ASSESMENT OF THE EFFICIENCY OF UV LIGHT EMITTING DIODES (UV-LEDS) FOR FRUIT JUICE PASTEURIZATION

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ABSTRACT

ASSESMENT OF THE EFFICIENCY OF UV LIGHT EMITTING DIODES (UV-LEDS) FOR FRUIT JUICE PASTEURIZATION

The objectives of this thesis are to evaluate the application of ultraviolet light emitting diodes (UV-LEDs) with various wavelengths for pasteurization of cloudy (CAJ) and clear apple juices (AJ), to assess the disinfection efficiency of combined UV-LED irradiation and mild heat (UV-LED+MH) treatments, to investigate the effect of individual and combined processes on the activity of enzyme, physicochemical and microbiological quality of AJ, to determine the shelf life of UV-LED irradiated juice during storage, to reveal the reactivation potential of target microorganism (*E. coli* K12) in juice medium with different optical properties, and to elucidate the mechanism of microbial inactivation by UV-LEDs.

280/365 nm UV-LED irradiation assisted by mild heat at 55ºC and 15 min exposure time enhanced the inactivation of *E. coli* K12 and polyphenoloxidase and satisfied the 5-log reduction pasteurization criterion in apple juice. UV-LED+MH treatment extended the shelf life of CAJ from 3 to 30 days and increased its total phenolic content. However, the color properties were slightly affected by ultraviolet treatment.

Subsequent photoreactivation of *E. coli* K12 after inactivation by UV-LEDs was observed in both CAJ and AJ. Contrarily, dark repair was repressed at 4ºC and 22ºC, furthermore, a decrease in the survival ratio was recorded in both medium.

It was revealed that the damage of DNA repair enzymes and the proteins that form the outer cellular membrane of bacteria was highly induced by UV-C light at 280 nm. Additionally, the bactericidal effect of 365 nm (UV-A) wavelength was attributed to enhanced production of reactive oxygen species (ROS) resulting in oxidative damage to cellular lipids, proteins and DNA. The combination of these two wavelengths provided more efficient disinfection than that of UV-C light used alone.

UV-LED irradiation assisted by mild heat has a potential to be used as an alternative technique to traditional thermal pasteurization process for juice products. This study leads to useful information for the future design of UV-LED treatment systems.

ÖZET

MEYVE SUYU PASTÖRİZASYONU İÇİN UV IŞIK YAYAN DİYOTLARIN ETKİNLİĞİNİN (UV-LED) DEĞERLENDİRİLMESİ

Bu tez, farklı dalga boylarında UV ışık yayan diyotların (UV-LEDs) ve bu dalga boylarının hafif ılımlı ısı uygulaması ile kombinasyonunun meyve suyu pastörizasyonunda uygulanabilirliğinin belirlenmesini, UV-LED ve hafif ılımlı ısıl işlemlerinin ayrı ayrı ve kombineli kullanımının meyve suyunun enzim aktivesi, fizikokimyasal ve mikrobiyal kalitesi üzerine olan etkisinin araştırılmasını, farklı optik özellikteki meyve sularına kontamine edilmiş hedef mikroorganizmanın reaktivasyon potansiyelinin incelenmesini ve UV-LED uygulamasının neden olduğunu mikrobiyal inaktivasyon mekanizmalarının aydınlatılmasını kapsamaktadır.

15 dakika boyunca 55°C'de hafif ısı ile desteklenen 280/365 nm UV-LED ışınlama uygulaması, *E. coli* K12 ve PPO enziminin inaktivasyonunu arttırmış olup ve elma suyu için 5D pastörizasyon kriterlerini karşılamıştır. UV-LED+MH uygulaması bulanık elma suyunun raf ömrünü 3 günden 30 güne uzatmış olup örneklerin toplam fenolik içeriğini arttırırken, optik özelliklerini olumsuz yönde etkilemiştir.

Hem bulanık hem de berrak elma suyu örneklerinde bulunan *E. coli* K12 hücrelerinde UV-LED (280/365 nm) uygulamasından sonra düşük seviyede fotoreaktivasyon gözlenmiştir. Ancak, hücrelerin karanlık onarım mekanizması 4ºC ve 22ºC'deki inkubasyonlarda çalışmamıştır.

DNA onarım enzimlerinin ve bakterilerin dış hücresel zarını oluşturan proteinlerin hasarının 280 nm'de UV-C ışığına yüksek oranda duyarlı olduğu ortaya çıkmıştır. Ayrıca, 365 nm (UV-A) dalgaboyunun biyolojik etkileri, reaktif oksijen türlerinin (ROS) üretimini arttırmış olup, bu ürünlerin hücre içindeki lipid, protein ve DNA üzerinde oksidatif hasara neden olmuştur. Bu iki dalga boyunun kombinasyonu, tek başına kullanılan UV-C ışığından daha verimli bir dezenfeksiyon sağlamıştır.

Ilımlı ısıl işlem uygulaması ile desteklenen UV-LED ışınlama, meyve suyu pastörizasyonu için kullanılan geleneksel termal pastörizasyon işlemine alternatif bir teknik olarak kullanılma potansiyeline sahiptir. Bu çalışma, gelecekte tasarlanacak olan UV-LED sistemlerini için yararlı bilgiler sağlayacaktadır.

This Ph. D. thesis is dedicated to
my dear grand parents
Zeliha and Mehmet YENİCE

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CHAPTER 1

INTRODUCTION

As an alternative to consumption of fruits, drinking fruit juices is easy to consume especially for very young, elderly and infirm people. It is known that consumption of fruit juices decreases the risk of chronic diseases, retards Alzheimer disease onset, slows down LDL oxidation, inhibits platelet aggregation, and prevents the development and progression of coronary artery diseases due to their antioxidant compounds (Borenstein et al. 2005, Keevil et al. 2000, Stein et al. 1999).

Fruit juices are also susceptible to microbial spoilage though they have acidic pH values. Observation of outbreaks caused by the consumption of unpasteurized fruit juices raised a question about the safety of acidic juices. The most common microorganisms found in fruit juices are acid-tolerant bacteria, yeasts and molds. However, *Salmonella* and *Escherichia coli* 0157:H7 outbreaks indicated the potential of fruit juices to carry pathogenic microorganisms (Cook et al. 1998). Foley et al. (2002) estimated the number of cases of illnesses associated with unpasteurized juices as 16000 to 48000 in a year.

Thermal pasteurization is the best-known technique in order to remove pathogens, reduce the number of spoilage microorganisms and inactivate the enzymes which decrease the quality of the products. However, use of high temperatures may cause some quality problems such as color, taste and flavor defects (Aguilar-Rosas et al. 2007, Choi and Nielsen 2005).

Increased trend towards fresh-like products forced the researchers to investigate alternative processing techniques (Basaran-Akgul et al. 2009, Tahiri et al. 2006). One of the alternative method to thermal pasteurization is UV-C radiation. Antimicrobial effect of UV-C light is very well-known, and this technique is used for disinfection of fruits surfaces, hospital equipment, water resources etc. (Begum, Hocking, and Miskelly 2009, Bintsis et al. 2000, Nigro et al. 1998, Pan et al. 2004). Inactivation mechanism of UV-C irradiation is based on the absorption of UV photons by the genetic material and the formation of dimers which inhibit the transcription and replication of the cell (Bolton et al. 2011, Koutchma 2009, Oguma et al. 2013). Despite the benefits of this non-thermal technology for food decontamination, some drawbacks should not be ignored. Following

the UV irradiation, many microorganisms may develop a mechanism to repair damaging effects of irradiation on their DNA structure (Zimmer, Slawson, and microbiology 2002). There are two main mechanisms to repair and cause formation of pyrimidine dimers on DNA: photoreactivation and dark repair (nucleotide excision) (Salcedo et al. 2007). Photoreactivation which is the simplest and oldest repair mechanism is carried out by a single enzyme i.e., photolyase. This enzyme reverses the damages by specifically binding to cyclobutene pyrimidine dimers (CPD) or 6–4 photoproducts via using the energy of light (Sinha, Häder, 2002). Unlike photoreactivation, dark repair is a multistep mechanism and light independent process. Two different dark repair mechanisms take place in microorganisms that are corresponding not to directly reversing the DNA damage but instead replacing the damaged DNA with new, undamaged nucleotides: base excision repair and nucleotide excision repair (Rastogi et al. 2010, Sinha, Häder, and Sciences 2002). Previous studies reported photoreactivation and dark repair mechanism including the effect of temperature, type of UV light, and exposure dose on the reactivation mechanisms of microorganisms. Photoreactivation and dark repair in pathogenic bacteria at different incubation temperatures following UV light treatment employing low pressure mercury lamps and medium pressure mercury lamps have been well studied in different medium e.g., water, apple juice, bovine milk etc. (Jungfer, Schwartz, and Obst 2007, Quek and Hu. 2008, Yin et al. 2015a, b, Zhou et al. 2017).

While the inactivation of natural occurring and inoculated microorganisms in fruit juices is well investigated, little is known about the effect of UV treatment on enzymes in fruit juices. Polyphenol oxidase (PPO) is a copper containing enzyme, which occurs in many fruits (Falguera et al. 2013). The enzyme catalyzes the oxidation of various phenolic substrates, whose polymerization leads to the formation of undesirable brown pigments and therefore has to be inactivated by processing to enhance the shelf life of the juice (Falguera et al. 2012).

Moreover, previous limited and controversial studies have been showed that UV-C light is inadequate on inactivation of certain enzymes associated with fresh juices including polyphenoloxidase (PPO), peroxidase (POD), lipoxygenase (LOX), and pectin methylesterase (PME) (Gayan et al. 2013, Müller et al. 2014, Noci et al. 2008, Orlowska et al. 2014). PPO is responsible for enzymatic browning which adversely affects the color of juice. In general, the inactivation of enzymes which depend on UV dose, wavelength, and juice composition. is successfully achieved after exposure to light emitted in the range of 250-740 nm (Falguera et al. 2012).

Generally low or medium pressure mercury vapor lamps are used for UV-C irradiation, first one emitting predominantly monochromatic UV irradiation at 254 nm and second ones emitting polychromatic UV irradiation in the wavelengths range from 200 to 400 nm. However, these lamps contain mercury which is known to have toxic effect for both environment and human body (Mori et al. 2007). Additionally, UV reactors are required to be designed according to the shape of the lamps. These lamps are mostly large in size and take up too much space (Chevremont, Farnet, Sergent, et al. 2012). Moreover, UV lamps are not resistant to shock and have a low life span (approximately 4,000-10,000 hours). Therefore, it is necessary to design new sterilization equipment in various sizes which do not contain toxic substances and have low energy consumption rate (Crawford et al. 2005, Hamamoto et al. 2007).

In that regard, the UV disinfection using LEDs (Light Emitting Diodes) seems a promising technology. UV-LEDs are created by connecting p-type and n-type semiconductors that move electrons into positively charged holes between these two materials. Light is generated when the electrons and holes recombine at a junction. The wavelength of light depends on the type of material that is used for those semiconductors (i.e. indium gallium nitride for light in the visible range, and aluminum gallium nitride and aluminum nitride for UV range) (Bowker et al. 2011). They have a very compact, shock-resistant and robust design. They also can be used for sterilization of narrow spaces and allow saving space due to their small sizes. UV-LEDs do not need a warm-up time in contrast to traditional UV-C lamps. Hence, they consume less energy. It was also reported that UV-LEDs have relatively longer life time exceeding 100,000 hours. Most importantly, they do not contain any toxic substances which are harmful for human health and the environment. They are capable of emitting UV light at multiple individual wavelengths. Besides, it is possible to use the combination of different UV-LEDs emitting light at different wavelengths.

