and the light meter both attest to a dimming of the LEDs, but the alcohol production by the yeast do not bare the same result. The different levels of illumination, when taken together, are not statistically different (Figure 10). When just a few illumination values are considered (Figure 11) the difference between full illumination and the dark control are more apparent and statistically significant. Dropping values out of a data set is not typically an acceptable method to achieve statistical significance. What makes this data set unique is that the values are not truly a continuum of intensity. The LEDs are full intensity, just flashing, so the measured intensity difference is not a true difference. The overall energy impacting the ferment

is different and increasing along the continuum, but how the light is being applied is not a continuum. Comparing these results to those produced by a true continuum of intensity will demonstrate the difference.

Dimming by Resistance

Dimming LED's using resistors is true dimming, the total intensity is decreased, not just a perceived decrease. The LEDs were set to match the light intensity of the PWM dimmed LEDs because the PWM values are quantized by the digital processor, where the resistor based dimming can be fined tuned to the PWM value because they are analog in nature. The intensity values were matched using the light meter.

The first noticeable difference between the two methods of intensity control is that the results for the resistor-based dimming are statistically significant (Figure 12, Table 8). The full intensity LED still produced the most ethanol with the amount decreasing when the light level was decreased by one-half and one-quarter. When the light levels were decreased further, the alcohol production was lower than the dark control. This may imply that there is a reverse effect from higher intensity illumination.

The pooled data for the two dimming methods (Figure 13,Figure 14, Table 9) confirm this finding. The resistor-based dimming represents a true spectrum; the effects on the ferment change with a change in resistor setting. The implications of this, combined with the light color, are three-fold:

1) Light wavelength and intensity do have an impact on fermentation.
2) The method of light control directly and quantitatively changes the effect on fermentation.

3) The increasingly common use of high intensity LEDs, controlled by PWM light control, may have serious impacts on chemical and biological samples or organisms.

The resistor-based dimming effects increased smoothly as light intensity was increased, where the PWM-based dimming did not change significantly until higher intensities. A small change in a digital setting may not yield a significant perceived change in light brightness, but the effects on chemical and biological reactions may be significant.

Constant Wavelength / Constant Intensity / Varied Time – Periodic Timing

It is well understood that microbial growth during fermentation goes through stages, termed lag phase, logarithmic phase, stationary phase, and death phase. Occasionally "deceleration phase" is included between logarithmic and stationary phases. Each of these phases represent unique cell growth and division periods with unique nutrient needs and availabilities. In a standard alcoholic fermentation, the lag phase represents the only time where aerobic fermentation may take place. This is also the period where a newly inoculated yeast culture acclimates to the new fermentation medium. By probing the timing of light in terms of when and how long light is applied, we may identify some aspects of the pathway that light affects fermentation. In terms of industry application, focusing the application of light to a critical timeframe results in less cost and possibly great efficacy.

Discrete Block of Illumination

The first trial of illumination timing focused on the "when" portion of light application. As far as the fermentation stages of yeast are concerned, it is not known when a certain stage has been reached until the fermentation is already there. Instead of trying to match illumination to fermentation stage, light application was determined by the number of desired segments and the length of duration. Initial tests showed that the fermentations reached stationary phase

approximately 60 hours post-inoculation. Based on the number of digital controls for turning the lights on/off and stationary phase, 8 timing blocks of 9 hours, were used, along with constant illumination and a dark control. Although the exact timing of the phases was not measured, it is expected that this included a sample completely in the lag phase and a sample completely in the stationary phase.

Constant illumination still produced the most ethanol and was statistically significant compared to the dark control, but an illumination period in late lag phase/early log phase produced nearly the same amount of ethanol (Figure 15, Figure 16, Table 11). Light at the very beginning of fermentation negatively affected the production of ethanol. The combination of near-UV light and molecular oxygen at the start of fermentation may have had a deleterious oxidative effect on microbial cells. This is also the stage where the is the lowest number of cells and the solution is most clear, so the light available to each unique cell would be considerably higher than in latter stages.

Duration of Illumination

Noting that the individual blocks of illumination may not represent each stage of fermentation and considering that illumination seemed to have an increasing effect at least up to 18 hours post-inoculation, experiments in light duration were performed. In this experiment, all fermentations were illuminated from the beginning, with illumination terminating after a set amount of time. Considering a 6-day time to completion for the fermentation, including constant illumination and a dark control, 11 timing blocks were chosen, each increasing by 14 hours of illumination (Table 10).