There are limited numbers of studies related to the use of UV-LEDs for water disinfection. Chatterley and Linden (2010) reported that most of those data were available for LEDs emitting light at UV-A range. However, UV-C LEDs were also indicated to be preferred for this purpose (Bowker et al. 2011, Chevremont, Farnet, Coulomb, et al. 2012, Chevremont, Farnet, Sergent, et al. 2012, Hamamoto et al. 2007, Li et al. 2010, Würtele et al. 2011). Moreover, combination of UV-A and UV-C LEDs was used in some studies (Aoyagi et al. 2011, Chevremont, Farnet, Coulomb, et al. 2012). Bowker et al. (2011) indicated that emitted UV light at 275 nm resulted in much higher microbial inactivation.

This is due to the fact that protein absorption spectrum reaches the maximum near 280 nm and thus enzymes become more sensitive to inactivation at these wavelengths. Moreover, at a wavelength range of 200 to 280 nm (UV-C) and 280 to 315 nm (UV-B), it has a damaging effect on DNA replication and transcription. Direct exposure to UV-C or UV-B results in pyrimidine dimers, pyrimidine hydrates, or cross-links between proteins and DNA. Hence, it is capable of inactivating a variety of pathogens such as bacteria, viruses, fungi, protozoa, and other pathogenic organisms (Liu et al. 2015). Furthermore, it is known that UV-A radiation mechanism is based on the inactivation of microorganisms by damaging proteins and producing hydroxyl and oxygen radicals which destroy cell membrane and other cellular components (Chevremont, Farnet, Coulomb, et al. 2012). Although DNA damage caused by UV-C radiation can be repaired by the enzyme photolyase, there is no possibility to repair the damage to bacterial membranes by UV-A radiation. Chevremont, Farnet, Coulomb, et al. (2012) showed that coupling UV-A and UV-C could be paired by using the germicidal effect of UV-C and greater penetrating ability of UV-A. They also found that use of coupled wavelengths 280/365 nm and 280/405 nm caused total disappearance of fecal enterococci, total coliforms and fecal coliforms in the effluent. Besides lack of possibility to repair the damage in the bacterial membranes occurred after UV-A exposure increased the efficiency of microbial inactivation.

Therefore, the main objective of this Ph. D. thesis was to investigate the assessment of applicability of UV-LED irradiation for pasteurization of fresh fruit juices with respect to microbial safety, enzymatic activity and product quality. Second aim of the study was to determine the effect of different wavelengths and their combinations (UV-A and UV-C) on the mechanism of microbial inactivation by elucidation of the formation of DNA breakages, protein and lipid oxidation, ROS generation in cell.

CHAPTER 2

LITERATURE REVIEW

2.1. Fruit Juice

Fruit juices are described as unfermented but fermentable products obtained from fresh, ripe and healthy fruits (Republic of Turkey Ministry of Agriculture and Rural Affairs General Directorate of Protection and Control, 2006).

2.1.1. Value of Fruit Juices

Popularity of fruit juices is being raised due to their nutritional value and various health benefits. It was declared that consumption of fruit juices in Turkey increased by 90% between years 1994 and 2004. The consumption rate was reported to be 6.4 L/person in 2006 (Republic of Turkey Under secretariat of the Prime Ministry for Foreign Trade Export Promotion Center, 2007). However, it reached to 12 L/person for the year 2010 (Akdağ, 2011).

As an alternative to consumption of fruits, drinking fruit juices is easy especially for very young, elderly and infirm people. It is known that consumption of fruit juices decreases the risk of chronic diseases, retards Alzheimer disease onset, slows down LDL oxidation, inhibits platelet aggregation, and prevents the development and progression of coronary artery diseases due to their antioxidant compounds (Stein et al. 1999, Borenstein et al. 2005, Keevil et al. 2000). Also, processing of liquid foods is simpler than that of solid products. Thus, all safety and quality requirements can be readily satisfied (Hakguder 2009).

2.1.2. Microbial Safety and Quality of Fruit Juices

Fruit juice quality and safety highly depend on the raw material, processing conditions, packaging material and storage conditions. These factors may cause microbiological, enzymatic, physical and chemical alterations (Souza et al. 2004).

The main groups of enzymes found in fruits and vegetables are peroxidases (POD), pectinases, especially pectinmethylesterases (PME), polyphenoloxidases (PPO) and lipoxygenases (Cemeroğlu 2004). PME and PPO affect colour and viscosity of fruit juices (Zhang et al. 2011). PME enzyme cannot be completely inactivated by pasteurization due to its heat resistant fraction (Kim and Tadini, 1999). On the other hand, peroxidases are the most heat resistant enzymes which cause color changes and flavor defects (Cemeroğlu 2004).

Fruit juices are also susceptible to microbial spoilage though they have acidic pH values. Observation of outbreaks caused by the consumption of unpasteurized fruit juices eliminated the concept of acidic juices are safe. The most common microorganisms found in fruit juices are acid-tolerant bacteria, yeasts and molds. However, recent *Salmonella* and *Escherichia coli* 0157:H7 outbreaks indicated the potential of fruit juices to carry pathogenic microorganisms (Cook et al. 1998). Foley et al. (2002) estimated the number of cases of illnesses associated with unpasteurized juices as 16000 to 48000 in a year.

As a result of these outbreaks related to juice products Food and Drug Administration (FDA) proposed new regulations for the pasteurization of fruit juices (FDA 1998). According to this, a 5-log reduction in the most pertinent microorganism must be attained by processing (FDA 1998). Currently, *Salmonella* is generally accepted as the pertinent pathogen in citrus juices, whereas *Escherichia coli* O157:H7 as well as *Cryptosporidium parvum* are both considered pertinent for apple juice.

2.2. Preservation Methods

Preservation methods can be classified into two main groups as thermal and nonthermal techniques.

2.2.1. Thermal Pasteurization

Thermal pasteurization is the best-known technique in order to remove pathogens, reduce the number of spoilage microorganisms and inactivate the enzymes which decrease the quality of the products (Aguliar-Rosas et al. 2007). Pasteurization describes a mild heat treatment which is applied at temperatures below 100°C (Silva and Gibbs 2004). Thermal pasteurization criteria for fruit juices change between 90-95°C for 15-60

seconds (Cemeroğlu 2004). However, application of high temperatures may cause some quality deteriorations, such as colour and flavour defects, in addition to the degradation of nutritional value of the products (Aguilar-Rosas et al. 2007, Choi and Nielsen 2004).

2.2.2. Nonthermal Methods

Consumers' increased trend towards fresh-like juices and recent outbreaks related to the consumption of unpasteurized products forced the researchers to develop alternative non-thermal processing techniques which can maintain the quality and safety of the juice products (Tahiri et al. 2006, Basaran-Akgul et al. 2009).

Some of the non-thermal processing techniques are; UV irradiation and ionizing irradiation, addition of antimicrobial agents, pulsed electric field (PEF) and high hydrostatic pressure (HHP) applications (Bates et al. 2001).

Although addition of antimicrobial agents provides desired microbial reduction, the main disadvantages of this technique are the requirement of longer application time and loss of ascorbic acid (Opstal et al. 2006, Basaran-Akgul et al. 2009). Besides, antimicrobial agents have no effect on the inactivation of quality degrading enzymes.

On the other hand, PEF technique is based on the use of high electric field pulses (El-Hag et al. 2006). It is indicated that PEF system caused lighter colour in processed orange juice during storage. The inactivation of natural flora esp. yeasts is not effective by PEF technique (Min et al. 2003, El-Hag et al. 2006, Marselles-Fontanet et al. 2007). Moreover, industrial application of this system is reported to be problematic from the economical point of view (Tahiri et al. 2006).

Use of high pressures, such as 100-1000 Mpa, is the main concept of HHP method (Morris et al. 2007). It is shown that HHP application causes browning, increase in pH value and temperature. The temperature is increased as 3°C for every 100 Mpa in white grape juice. However, it is less efficient in the inactivation of spores and high pressures are required for the inactivation of vegetative cells (Daoudi et al. 2002, Tahiri et al. 2006, Morris et al. 2007).

On the other hand, undesirable sensorial quality changes are observed in the products treated by ionizing irradiation. For example, orange juice samples after ionizing irradiation are reported to become inappropriate for consumption (Foley et al. 2002).

2.3. UV Light

UV-C light irradiation is another nonthermal processing method which has been used to disinfect water for many years. It is reported to be effective on the inactivation of bacteria, viruses, protozoa and algea (Begum et al. 2009). FDA has approved the use of UV-C light as a germicidal agent for the disinfection of fruit juices (FDA 2000).

2.3.1. Description of UV Light

Ultraviolet light is a part of electromagnetic spectrum ranges between 100-400 nm (Miller et al. 1999, Bintsis et al. 2000, Begum et al. 2009).

The spectrum can be divided into four groups based on wavelengths (Fig. 2.1). The subgroups are;

- UV-A (320 to 400 nm)
- UV-B (280 to 315 nm)
- UV-C (200 to 280 nm)
- UV-V (100 to 200 nm)

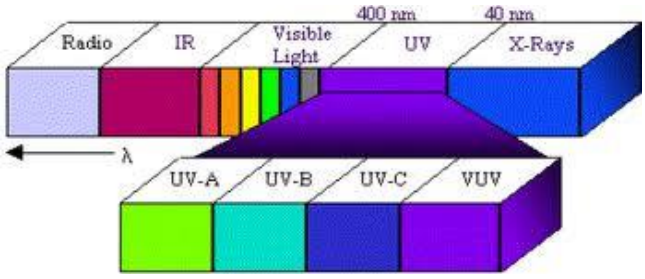


Figure 2.1. Electromagnetic spectrum
(Source: Aqualight, 2018)

The highest germicidal effect of UV light is reported to be observed between 250 and 270 nm (UV-C region) (Tran and Farid 2004, Guerrero-Beltran and Canovas 2005). Koutchma (2009) and Oteiza et al. (2010) declared that the most efficient inactivation can be obtained at 253.7 nm due to the maximum absorption of UV photons by the genetic material at this specific wavelength.

Low or medium pressure mercury vapor lamps are used for many years in UV light treatment (Koutchma 2009).

2.3.2. Antimicrobial Mechanism of UV Light

Bacteria are very sensitive to UV irradiation, because of their small size, short generation time and inadequate UV-protective pigmentation. Bacterias' UV sensitivity depends on wavelength. UV radiation at different regions (UV-A, UV-B and UV-C) is absorbed by different biomolecules such as DNA, proteins and lipids.

Biological inactivation mechanism of UV-A is based on damaging lipids, proteins and DNA via the photooxidation of O_2. It increases the production of reactive oxygen species (e.g. H_2O_2, $O^{2'}$ and OH) and causes breakage of single-strand in DNA (Santos et. 2013). UV-C irradiation is directly effective on DNA or RNA pyrimidine bases and cause formation of cyclobutane thymine dimers without any intermediate steps (Chatterley and Linden 2010). Incident light causes the formation of dimers on the same DNA strand which inhibits the transcription and replication of the cell (Figure 2.2). Although DNA damage caused by UV-C radiation can be repaired by the enzyme photolyase, there is no possibility to repair the damage to bacterial membranes by UV-A radiation (Bolton et al. 2003, Oguma et al. 2002, Donahue et al. 2004).

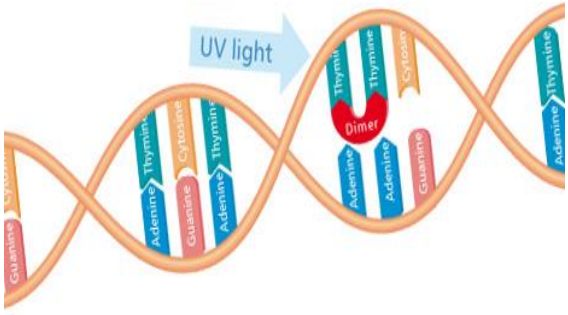


Figure 2.2. Effect of UV-C light on DNA.

Exposure to UV-B causes not only direct DNA damage by inducing the formation of DNA lesion, but also oxidative stress on lipid and proteins of microorganisms (Santos et al. 2013).

2.3.3. Applications of UV Light

UV-C light treatment has been used to disinfect water effluent, potable water and water for swimming pools for many years (Begum et al. 2009). It is also used for surface disinfection of foods, decontamination in hospitals, pharmaceutical industry and public buildings (Begum et al. 2009, Pan et al. 2004, Nigro et al. 1998).

There are also many applications of UV-C irradiation on different fruit juices (Torkamani and Niakousari, 2011, Gabriel, 2012, Azhuvalappil et al. 2010, Koutchma et al. 2004, Choudhary and Bandla, 2012). It can also be applied decontamination of the surface of the foods such as ready to eat meats, banquettes, shell eggs, fresh cut fruits and whole fruits (Romo, 2004; Manzocco et al. 2011; Chan et al. 2009). UV-B irradiation has been applied during and after harvesting of the plants. It creates abiotic stress on fruit and vegetables allowing to increase the levels of secondary metabolites such as phenolic acids and flavonoids content. It is observed that treatment of UV-B irradiation increases the antioxidant capacity and induced the production of phenolic content of tomato, fresh cut carrot, black currant fruit (Lui et al. 2011, Huykes et al. 2007, Gonzalvez et al. 2013).

Only a few studies indicated that UV-A treatment can be used for disinfection of fruit juice and water (Lian et al. 2010; Hamomota et al. 2007; Mori et al. 2007). Nowadays, UV-A treatment is usually coupled with UV-B and UV-C light. UV-A and UV-B have effects on enzyme and phenolic content of fruit and vegatables (Aiamla-Or et al. 2009), whereas coupling them with UV-C light treatment can be used in disinfection of wastewater (Chevremont et al. 2012).

2.3.4. Advantages and Disadvantages of UV Light

One of the most important advantages of the UV light processing is the low cost of the operation and ease of use (Bintsis et al. 2000). The equipment needs less space than the ones used in other methods (EPA 1999). Also, UV light is effective against most type of microorganisms (Bintsis et al. 2000). It is a physical treatment which does not cause chemical residues. Hence, it is not harmful to humans or aquatic life (EPA 1999, Canitez 2002).

However, lack of penetration in highly absorptive media or in the case of existence of organic matters, and reduced effectiveness due to the presence of the suspended

particles are the disadvantages of this technique (EPA 1999). Additionally, it does not have a significant effect on the quality degrading enzymes in fruit juice products.

2.4. UV-LED Irradiation

Use of UV light emitting diodes (UV-LEDs) is a relatively new method to generate UV light. Theoretical background, applications and superiority of this new technology over the traditional one will be discussed below.

2.4.1. Theoretical Background

UV-LEDs are created by connecting p-type and n-type semiconductors that move electrons into positively charged holes between these two materials (Fig. 2.3).

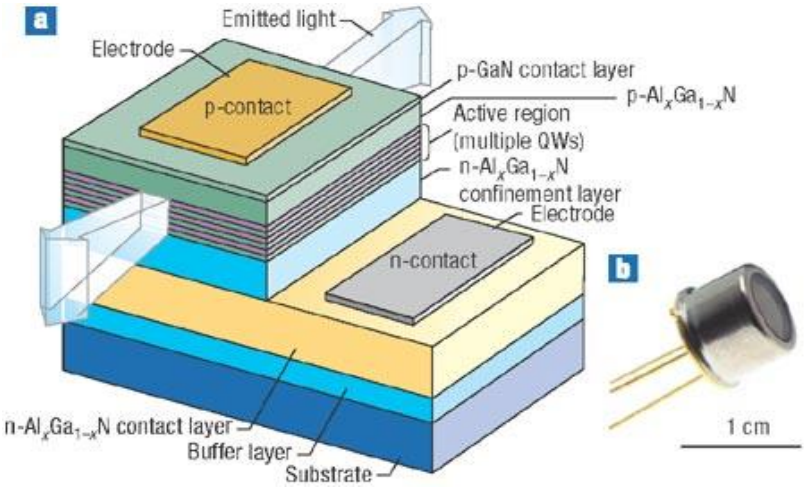


Figure 2.3. A typical UV-LED's structure
(Source: Aqualight, 2018)

Light is generated when the electrons and holes recombined at a junction. The wavelength of the light depends on the type of material that is used for those semiconductors (i.e. indium gallium nitride (GaN) for light in the visible range, and aluminum gallium nitride and aluminum nitride for UV range) (Bowker et al. 2011).

2.4.2. Applications of UV-LED Irradiation

There are many applications of GaN-based blue, green, and white light emitting diodes (LEDs) like backlighting of mobile phones, laptops and TVs, street lighting, and car headlight (Techneau, 2010). UV-LEDs are also used for;

- Water disinfection (UV-C, UV-A)
- Sensing (UV-C, UV-A)
- UV curing (UV-B, UV-A)
- Medical treatment (e.g. treatment of psoriasis, UV-B)
- Tanning (UV-B)
- Lithography (UV-A)
- Non-line-of-sight communication (UV-C)
- Sterilization (e.g. medical, UV-C)

In literature there are limited numbers of studies related to the use of UV-LEDs for water disinfection purpose. Chatterley and Linden (2010) reported that most of those data are available for LEDs emitting light at UV-A range. However, UV-C LEDs are also preferred for this purpose (Li et al. 2008, Chevremont et al. 2012a, Chevremont et al. 2012b, Bowker et al. 2011, Würtele et al. 2011, Hamamoto et al. 2007). Moreover, combination of UV-A and UV-C LEDs was used in some studies (Aoyagi et al. 2011, Chevremont et al. 2012a).

Chevremont et al. (2012a) showed that coupling UV-A and UV-C could be paired by using the germicidal effect of UV-C and greater penetrating ability of UV-A. They also found that use of coupled wavelengths 280/365 nm and 280/405 nm caused total disappearance of fecal enterococci, total coliforms and fecal coliforms in the effluent. Besides lack of possibility to repair the damage in the bacterial membranes occurred after UV-A exposure increased the efficiency of microbial inactivation (Chevremont et al. 2012a).

2.4.3. Comparison of UV-LEDs and Traditional UV Lamps

Table 2.1 shows the main differences between UV-LEDs and traditional UV lamps. According to that the mercury-vapor lamps contain mercury, which is toxic to the environment as well as to the human body. On the other hand, a UV-LED is an

environment-friendly sterilizer. It does not contain any toxic substances, so it does not have harmful effects (Mori et al. 2007). Additionally, traditional UV lamps are fragile, non-durable to shock, less energy efficient and large in size. Sterilizers using UV lamps must be designed according to the shape of the lamps which take up a lot of space. It was also speculated that these lamps have a short life span of approximately 4000-10000 hours (Chevremont et al. 2012a). Hence, the cost of a UV device equipped with mercury lamps were reported to be relatively higher (Hamamoto et al. 2007). Moreover, low pressure mercury lamps are known to emit nearly monochromatic UV light at a wavelength of 254 nm whereas UV-LEDs offer the possibility to use preferred wavelenght (Crawford et al. 2005).

Table 2.1. Differences between UV-LEDs and traditional mercury vapor UV lamps.

UV-LEDs	**Traditional Mercury Vapor UV Lamps**
Do not contain toxic substances	Contain mercury
Robust design	Fragile
Compact size	Take up a lot of space
Longer life time	Short life span
Use different light source emitting UV light at different wavelengths (UV-A, UV-B, UV-C)	Emit monochromatic UV light at 254 nm (UV-C)

In contrast to traditional UV lamps, UV-LEDs were shown to have a robust design, compact size, higher energy efficiency, reduced frequency of replacement due to long life time of nearly 100000 hours since they do not need a warm-up time (Chevremont et al. 2012b, Vilhunen et al. 2010). Furthermore, using UV-LEDs will provide the opportunity to develop small, space-saving sterilization equipment. UV-LED devices can sterilize small and narrow spaces due to their compact size (Mori et al. 2007). Therefore, design of a new sterilization equipment of low energy consumption which is in various shapes and sizes without using harmful substances is possible using UV-LEDs.

Another advantage of UV-LEDs can be the possibility to compose a UV-LED array at different wavelengths in order to enhance the germicidal effect. Such a system will hold for the potential to design sterilization units which target a wide range of

pathogens or the main pathogens specific for the product of concern (Chatterley and Linden 2010).

CHAPTER 3

UV-LED IRRADIATION OF APPLE JUICE

Fruit juice consumption has been rising in the last decades due to their nutritional properties (Bates et al., 2001). Fruit juice consumption has been rising in the last decades because of their nutritional properties (Bates et al. 2001). Increased trend towards fresh-like products forced the researchers to investigate alternative processing techniques (Basaran-Akgul et al. 2009; Tahiri et al. 2006). One of the alternative methods to thermal pasteurization is UV-C radiation. Antimicrobial effect of UV-C light is very well known, and this technique is used for disinfection of fruit surfaces, hospital equipment, water resources etc. (Begum et al. 2009, Bintsis et al. 2000, Nigro et al. 1998, Pan et al. 2004). Inactivation mechanism of UV-C irradiation is based on the absorption of UV photons by the genetic material and the formation of dimers which inhibit the transcription and replication of the cell (Bolton and Linden 2003, Koutchma 2009, Oguma et al. 2002). On the other hand, many microorganisms can repair damages on their DNA caused by UV-C irradiation by means of two different mechanisms depending on the light availability such as photoreactivation and dark-repair (Chevremont et al. 2012a, Oguma et al. 2002).

Generally low or medium pressure mercury vapor lamps are used for UV-C irradiation, first one emitting predominantly monochromatic UV irradiation at 254 nm and second one emitting polychromatic UV irradiation in the wavelengths range from 200 to 400 nm. However, usage of these lamps has some limitation. For example, these lamps contain mercury which is known to have toxic effect for both environment and human body (Mori et al. 2007). The UV systems are also mostly large in size and take up too much space (Chevremont et al. 2012a). Therefore, it is necessary to design new disinfection equipment in various sizes which do not contain toxic substances and have low energy consumption rate (Crawford et al. 2005, Hamamoto et al. 2007).

UV disinfection using Light Emitting Diodes (LEDs) seems a promising technology owing to their compact, shock-resistant and robust design. They can also be used for disinfection of narrow spaces due to their small sizes. UV-LEDs do not need a warm-up time in contrast to traditional UV-C lamps. Hence, they consume less energy. It was also reported that UV-LEDs have relatively longer life time exceeding 100,000 h

(Chevremont et al. 2012a). Most importantly, they do not contain any toxic substances which are harmful for human health and the environment. They are capable of emitting UV light at various wavelengths. Besides, UV-LEDs of different wavelengths can be combined to enhance the disinfection efficiency.

The objectives of this chapter were;

i) to investigate the potential of UV LEDs irradiation for non-thermal processing of clear (AJ) and cloudy apple juices (CAJ),

ii) to study the inactivation efficiency of UV-LEDs on quality reducing enzymes such as PPO in apple juices,

iii) to evaluate the subsequent reactivation potential of target microorganism, i.e. *E. coli* K12, in apple juice after UV-LEDs irradiation.