Illumination in all instances produced more ethanol than the dark controls, but of note is that the fermentation illuminated for only the first 42 hours post-inoculation produced more

ethanol than even the constant illumination samples. This roughly corresponds to the lag and log phases of fermentation, approaching the deceleration/stationary phases. Continued illumination still produced more ethanol than the dark control, but less than the constant illumination control.

These two experiments taken together indicate a path toward maximal ethanol production and some insight as to the mechanism of light enhancement. Decreased ethanol production at the very beginning of fermentation may be the result of oxidative stresses. Those are removed due to depletion by aerobic fermentation. The light driving increased ethanol production in late lag phase and early to mid-log phase indicates it is related to cell metabolism, specifically nutrient uptake, and cell division. Continued illumination decreasing maximal ethanol production indicates that the overall scheme of illumination may be seen as a stressor to yeast metabolism, with careful application of light balancing between driving ethanol production and driving cell defense or repair. To be clear, the same amount of nutrients and carbohydrates were available to all fermentations. The inoculated and mixed culture was divided into the different treatments and all treatments fermented to completion. Therefore, the total allocation of carbohydrate to metabolic function is what changed.

For this dissertation it is assumed that the most efficient fermentation would lead to the highest alcohol level and cell challenges and stressors will lead to decreased levels of ethanol. As noted in the literature, illumination tends to make the cell wall of yeast “leaky”, allowing for easier uptake of nutrients. A weakened membrane in otherwise healthy and actively dividing yeast, in the presence of abundant nutrients, may be what allows increased fermentation efficiency. Continued illumination past log phase and into the stationary phase may shift the metabolism away from ethanol production to cell maintenance and repair, decreasing overall ethanol production. It may even be possible that under lowered fermentation rates toward the end

of fermentation diffusion of oxygen back to the fermenter may cause a diauxic shift in metabolism.

Constant Wavelength / Constant Intensity / Varied Time – RTS

To determine what type of interaction or reaction might be driving the metabolism response, discrete timing of light was used. The light timing scheme used was based on condensing Figure 3 which demonstrates reaction rates of chemical and biological species.

Light Duration	**Chemical/Biological Reaction**
1 Millisecond	Fluorophore Excited States, Radicals, Enzymatic Reactions
10 Milliseconds	Radicals, Enzymatic Reactions (fast)
100 Milliseconds	Radicals, Enzymatic Reactions (average)
1 Second	Enzymatic Reactions (slow)
1 Minute	Enzymatic Reactions (complex), Protein Turnover
1 Hour	Protein Turnover, *Saccharomyces* Generation Time (extreme)
2 Hours	*Saccharomyces* Generation Time (average)
12 Hours	Circadian timing

Table 17. Timescales of select chemical and biological reactions.

The illumination was performed by pulsing the LEDs for the time listed (e.g. 1 minute on / 1 minute off, 2 hours on / 2 hours off) with the idea being that a specific reaction being the major contributor to ethanol production would require light for a certain amount of time. Shorter durations would not support or drive the reactions, longer exposure might saturate the reaction/fluorophore and lead to a decrease in overall ethanol production.

The data present in Figure 19 demonstrate that this date is “noisy”. There is a scatter of ethanol production levels both above and below dark controls, even within the same replicate

treatments. The two that did not follow this pattern were constant illumination and 2-hour illumination. They had very consistent responses to illumination across replicates. This demonstrates that illumination may be impacting many facets of yeast metabolism, and that flashing of light maybe deleterious to yeast health. Regardless of timing, all these treatments (save for constant on/off) received the same *quantity* of light and spent the same amount of time illuminated throughout the total fermentation (50%) but had significantly varied responses. Constantly illuminated ferments produced more ethanol than dark control, but fermentations following yeast generation time (2 hours) produced more, and fermentations under 12-hour illumination produced significantly more (Figure 20) along with 100 ms timing ferments. These last two produced more scattered results, but also produced the most ethanol of any treatment tried.