For this purpose, a UV-LED device was constructed for static tests. UV-LEDs with peak emissions at 254 nm 280 nm and combined emissions at 254/365, 254/405, 280/365, 280/405 and 254/280/365/405 nm were applied to apple juice samples (cloudy and clear). The microbial effectiveness of UV light treatment was tested by inoculating *E. coli* K12 into AJ and CAJ. In addition, the effect of UV-LED irradiations on the activity of polyphenol oxidase (PPO) enzyme in CAJ was investigated. Moreover, the effect of UV-LED irradiations on the photoreactivation and dark repair ability of *E. coli* K12 in cloudy (CAJ) and clear apple juice (AJ) during the 6 h incubation time at ambient (22ºC) and refrigerated (4ºC) temperatures were explored.

3.1. Materials and Methods

3.1.1. Preparation of Apple Juice Samples

For the production of cloudy apple juice (CAJ), apples of the Starking Delicious variety were purchased from a local supermarket (Tesco-Kipa) in Izmir, Turkey. Apple juice was obtained by following steps shown in Figure 3.1. Apples were washed under tap water and patted dry and squeezed using a household table top juice extractor (Arçelik Robolio, İstanbul). After the extraction step, the juice was strained through a sterile gauze strip and centrifuged at 400 rpm for 5 min to remove the big particles and foams in the juice. Then, the cloudy apple juice (CAJ) was obtained and stored in the refrigerator until the processing time.

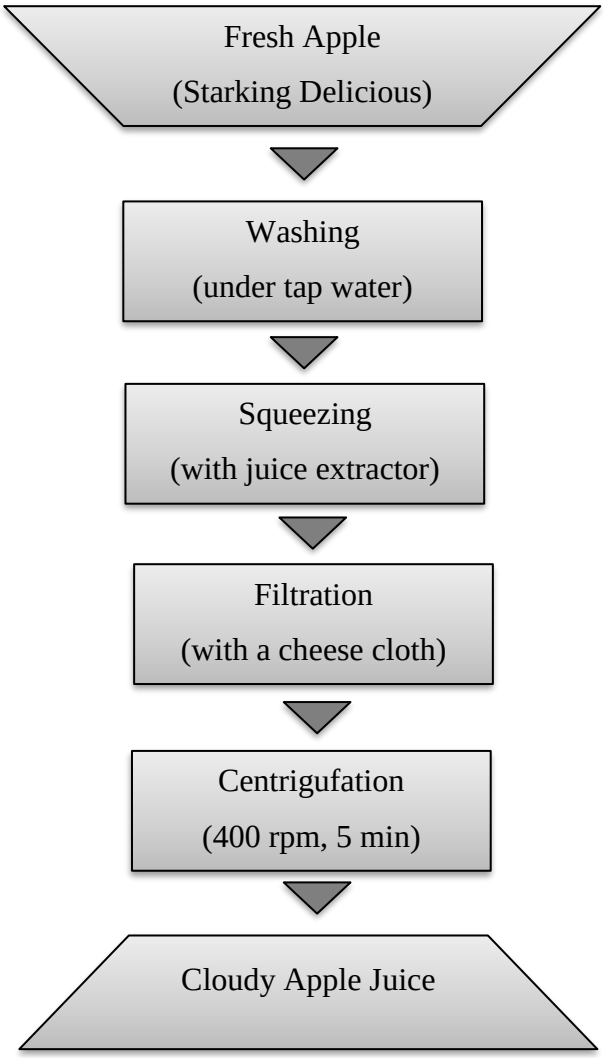


Figure 3.1. Flow diagram of cloudy apple juice production

Pasteurized clear apple juice (AJ) in bottles (Dimes, Kemalpaşa, IZMIR) was purchased from a local market in Izmir, Turkey. AJ does not contain any citric acid and other preservatives. Background flora of pasteurized samples was tested by surface plating on Plate Count Agar (PCA, Sigma-Aldrich, St. Lous, MO, USA) for enumeration of total aerobic bacteria, and yeast and molds were counted by spreading on Potato Dextrose Agar (PDA). Moreover, coliform bacteria were counted on Violet Red Bile Agar (VRBA) by using spreading method prior to UV treatment.

3.1.2. Measurement of Physical, Chemical, and Optical Properties

Prior to UV-LED irradiation some physical, chemical and optical properties including pH, total soluble solid (°Brix), titratable acidity, ascorbic acid content, color, the absorption coefficient of AJ and CAJ samples were measured.

3.1.2.1. pH

The pH values of the samples were carried out by a bench top pH meter (HANNA Instruments, United States) at room temperature (22 ºC). Before the measurement of the pH of the samples, calibration of pH meter was done with using standard pH buffer solutions of pH 7.0 and pH 4, respectively. then the pH probe was immersed in volume of 20 mL sample and the value was recorded.

3.1.2.2. Titratable Acidity

Titratable acidity of the samples was assessed according to the method proposed by Cemeroğlu, Karadeniz, and Özkan (2004). An appropriate amount of sample (10 mL) was titrated against standardized 0.1 N NaOH up to pH 8.1 which is phenolphthalein end point. The acidity of the sample was calculated by the following formula;

$$TA, \% = \frac{(V)(f)(E)\ 100}{M} \tag{3.1}$$

V; represents the volume of 0.1 N NaOH used up during titration (mL),
f; is the normality factor,
E; is the milliequivalent weight of malic acid (g),
M; is the volume of the sample (mL).

3.1.2.3. Determination of Total Soluble Solid Content

Mettler-Toledo RE40D Benchtop Refractometer (AEA Investors Inc., USA) was used to determine the total soluble solids (°Brix) content of the juice samples. A few drops of the sample were put into the reading cell and the results were recorded at 22ºC.

3.1.2.4. Ascorbic Acid Level

Boehringer enzymatic method proposed by Danielczuk et al. (2004) was used for ascorbic acid determination. For this purpose, test kits (R-Biopharm, Roche, Germany, catalog no. 10 409 677 035) were utilized. In this method, the absorbance of MTT-formazan, which is produced by the reaction of reducing substances and tetrazolium salt MTT (3-(4,5-dimethyl thiazolyl-2)-2,5- diphenyltetrazolium bromide) in presence of electron carrier PMS (5-methylphenazinium methosulfate) at pH 3.5, is measured at 578 nm. The absorbance difference between the sample and sample blank, of which ascorbic acid is removed by the enzyme ascorbic acid oxidase, yield the quantity of ascorbic acid in the sample (mg L-ascorbic acid/L sample solution).

3.1.2.5. Color

Color parameters of the samples were measured by Konica Minolta CR 400 Chromometer (Konica Inc. Japan). Measurement were expressed as CIE color parameter such as L* (lightness-darkness), a* (redness-greenness), b* (yellowness-blueness). The three-dimensional color space image of the L*, a* and b* value was shown in Figure 3.2. The value of L* indicates the lightness of the sample that changes from 0 (dark) to 100 (light), a* value represents the color changes from green (-) to red (+), b* value is the color changes from blueness (-) to yellowness (+). The total color difference (ΔE) and browning index (BI) (Palou et al. 1999) of the samples were calculated from the Equations 3.2 and 3.3:

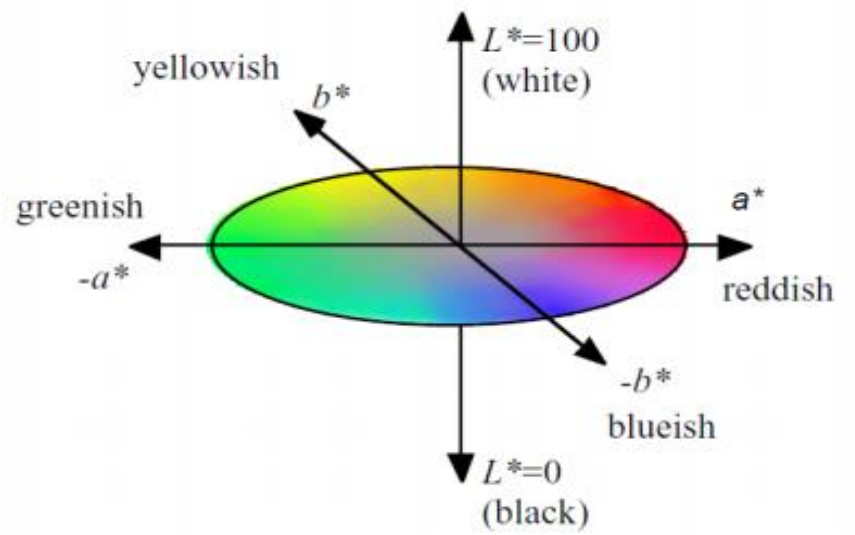


Figure 3.2. CIELAB color space

(Source: Molino et al. 2013)

$$\Delta E = \sqrt{\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2} \tag{3.2}$$

$$BI = 100 * \frac{\left(a^* + 1.75L^*\right)}{\left(5.645L^* + a^* - 3.012b^*\right)} - 0.31 \over 0.172 \tag{3.3}$$

3.1.2.6. Absorption Coefficient

The absorbances of the juice samples were measured by using Cary 100 UV-Visible Spectrophotometer (Varian, USA) at different wavelengths ranging between 250-410 nm. Different dilution factors were applied (1:10, 1:25, 1:50, 1:100, 1:250, 1:500 and 1:1000). Absorption coefficients at each individual wavelength were estimated from the slope of the graph by plotting absorbance values against sample concentrations (Hakgüder 2009).

3.1.2.7. Turbidity

HACH 2100AN IS Turbidimeter was used for measurement of the turbidity of juice samples. Forty-five mL of the sample was placed into the glass cuvette of the

equipment and the turbidity was recorded in terms of Nephelometric Turbidity Unit (NTU) within 2 or 3 min.

3.1.3. Microbiological Analysis

3.1.3.1. UV-LED Irradiation System

Static UV-LED unit was designed by considering the similar systems used in the study of Chevremont, Farnet, Coulomb, et al. (2012). Four UV-LEDs (8.33 mm diameter) (manufactured by SETI Sensor Electronic Technology Inc., Columbia, SC, USA) emitting light at 254, 280, 365 and 405 nm were used throughout experiments. These LEDs could be used together or separately, and the electronic circuit was connected to a customized four channel power supply. Each UV-LED was operated with a constant forward current of 20 mA. The forward voltage of each UV-LED at 20 mA was 6.50, 5.8, 5.50 and 3.80 V for the 254, 280, 365 and 405 nm UV-LEDs, respectively. The optical output power of each UV-LED was 0.20, 0.60, 0.40 and 10 mW for the 254, 280, 365 and 405 nm UV-LEDs, respectively.

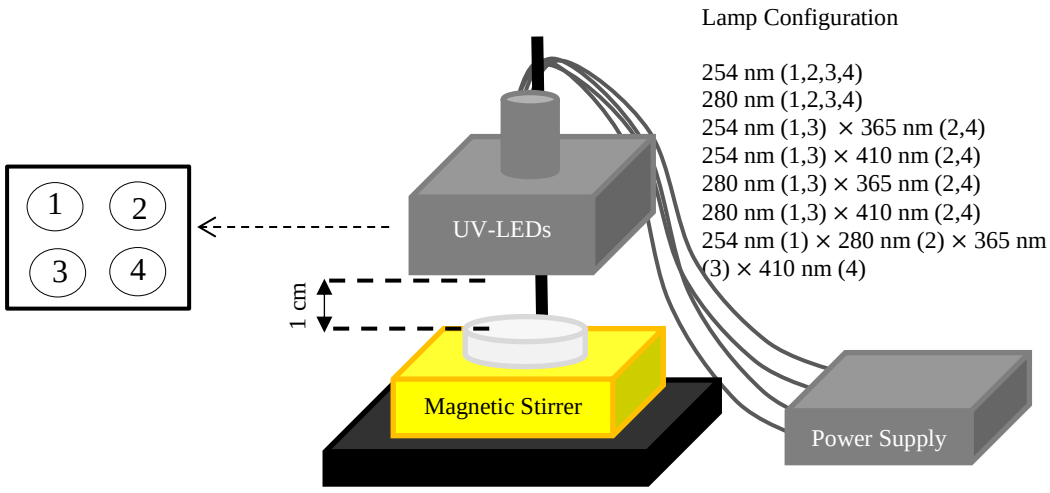


Figure 3.3. Static UV-LED system

A scheme of the experimental device is shown in Figure 3.3. The UVLEDs were fixed facedown, 1 cm above the surface of a sample (3 mL) placed in a sterilized Petri dish (55 mm diameter). The sample depth was 0.15 cm. The depth was calculated from

the ratio of the sample volume and the surface area of a Petri dish. An adequate stirring was applied during treatment in order to ensure equal distribution of UV dose through the sample. The edge effects caused by stirring are avoided by using the smallest possible sample volume (Bolton and Linden, 2003). UV exposure was performed at room temperature (25 °C).

3.1.3.2. UV Intensity and Dose Calculation by Using Ferrioxalate Actinometrical Method

UV dose was calculated from the product of incident UV intensity and exposure time. Incident UV intensity emitted by each LED was measured by ferrioxalate actinometry (Chevremont, Farnet, Sergent, et al. 2012). For the actinometric determination, potassium ferrioxalate was chosen as a chemical actinometer since it was used in wavelength ranges from UV to visible regions (Jankowski et al. 1999). Three millimeters of 12 mM potassium ferrioxalate solution were irradiated with each LED for 10, 20, 30 and 40 min. At the end of each irradiation, 0.5 mL phenanthroline buffer solution (0.1%: 225 g $CH_3COONa{\cdot}3H2O$, 1 g of phenanthroline in 1 L of H_2SO_4 0.5%) was added into the cells and then the mixture was stirred in the dark for 30 min. The ferrous ions produced during the photolysis of potassium ferrioxalate solution formed a red complex with phenanthroline. The absorbance of this complex at 510 nm was measured immediately by a spectrophotometer (Shimadzu Co. Ltd., Japan). The number of Fe^{2+} ions in the solution can then be determined from the following equation:

$$n_{Fe^{2+}} = (N * V_1 * V_3 * DO_{510})/(V_2 * l * \varepsilon * 10^3) \quad (3.4)$$

where N is the Avogadro number, V_1 the volume of the irradiated solution (mL), V_2 the volume of V_1 taken for analysis (mL), V_3 the final volume after dilution of V_2 (mL), DO_{510} the absorbance of the complex measured at 510 nm in the V_3 solution, ε the coefficient of molar extinction of the complex phenanthroline-Fe^{2+} (ε complex, 510 nm=11,100 $mol^{-1}.L.cm^{-1}$) and l the optical path length (cm).

The number of Fe^{2+} ions is used to calculate the incident photon flux (photons.s^{-1}) using the following formula:

$$P_{0,\lambda} = n_{Fe^{2+}}/(\Phi_{AC} * \Delta t) \quad (3.5)$$

where φ_{AC} =1.25 is the quantum yield of the actinometer at the wavelength of 280 and 365 nm and t the irradiation time (s).

The incident photon flux is used to calculate the power emitted by each LED:

$$P_e = (h * c)/\lambda)/P_{0,\lambda} \quad (3.6)$$

where h (J.s) is the Planck's constant, c is the celerity of light ($m.s^{-1}$) and λ the wavelength (m).

The luminous flux emitted is expressed in Watts. Dividing this result by the irradiated surface may allow to determine the incident light intensity (W/cm^2).

Average UV intensity (average fluence rate or irradiation) passed through the juice samples was calculated according to Beer-Lambert law given in Equation 3.7 (Unluturk et al. 2008);

$$I_{avg} = I_0 * (1 - e^{-A_e * L})/(A_e * l) \quad (3.7)$$

I_0 is the incident intensity of the sample surface (mW/cm^2), A_e is the absorbance coefficient of the samples (cm^{-1}) and l is the path length of the samples (cm) in the Equation 3.6. UV dosage (D, mJ/cm^2 or $mW.s.cm^{-1}$) applied to the samples was computed by multiplying average UV intensity of the samples (I_{avg}, mW.cm-^{2}) with UV exposure time (t, s) according to the Equation 3.8;

$$D = I_{avg} * t \quad (3.8)$$

3.1.3.3. Target Microorganism and Growth Conditions

Koutchma et al. (2004) reported that *E. coli* K12 has almost the same UV light sensitivity compared with *E. coli* O157:H7. Therefore, experiments were performed using non-pathogenic strain of *E. coli* K12 (ATCC 25253) as a surrogate of pathogenic *E. coli* O157:H7 in this study.

The *E. coli* K12 (ATCC 25253) strain was cultured from −80 °C lyophilized vials, enriched in a test tube containing nutrient broth (NB, Merck, Darmstadt, Germany) and incubated overnight (17-24 h) at 37 °C to reach the stationary phase. Prior to UV-LEDs irradiation process, *E. coli* K12 cells were adapted to acidic conditions of apple juice. For this purpose, three hundred microliter cells cultivated from the stationary phase was used to inoculate 30 mL pre-pasteurized cloudy apple juice (pH 4). Then the cells were incubated in an orbital shaker (Thermo Electron Corp., Ohio, USA) for 6 h at 200 rpm and 37ºC. Stock cultures were prepared from the acid adapted cells and stored in vials containing 20 % glycerol at -80°C.

100 μL of stock culture was inoculated into a test tube containing 10 mL Nutrient Broth in order to prepare a subculture. Test tubes were incubated for overnight at 37°C to allow the culture to reach a stationary phase. For the inactivation studies, samples were spiked with this stationary phase culture at a concentration of 10^7 CFU/mL.

3.1.3.4. Determination of Thermal Inactivation Parameters of *E. coli* K12 in Cloudy Apple Juice

Fresh apple juice, prepared from Starking Delicious variety of apples, was first pasteurized for removal of natural background microflora. FDA issued a the 5-log pathogen reduction performance standard. The 5-log pathogen reduction performance standard required by the regulation means that juice processors must treat the juice using a process that will achieve at least a 100,000-fold decrease in the number of microorganisms. The 5-log pathogen reduction must be accomplished for the "pertinent microorganism," which is the most resistant microorganism of public health significance that is likely to occur in the juice, e.g., *E. coli* O157:H7. For apple juice at pH values of 4.0 or less, FDA recommends the following thermal processes (FDA, 1998):

- 71.1°C for at least 6 seconds
- 73.8 °C for at least 2.8 seconds
- 76.7 °C for at least 1.3 seconds

Furthermore, 71.7°C for 15 seconds was also indicated to be appropriate. On the other hand, according to Cemeroğlu (2004), thermal treatment of apple juice at 96°C for

15-30 s was sufficient to reduce the pectolytic enzyme activity along with PPO activity and to decrease the microbial load to a safe level. Hence, freshly squeezed apple juice was pasteurized at different time-temperature conditions to achieve a 5-log CFU/ml reduction of *E. coli* K12 (Table 3.1). For this purpose, a 2.9 mL of CAJ was put into the sterile thin glass tube (diameter 0.5 cm) and the tubes placed into a water bath set at desired temperature changing from 70ºC to 90ºC. After the tubes reached to a desired temperature, 100 μL of acid adapted *E. coli* K12 (10^7 CFU/mL) was inoculated and applied heating at various times changing from 3 s to 480 s. *E. coli* K12 cells were counted by surface plating method on Tryptic Soy Agar (TSA) medium containing 0.1% dihydrostreptomycin.

Table 3.1. Time-temperature combinations for thermal pasteurization of apple juice

Time (second)	**70°C**	**80°C**	**90°C**
3			X
10			X
15		X	
20			X
30	X	X	X
45		X	X
60	X	X	
120	X		
180	X		
240	X		
360	X		
480	X		

3.1.3.5. UV-LED Irradiation of Clear (AJ) and Cloudy (CAJ) Apple Juice

Different parameters potentially influence the UV inactivation of *E. coli* K12 in CAJ and AJ. The studied factors were the individual wavelengths and the wavelength combinations including 254, 280, 254/365, 254/405, 280/365, 280/405 and 254/280/365/405 nm; and the exposure time, i.e., 20- and 40-min. Pairs of UV-LED lamps were connected in parallel to the DC power source as depicted in Figure 3.1 (Bowker et al., 2011). The medium depth was fixed at 0.153 cm. Tandon et al. (2003) indicated that the suitable stirring was required to be applied for uniform light distribution. Besides, Bolton and Linden (2003) reported that stirring caused the changing of sample depth from middle to border of petri dish and it affected the absorption of UV light, conversely. Unluturk et al. (2010) proposed the use of minimum amount of sample

and showed that the depth at 0.153 cm of sample provided a homogeneous light distribution. Pre-pasteurized CAJ and commercial AJ samples inoculated with *E. coli* K12 were used as treatment medium for assessment of the microbial efficiency of UV-LEDs.

The temperatures of juice sample were recorded during the treatments using an Infrared Thermometer (IR-82, CEM, Shenzhen, China) attached to a Data Logger (DT 8891E, CEM, Shenzhen, China) to ensure that the maximum temperature reached in the medium was not lethal to the bacteria.

3.1.4. Polyphenoloxidase (PPO) Inactivation

The freshly squeezed CAJ samples were centrifuged at 10000 rpm for 15 min at 4°C (Universal 320 R, Hettich**,** Germany) and the supernatant was collected to test PPO activity. Activity of PPO was assayed by a spectrophotometric method (Bi et al. 2013) with some modifications. The method is based on the measurement of the absorbance of brown polymers formed when catechol is oxidized in presence of polyphenoloxidase at 410 nm. Catechol was used as the substrate, and 0.05 M catechol substrate solution was prepared with 0.2 M phosphate buffer (pH 6.5). All samples were analyzed by adding 0.5 mL centrifuged juice into 2.5 mL substrate solution. The increase in absorbance at 410 nm in 10 min was immediately monitored after incubation for 3 min using a UV-1800 spectrophotometer (SHIMADZU Co. Ltd., Japan), which was equipped with a Peltier Thermo-Statted cell holder, a water pump (Varian Co. Ltd., California, USA) to keep temperature at 30°C. An enzyme activity unit was defined as an increase of 0.1 in absorbance per minute. All the experiments were repeated three times. Residual activity of PPO was calculated from the following equation:

$$\text{Residual Activity of PPO, \%} = \frac{A_1 x 100}{A_0} \qquad (3.9)$$

A_1 is the enzymatic activity after UV-LED and thermal pasteurization treatment; A_0 is the enzymatic activity of untreated sample (freshly squeezed). Untreated and pasteurized CAJ samples were used as negative and positive controls.

3.2.5. Reactivation of *E. coli* K12 in Apple Juices

After UV-LED treatment of juice samples at combined 280 nm/365 nm wavelengths, photoreactivation and dark repair of *E. coli* K12 in CAJ and AJ were investigated. For photoreactivation experiments, 3 ml apple juice samples both CAJ and AJ were subjected to UV-LED irradiation at the combination of 280 nm/365 nm after then they were immediately placed into an orbital shaker (Thermo, Electron Corporation) equipped with two horizontally fixed daylight fluorescent lamps (T5, F8W/840, Philips) emitting light in the 400 nm to 750 nm wavelength range and shaken at 80 rpm for 6 h at two different temperatures, i.e., 4ºC and 22ºC. The distance between the surface of the test samples and the lamps was 27 cm. The illuminance (lx) was measured online with a radiometer with UVX-36 sensor (UVX, UVP Inc., CA, USA). The mean intensity of illuminance was 844 lx, which was similar to the recommended light level in supermarkets (Recommended Light Levels, 2015).