The scatter of the results tied to 100 ms light timing trial may implicate a protein-based mechanism for light interaction with the yeast. All known biological circadian clock mechanisms are initiated and controlled by proteins. The scatter of results in the 12-hour light timing trial may just reflect the 100 ms light timing trial, the circadian rhythm controlled by proteins. The scatter of results in different trials related to the yeast in the different trials not being exactly at the same stages of fermentation. This contrasts with the 2-hour trial data. If the light is acting a timing signal as mentioned above, tying the signal to roughly the generation time of yeast would put all the microbes in sync. Continued signaling at the generation time frame would keep all cellular processes in sync between generations as well. Within the 12-hour timing scheme there is enough time for 3 new generations of yeast. The newest members may not be maximally efficient at fermentation, and the oldest members may be at the end of their life.

Constant Intensity / Varied Wavelength – Apple Juice

Light conclusively influences fermenting yeast. Ethanol as a marker for fermentation efficiency shows that Blue-Near UV light can increase the amount of ethanol produced by yeast in a neutral synthetic media. Apple juice was selected as a commodity media for fermentation due to its relatively low absorbance and relatively simpler chemical profile and phenol content compared to grape juice. Apple juice samples were prepared the same as synthetic media samples and fermented under different wavelength LEDs. In contrast to the synthetic media, illumination by wavelengths from 400 nm to 733 nm had no statistically significant effect on ethanol production (Figure 21, Table 16). As samples were being prepared for analysis post-fermentation, it was noted that 470 nm illuminated fermentations had a strong odor. An informal sensory panel confirmed this finding, and a separate research project was undertaken to look at sensory impacts of light on apple juice fermentations. The samples fermented under blue/UV wavelengths (375 nm, 400 nm, and 470 nm) in this work and the 400 nm and 470 nm illuminated fermentations in the apple sensory project had significantly decreased color post fermentation.

The most striking finding, though, was that 375 nm fermentations produced very little alcohol, less than 1/3rd of the control samples (Table 16). In contrast to synthetic media, the 400 nm illuminated fermentations produced slightly less than the dark control, instead of the UV/near UV fermentations producing the maximal amount. The decrease in solution color post-fermentation is indicative of photobleaching of the apple juice phenolics. These two pieces of data are evidence that apple phenolics may act as photoreceptors for UV wavelengths and photosensitizers for the apple juice solution. Passing the absorbed energy from the phenolics to the microbes inhibited ethanol production to an extreme extent.

Chapter 5 – Conclusion and Future Work

Previous work has clearly demonstrated that light can influence fermentation by *Saccharomyces* sp. yeast. This work adds to that body of knowledge by showing that parameters of color, timing, and intensity independently and communally all have effects on the fermentation in terms of ethanol production, and in certain circumstances, net growth.

Blue to UV wavelengths of light, which have the broadest absorbance species and most intense absorbance spectrum have the strongest influence on the fermentations, but longer wavelengths should be considered in future investigations. For clear fermentation media or low phenolic fermentation media, UV illuminated fermentations under yeast generational timing or circadian timing will produce the most ethanol. In a choice between these two timing schemes, one has to balance the most ethanol produced (12 hour circadian timing) versus consistency (2 hour generational timing).

The sensory impacts of illuminated fermentations were noted immediately and found to be highly variable. Varied ethanol and ester production, along with complex flavor manipulation lead to an entirely separate research project, with sensory panels noting that illumination and color do effect fermentation outcomes and consumer preference.

Although no timing mechanism has ever been identified in *Saccharomyces*, light timing effects have been noted in previous publications and in this work. The effect of different timing schemes, wavelengths, and fermentation outcomes from this work imply a chromophore-based mechanism for development of a circadian pattern in yeast. The tight control of ethanol production seen under 2-hour illumination mirrored in the 12-hour timing scheme, but with higher variation seem to indicate that this timing control is related to DNA repair and replication

mechanisms. These mechanisms are protein-based with known photoreceptors that are evolutionarily linked to circadian clock proteins in higher organisms.

This research has led to several possible future scenarios. The most important of which is that there is no one specific illumination scheme (color/time/intensity) that fits all fermentation matrices. This will have to be determined experimentally for each solution and desired outcome. If that outcome is decreased microbial activity, there may be a pathway using light and naturally occurring phenolics to impart control.

For future work on fermentation, and even lighting in general, the type of dimming used should be seriously considered. Pulse-width modulation of high intensity LEDs instead of true dimming by current limiting devices (resistors, variacs) are not equal despite what industrial light meters report.

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