The same procedure was adopted for dark repair, except that the Petri dishes were immediately covered with aluminum foil and incubated at the same temperatures for 6 h. Number of bacteria in samples were counted periodically at 10, 20, 30, 40, 60, 90, 120, 150, 200, 250, 300 and 360 min. The enumeration was carried out by spread plating onto tryptic soy agar (TSA)incubating at 37°C for 18-24 h. The experiments replicated three times. The reactivation ratio of *E. coli* K12 in juice was calculated according to Equation 3.10 (Li et al., 2017).

$$\%\, reactivation = \frac{N_t - N_0}{N_{initial} - N_0} \times 100\% \qquad (3.10)$$

Where N_t is the number of *E. coli* K12 at incubation time *t* (CFU/mL), N_0 is the number of *E. coli* K12 immediately after UV treatment (CFU / mL) and $N_{initial}$ is the population of *E. coli* K12 before the treatment (CFU/mL).

3.1.6. Data Analysis

All the processes were repeated two times. The results of microbiological, physicochemical and optical measurements were expressed as their means and standard deviations. Measurements were done in duplicate. Regression analysis was applied by

using a commercial spreadsheet (Microsoft Excel, Redmond, WA, USA). One-way analysis of variance (ANOVA) was carried out to determine how significantly the independent variables (UV-LED and thermal processes) affect the dependent variables (microbiological, physicochemical and optical properties of apple juice) by using the Minitab 16 software program (Minitab Inc., State College, PA, USA). The means were evaluated in terms of Tukey comparison test with a 95% confident interval. Differences of data were significant for p-value is equal or less than 0.05 ($p \leq 0.05$).

3.2. Results and Discussions

3.2.1. Physical, Chemical and Optical Properties of Freshly Squeezed Cloudy and Clear Apple Juices

3.2.1.1. Physical and Chemical Properties

Some physical and chemical properties of fresh CAJ and AJ samples including pH, total soluble solid (Brix°), titratable acidity, ascorbic acid content were determined only before the UV-LED treatments depicted in Table 3.2.

pH and total soluble solid content (ºBrix) of freshly squeezed CAJ extracted from Starking Delicious variety of apples (CAJ) and AJ were 3.92; 3.99 and 13.63°; 13.42° Brix. These values showed similarity with previous studies. Murakami et al. (2006) were studied 15 different apple varieties. They observed that the pH and the ºBrix values of apple juice were in between 3.2 and 6.50; 9.80 and 16.90 °Brix. Falguera et al. (2011) were reported that pH and ºBrix values of Golden, Starking, Fuji and King David variety of apples were in the range of 3.48 and 4.35; 10.70 and 14.70°Brix. Also, titratable acidity values of CAJ and AJ samples were recorded as 0.33% and 0.18%. The differences of titratable acidity values between CAJ and AJ were caused by additional of citric acid to commercial apple juice (AJ). These data were in agreement with other studies reporting (Santhirasegaram, Razali, George, & Somasundram, 2015).

Ascorbic acid content of apple juice was determined to be 2.22 mg/L. The ascorbic acid content of apple juice obtained from Starking variety was found lower than the findings of Juarez-Enriquez et al. (2014) and Falguera et al. (2011). The main reason of this might be the usage of different methods for determination of ascorbic acid content in

these studies. In our study, measurements were done by ascorbic acid test kits based on Boehringer enzymatic method. Falguera et al. (2011) reported the ascorbic acid of Starking type of apples as 788.20 mg/L measured by an iodine titration method. Besides, Juarez-Enriquez et al. (2014) determined the ascorbic acid content as 211.75 mg/kg by using 2,6-dichloroindophenol method. On the other hand, the content of vitamin C in fruits and vegetables can be influenced by various factors such as genotypic differences, pre-harvest climatic conditions and cultural practices, maturity and harvesting methods, and postharvest handling procedures (Lee and Kader, 2000).

Table 3.2. Physical and chemical properties of fresh CAJ and commercial pasteurized AJ.

Juice Type	pH	Brix (°)	TA (%)	A.A (mg/L)
CAJ	3.92 ± 0.01^{a}	13.63 ± 0.04^{b}	0.33	2.22
AJ	3.99 ± 0.02^{b}	13.42 ± 0.00^{a}	0.18	-

Results were presented as "means± standard error". Least significant difference was determined by Tukey pairwise comparison test. Means that do not share the same letter are significantly different ($p < 0.05$). Abbreviations: TA (titrable acidity), A.A (Abscorbic acid content),

3.2.1.2. Optical Properties

Table 3.3 shows optical properties such as absorption coefficient, turbidity, CIE color parameters (L*, a*, b*) of CAJ and AJ.

Absorbance is an important factor that affects the UV light intensity and its penetrating ability to liquid materials. Absorption coefficients of CAJ and AJ were calculated from the slope of absorbance values. In this study, the average absorption coefficients of CAJ and AJ were determined as $28.53_{(254nm)}$; $5.91_{(254nm)}$ cm^{-1} which are in a good correlation with the ones obtained by Gayan et al. (2013) (28.54 cm^{-1}).

Turbidity of the samples was changed greatly because of suspended particles. Although CAJ had many suspended solids in its composition and higher turbidity value as 1619 NTU, AJ was processed with a clarification step where suspended particles were removed. This step was resulted in less turbid juice as 4.43 NTU.

The color parameters (L*, a*, b*) of apple juice were determined 25.43; 1.60; 6.71. The results were comparable with the ones obtained by Abid et al. (2013), Noci et al. (2008), Cominiti et al., (2012). The L*, a*, b* values were in range of 19.27 and 25.40;

-0.72 and 1.33; 3.99 and 6.75. The color parameters of juice samples were dissimilar although they were made of same kind of fruits. L* values of the juices indicated that CAJ (L*: 25.43) has a less light color than pasteurized AJ (L*: 29.49). In contrast, b* value of the AJ as 6.12 was lower than CAJ due to carotenoid content in the juice composition. AJ had less carotenoid pigment comparing to CAJ due to carotenoid degradation caused by pasteurization process (Fratianni et al. 2010). Parameter a* that is responsible for redness of the juice was lower in the AJ. The loss of redness by a decrease of a* value was attributed to the breakdown of anthocyanin pigments during treatment.

Table 3.3. Optical properties of fresh CAJ and commercial pasteurized AJ.

Juice Type	Absorption Coefficient 254_{nm} (1/cm)	Turbidity (NTU)	Color		
			L*	a*	b*
CAJ	28.53	1619 ± 46^{b}	25.43 ± 0.09^{a}	1.60 ± 0.12^{b}	6.71 ± 0.06^{b}
AJ	5.91	4.43 ± 0^{a}	29.49 ± 0.02^{b}	-0.28 ± 0.01^{a}	6.12 ± 0.00^{a}

Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. Means that do not share the same letter are significantly different ($p < 0.05$). Abbreviations: NTU (Nephelometric Turbidity Units), L* (brightness–darkness), a* (redness–greenness), b* (yellowness–blueness), ΔE (total color difference).

3.2.2. Thermal Pasteurization of Cloudy Apple Juice

Initial microbial load of freshly squeezed cloudy apple juice including total aerobic bacteria (TAC), yeast and molds counts (YMC) were 3.4 and 3.2 log CFU/ml. No coliform bacteria were observed in fresh apple juice samples. In order to investigate the microbial efficiency of UV-LEDs for cloudy apple juice, pasteurization of CAJ was required prior to inoculation of target microorganism, i.e. *E. coli* K12. For this purpose, freshly squeezed apple juice was pasteurized at different time temperature combinations to reach the 5 log CFU/ml reduction of *E. coli* K12. Survival number of the cells following the treatments were shown in Table 3.4. Heat treatments for 60 seconds or less at 70°C were insufficient to reach 5 log reductions in the number of *E. coli* K12. The applications of heat treatment at 70°C for longer than 60 s, and the other heat treatments at 80°C and 90°C resulted in 6.78 log CFU/ml reduction. According to these results, the thermal treatment conditions, e.g. 70°C-100 s, 70°C-120 s, and 80°C-15 s were found to be sufficient to achieve 5-log reduction of *E. coli* K12 in apple juice. Moreover, at these process conditions, TAC and YMC were found to be below the detection limits. Mazzotta (2001) reported that the commercial pasteurization conditions for fruit juices are90 °C for

2 s and 84 °C for 20 s. However, the heat treatment of juice at these high temperatures often causes quality loss. It was observed that when the pasteurization temperature was higher than 80°C, the color of apple juice samples was significantly affected (Table 3.5) supporting this finding. Recently, a mild heat pasteurization (between 70ºC and 72°C) have begun to be applied to keep the quality of fruit juices high (Timmerman and Lorenzo-Seva 2011). The FDA has also recommended a minimum temperature (71.1°C) and time (3 s) for pasteurization of food products having the pH range of 3.6 and 4.0 (FDA 2004). Therefore, 70ºC and 120 s were chosen as the thermal processing conditions for apple juice in this study.

Table 3.4. Reduction number (log CFU/ml) of inoculated *E. coli* K12 in CAJ pasteurization at different time-temperature combinations

Time (s)	70°C	80°C	90°C
3			6.78
10			6.78
15		6.78	
20			6.78
30	3.018	6.78	6.78
45		6.78	6.78
60	3.49	6.78	
120	6.78		
180	6.78		
240	6.78		
360	6.78		
480	6.78		

Table 3.5. Color changes of CAJ subjected to thermal pasteurization applied with different time and temperature conditions

	70ºC, 100 s			**70ºC, 120 s**			**80ºC, 15 s**		
	L*	**a***	**b***	**L***	**a***	**b***	**L***	**a***	**b***
Untreated CAJ	25.35[a] ±0.16	2.50[b] ±0.03	7.86[a] ±0.12	25.46[a] ±0.09	1.60[b] ±0.13	6.71[a] ±0.06	28.96[a] ±0.16	2.38[b] ±0.32	6.83[a] ±0.27
Pasteurized CAJ	35.92[b] ± 0.17	0.56[a] ±0.09	15.50[b] ±0.28	28.12[b] ±0.19	-0.45[a] ±0.13	7.16[b] ±0.02	33.71[b] ±0.40	-0.49[a] ±0.15	9.30[b] ±0.34

[a-b]values in a column with the same superscript are not significantly different by Tukey's test ($p<0.05$)

3.2.3. UV-LED Irradiation of Apple Juice

3.2.3.1. Determination of the UV Exposure Time

The UV exposure time, dose and wavelengths required for the 5-log inactivation of *E. coli* K12 in CAJ and AJ samples were determined. The effect of four UV-LEDs emitting light at 254 nm on the inactivation of *E. coli* K12 in CAJ is presented in Figure 3.4. A linear decrease in the logarithmic reduction curve of *E. coli* K12 was observed when CAJ was treated up to 35 min. The exposure time that was applied longer than 35 minutes did not change the inactivation efficiency of the lamps significantly. UV-LED irradiation applied at 254 nm for 40 min was resulted in 3.70 log CFU/mL reduction of *E. coli* K12 in CAJ (1909.50 NTU, 15.43_{254nm} cm^{-1}), while the UV-LED applied for 50 min provided 3.77 log CFU/mL reduction. The inactivation efficiencies of 40 min and 50 min exposure times were not significantly different from each other ($p>0.05$). This could be a result of tailing effect of UV-LED irradiation. Tailing may have arisen from the aggregation of microorganisms (Koutchma 2009, Li et al. 2017, Oguma et al. 2013). UV-LED processing required a long exposure time to reach 5 log inactivation of *E. coli* K12 in CAJ and therefore led to the higher opportunity for microbial aggregation. The tailing phase was also observed in the other studies using UV-LEDs. Oguma et al. (2013) investigated inactivation efficiency of *E. coli* by combined emissions at 265/280, 265/310, 280/ 310 and 265/280/310 nm with a flow-through reactor. They observed a tailing at high fluences, regardless of the emission wavelengths.

The color of the juice samples was adversely affected when the treatment time exceeded 40 min. Thus, the maximum exposure time for UV-LED trials was determined as 40 min. UV dose was estimated from the exposure time (40 min) and incident UV intensity (irradiance). Incident UV intensity (irradiance) emitted by each UV-LED lamp (in mW/cm^2) was measured by ferrioxalate actinometry method and shown in Table 3.6. the applied UV-dosages to CAJ and AJ samples were also depicted in Table 3.7 and 3.8. Nadir et al. (2003) applied 300 mJ/cm^2 UV dose at 0.32 mW/cm^2 incident UV intensity for 16 min in order to achieve 4.2 log CFU/mL reduction of *E. coli* O157:H7 (ATCC 35150) in apple juice (0.02% transmittance) samples of 0.1 cm in depth. They irradiated the juice samples using a collimated beam apparatus consisted of a low-pressure mercury UV lamp with peak radiation at 254 nm. In another study, apple juice (absorption

coefficient 5.81 cm^{-1}) inoculated with *E. coli* K12 was treated with UV-C irradiation and 4.6 log_{10} CFU/mL reduction was obtained (Caminiti et al. 2011). In our study, 32.17 mJ/cm^2 UV dose at 0.042 mW/cm^2 average UV intensity for 40 min was resulted in 3.70 log CFU/mL reduction of *E. coli* K12 in CAJ of 0.15 cm in depth using four UV-LEDs emitting light at 254 nm (Fig. 3.3).

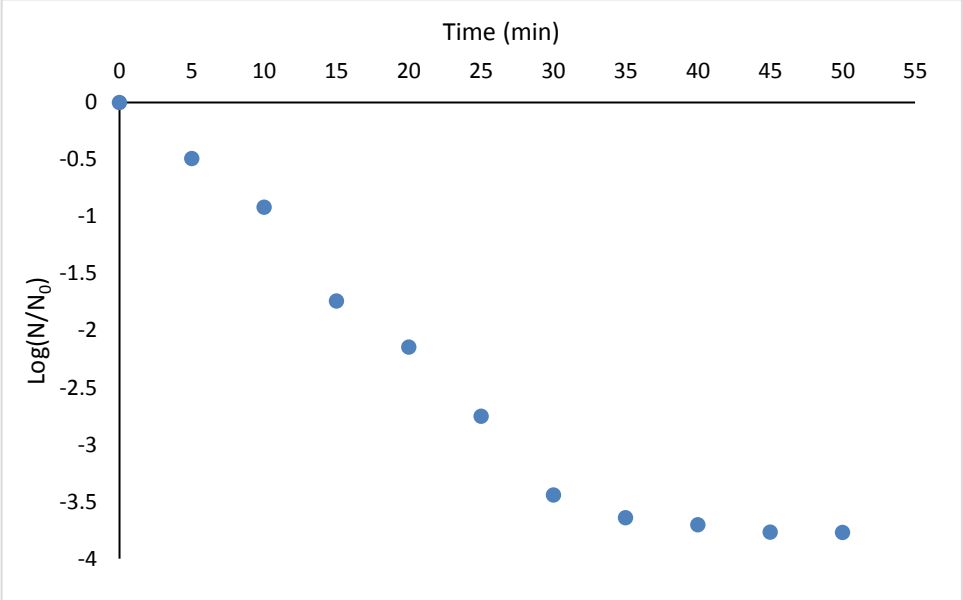


Figure 3.4. The effect of four UV-LED lamps emitting light at 254 nm on the inactivation of *E. coli* K12 in CAJ.

Table 3.6. Measured UV intensities of different UV-LEDs and UV Dosages applied to CAJ and AJ samples

	UV intensity	**UV Dose (mJ/cm^2)**			
	(mW/cm^2)	**CAJ**		**AJ**	
Treatments		**20 min**	**40 min**	**20 min**	**40 min**
254 nm (4 Lamps)	0.042	16.08	32.17	32.81	65.62
280 nm (4 Lamps)	0.045	14.08	28.17	37.28	74.56
254 nm (2 Lamps)	0.023	8.83	17.67	18.02	36.04
280 nm (2 Lamps)	0.026	8.15	16.31	21.58	43.17
365 nm (2 Lamps)	0.051	44.85	89.69	57.16	114.32
405 nm (2 Lamps)	0.021	20.44	40.88	24.12	48.25

3.2.3.2. UV-LEDs Irradiation of Apple Juices

The inactivation effects of different wavelengths including 254, 280, 254/365, 254/405, 280/365, 280/405 and 254/280/365/405 nm on *E. coli* K12 in CAJ and AJ for 20- and 40-min exposure times were examined using the static bench top UV-LEDs system. The turbidity and absorption coefficients of CAJ used in this part of study were 908.5 NTU, 24.71_{254nm} cm^{-1}, 19.18_{280nm} cm^{-1}, 4.24_{365nm} cm^{-1} and 2.60_{405nm} cm^{-1}, respectively. These values were different than those used in previous experiments. This was because apple juice samples used in the experiments were freshly prepared using apples purchased from the market at different time intervals. Thus, climate and seasonal changes cause a difference on the composition of apples and apple juice. Besides, the filtration of processes applied in obtaining cloudy juice may also have an effect on the physical properties of samples. For AJ, turbidity and absorption coefficient were measured as 4.43 NTU and 5.91 cm^{-1}, respectively. The logarithmic reductions of *E. coli* K12 in apple juice samples exposed to UV light emitted at different wavelengths and exposure times were demonstrated in Table 3.7. The results showed that the longer exposure time or higher UV dose increased the inactivation efficacy of individual and combined wavelengths on *E. coli K12* in both juice types. The highest inactivation achieved in 20 min using four UV-LEDs at 280 nm was 1.63 ± 0.09 log CFU / mL in the CAJ and 4.00 ± 0.02 log CFU / mL in AJ. Whereas, when the exposure time was increased to 40 min at 280 nm, the reduction of *E. coli* K12 in CAJ was maximum reaching to 2.0 log CFU/mL in CAJ, and 4.40 log CFU/mL in AJ. For the same treatment time, the combination of lamps emitting light at 280 and 365 nm (2 lamp/2lamp) resulted in 1.98±39 and 3.76±0.07 log CFU/mL reductions in CAJ and AJ, respectively. It was concluded that UV-LED irradiation had the highest inactivation efficiency in AJ than that of CAJ. This may be due to the fact that colored compounds, organic substances and suspended solids present in cloudy liquids reduce the penetration depth of UV light. Therefore, UV efficiency is lower in cloudy juices compared to clear ones (Koutchma 2009). Furthermore, 280/365 nm (2 lamp/2lamp) wavelengths resulted in similar reductions of *E. coli* K12 in CAJ and AJ. In other words, the log reductions achieved by using 280/365 nm were not statistically different from the treatments using 4 UV-LEDs emitting light at 254 nm ($p>0.05$).

Table 3.7. Logarithmic reductions (log CFU/mL) of *E. coli* K12 in CAJ and AJ exposed to UV-LED irradiation at different wavelength for different exposure times

		Wavelengths (nm)						
Juice Type	**Time (min)**	**254**	**280**	**280-365**	**280-405**	**254-365**	**254-405**	**254-280-365-405**
CAJ	**20**	1.10± 0.01^{abA}	1.63± 0.09^{cA}	0.94± 0.21^{abA}	0.97± 0.22^{abA}	0.60± 0.09^{abA}	0.37± 0.08^{aA}	1.05± 0.32^{abA}
	40	1.64± 0.05^{abB}	2.00± 0.06^{bB}	1.98± 0.39^{bA}	1.26± 0.09^{aA}	1.38± 0.11^{abB}	1.18± 0.34^{aA}	1.72± 0.33^{abA}
AJ	**20**	3.58± 0.04^{bA}	4.00± 0.02^{cA}	3.54± 0.08^{bA}	3.42± 0.02^{bA}	1.79± 0.06^{aA}	3.26± 0.03^{bA}	3.35± 0.26^{bA}
	40	3.92± 0.15^{bA}	4.40± 0.14^{cA}	3.76± 0.07^{bA}	3.58± 0.03^{bB}	3.22± 0.16^{aB}	3.77± 0.08^{bB}	3.66± 0.15^{bB}

Results were presented as "means± standard error". Least significant difference was determined by Tukey pairwise comparison test. $^{A-B}$: Values within each exposure time followed by the same letter are not significantly different ($P > 0.05$). $^{a-c}$: Values within each wavelength and their combinations followed by the same letter are not significantly different ($P > 0.05$).

Moreover, the germicidal effectiveness of 280 nm and 280/365 nm UV-LEDs in both juice medium were higher than that of 254 nm LEDs. This may be due to the fact that the UV light emitted at different UV ranges has different inactivation mechanisms. UV light at 254 nm directly affects the DNA of microorganisms by causing the formation of cyclobutane thymine dimers, leading to cell damage and resulting in the death of the cells (Santos et al. 2013). Whereas UV light at 280 nm is effective both on DNA s and protein type of molecules in microorganisms (Yin et al. 2015b). On the other hand, 365 nm wavelength has a different damaging effect on bacteria. UV-A photons are absorbed by photosensitizer cellular structures such as chromophores and the protein synthesis might be inhibited as a result of formation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Ravanat et al. 2001, Chatterley and Linden 2010). Chevremont et al. (2012a) was reported similar findings for coupled 280/365 and 280/405 nm wavelengths. They obtained significant reduction on mesophilic bacteria in waste water when compared to 254 nm wavelength. Additionally, Bowker et al. (2011) indicated that UV-LEDs emitting light at 275 nm resulted in much higher microbial inactivation. This might be due to the fact that protein absorption spectrum may reach the maximum near 280 nm and thus enzymes become more sensitive to inactivation by UV light emitted at these wavelengths (Chevremont, Farnet, Coulomb, et al. 2012).

The UV-LEDs with combined emissions at 254/365 nm and 254/405 nm did not increase the inactivation efficacy of 254 nm on *E. coli* K12 in CAJ and AJ. For instance, 40-minute exposure of CAJ by UV-LEDs at 254 nm using 4 lamps resulted in 1.64±0.05 log CFU/mL reduction of *E. coli* K12.Whereas, its combination with 365 nm and 405 nm

were resulted in 1.38±0.11 and 1.18±0.34 log CFU/mL reductions, respectively. The decline in logarithmic reductions could be explained by the role of 365 nm and 405 nm wavelengths in repair mechanism of the cell. It is known that the photolyase enzyme which reverses the damages by specifically binding to cylobutane pyrimidine dimers, absorb the light in the range of 310-400 nm, additionally, gives major peak near around at 375 nm which is close to 365 nm (Kim et al. 1994). As a result, it could be speculated that 365 nm might play a role on stimulation of the photoreactivation mechanism of cells and reduce the inactivation efficacy of UV-LED treatment. The same findings were observed in combination UV-LEDs' at 280 nm and 405 nm.

The results showed that UV-LEDs emitting light at different wavelengths have different inactivation efficiency on *E. coli* K12 in different optical medium, i.e., clear and cloudy apple juice, and the individual and combined emissions did not satisfy FDA`s 5-log reduction criterion for juice pasteurization. However, it was found that UV-LEDs with peak emission at 280 nm and combined emissions at 280/365 nm had the highest inactivation efficiency than other UV-LEDs.

Figure 3.5 shows the temperature of CAJ exposed to UV-LED at different wavelengths. During the process, room temperature was kept constant at 24.5°C. The maximum temperature increase was approximately 2°C in the treatment using 4 lamps emitting light at 280 nm. In other wavelengths conditions the temperature was fluctuated between 24 and 24.6°C. In the literature, many studies have mentioned the heating problem during the treatment of conventional UV-C irradiation produced by mercury vapor lamp. Because these lamps operate at high voltage (Chevremont, Farnet, Coulomb, et al. 2012, Taniyasu, Kasu, and Makimoto 2006, Vilhunen et al. 2009). In contrast, light-emitting diode (LED) is a semiconductor p–n junction device that emits light in a narrow spectrum, produced by a form of electroluminescence (Asif Khan 2006, Crawford et al. 2005, Hu et al. 2006). AlGaN and AlN are materials for deep UV LEDs. LEDs are free of toxicants (e.g., mercury) and consume less energy than traditional lamps. As LED transmits greater amount of energy into light and wastes little energy as heat. Thus, the heating effect of UV-LEDs on the inactivation of target microorganism in CAJ was ruled out.

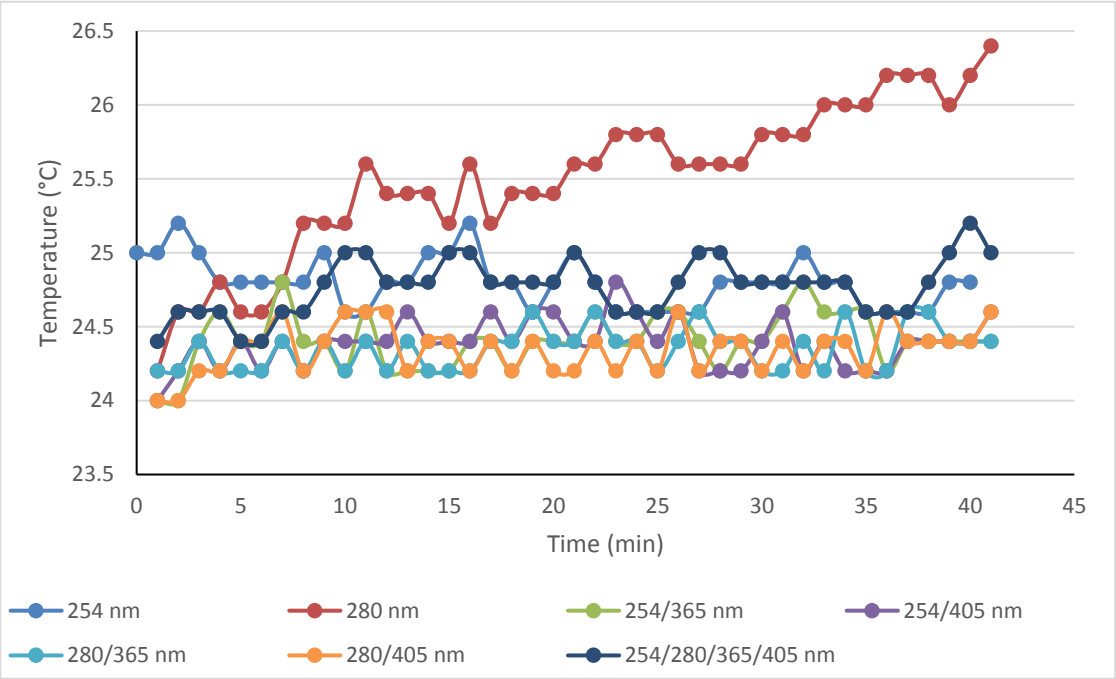


Figure 3.5. Temperature profiles of CAJ samples during UV-LED treatments

3.2.4. Polyphenoloxidase Inactivation by UV-LEDs

The stability of juice quality depends on the microbial activity as well as enzyme activity. One of the quality degrading enzymes in fruit juices is the polyphenoloxidase (PPO). PPO is responsible for enzymatic browning which adversely affects the color of juice. Table 3.8. summarizes the residual PPO activity in freshly squeezed CAJ following the UV-LED irradiation at different wavelengths such as 254, 280, 254/ 365, 254/405, 280/365, 280/405 and 254/280/365/405 nm after 40 min exposure time at 24°C.Thermal pasteurization was conducted at different temperatures for different duration times, i.e., 70ºC-100s, 70ºC-120 s. and 85ºC-15 s.

The results indicated that application of neither thermal pasteurization nor UV-LED irradiation provided a complete inactivation of PPO in CAJ. The thermal pasteurization at 85ºC for 15 s was able to reduce PPO activity to 27.37±0.17 %. When the temperature decreased to 70ºC and time increased to 100 s, and 120 s the residual activity of PPO was recorded as 25.76±1.38 % and 22.34±0.06 %. It was observed that applied temperature and time combinations were not enough to fully degrade PPO activity. Our results showed similarity with previous studies in the literature. Krapfenbauer et al. (2006) was investigated the effect of heating conditions considering high-temperature (60-90 °C) and short-time (20-100 s) combinations on the activity of

PPO in juice obtained from eight different varieties of apple. They indicated that heating at 70ºC was not enough to inactivate PPO completely, whereas heating at 80ºC and 90ºC after 20 s provided a complete inactivation.

The residual activity of PPO in CAJ subjected to UV-light at 254 nm (4 L) and 280 nm (4 L) wavelength for 40 min was reduced to 70.43% and 56.35%, respectively. It was clearly observed that 280 nm UV-LEDs provided a higher inactivation of PPO in CAJ than that of 254 nm UV-LEDs. The reason for this might be the degradation effects of 280 nm wavelength on protein type of compounds in plants. In addition to these, it was reported that the enzyme inactivation using UV-C light depends on the juice matrix and its composition (Müller et al. 2014). High absorption coefficient of cloudy apple juice indicated that the penetration depth of the UV-C light at 254 nm in juice was very small, thus the enzyme inactivation was low. Orlowska et al. (2014) was observed an increment of PPO residual activity by 14.5% after UV treatment of apple cider at 1.5 kJ/L UV dose. This effect was as a result of releasing the enzyme from plant tissues since disruptive effects of the hydrodynamical stresses on the biological tissues caused fragmentation and formation of small particles. Noci et al. (2008) reported that UV treatment of apple juice for 30-min had no noticeable effect on the enzymes such as pectinmethylesterase (PME), polyphenoloxidase (PPO) and peroxidase (POD).

On the other hand, combination UV-LED emitting light using UV-C rays at 254 nm and 280 nm and UV-A rays at 365 nm and 405 nm wavelengths showed a better inactivation efficiency on PPO enzyme than the one used only UV-C rays. The residual activity of PPO following 254/365 nm and 254/405 nm UV-LED irradiations was about 50% and 47%. Similarly, the coupling of UV-LEDs at 280 with 365 nm and 405 nm provided the residual activity of 32 % and 34 %. Limited and often controversial information is available in the literature regarding the effect of UV light emitted in the UV-B and UV-A bands on the activity of certain enzymes associated with fresh juices including polyphenol oxidase (PPO). Müller et al. (2014) investigated the effect of UV-C and UV-B irradiation on PPO activity of apple (52.4 cm^{-1}) and grape (43.5 cm^{-1}) juice by means of UV-C processing unit equipped with a Dean flow reactor. Even though, they did not observe a significant effect on PPO enzyme activity, a reduction of PPO activity of more than 20% and 40% was reported in apple and grape juices subjected to UV-B (290-315 nm) light, at UV dose of 100.48 kJ L^{-1}. Falguera, Pagán, and Ibarz (2011) irradiated fresh apple juices for 120 min using a high-pressure mercury UV lamp (400 W,

emitting light between 250 and 740 nm), They were able to inactivate polyphenol oxidase in 100 min, while peroxidase was completely destroyed in only 15 min.

Table 3.8. Residual Activity (%) of PPO in CAJ treated with thermal pasteurization and UV-LED irradiation

		Residual Activity (%)
Thermally Pasteurized CAJ	70°C 100 s	25.76 ± 1.38^{B}
	70°C 120 s	22.34 ± 0.06^{A}
	85°C 15 s	27.37 ± 0.17^{B}
UV-LED Irradiated CAJ (40 min)	254 nm	70.43 ± 3.38^{d}
	280 nm	56.35 ± 5.38^{c}
	254/365 nm	50.01 ± 1.62^{b}
	254/405 nm	47.76 ± 1.79^{b}
	280/365 nm	32.58 ± 0.77^{a}
	280/405 nm	34.38 ± 1.61^{a}
	254/280/365/405 nm	42.87 ± 1.45^{b}

Results were presented as "means± standard error". Least significant difference was determined by Tukey pairwise comparison test. $^{A-B}$: Values within each thermally pasteurized CAJ followed by the same letter are not significantly different ($P > 0.05$). $^{a-d}$: Values within each UV-LED Irradiated CAJ (40 min) followed by the same letter are not significantly different ($P > 0.05$).

3.2.5. Reactivation Behavior of *E. coli* K12 in Cloudy and Clear Apple Juices

3.2.5.1. Photoreactivation

Photoreactivation of *E. coli* K12 in cloudy apple juice (CAJ) and clear apple juice (AJ) after 280/265 nm UV-LEDs irradiation as a function of incubation time at different reactivation temperatures (22 and 4 ˚C) was demonstrated in Figure 3.6, 3.7 and 3.8.

Illumination of CAJ after 280/365 nm UV-LEDs irradiation from 20 min up to 90 min at 22˚C led to a distinct increase in the recovery of the cells in CAJ, i.e., approx. 0.6 log (Fig. 3.6a). However further prolongation of the illumination time gave a weak improvement in recovery. On the other hand, under the same illumination condition, the maximum recovery was 1.12 log CFU/mL obtained at 60 min in the clear apple juice (AJ) (Fig. 3.6c). For 1.12 log recovery, the percentage photoreactivation was 0.6% and it did not exceed the 1% (Fig. 3.7a). The photoreactivation percentages was the lowest for CAJ

illuminated at 22˚C for 6 h. However, it was concluded that the cells in AJ and CAJ kept their viability at the end of 6 h of incubation period under light at 22ºC (Fig. 3.8). Li et al. (2017) also reported higher photoreactivation percentages in water after 280 nm LEDs irradiation compared to 265 nm.

Photoreactivation was periodically repeated in CAJ incubated at 4˚C for 6 h between the periods of 20 - 90 min, and 120-360 min (Fig. 3.6b). There was no distinct induction period, the growth phase, the stabilization phase and decay period. However, thirty min of illumination of AJ after 280/365 nm UV-LEDs irradiation at 4˚C resulted in the highest recovery, i.e. ~1 log (Fig 3.6d). The maximum percentage photoreactivation exceeded the 1%, e.i, %1.2 (Fig. 3.7b). But prolongation of the illumination time caused a reduction in the recovery of cells. It was concluded that photoreactivation mechanism of the cells was not effective when the UV-LED treated cells in CAJ and AJ was incubated at 4ºC for 6 h. In other words, the UV light induced cell damages were not fully recovered in 6 h. Interestingly, the negative reactivation of *E. coli* K12 in apple juice samples was observed following to exposure to 280/365 nm UV-LEDs irradiation (Fig. 3.8). Similarly, Kashimada et al. (1996) observed a decrease of survival in the experiments with sunlight. This disinfection was attributed to the sunlight which penetrated through the glass tube. It was speculated that the visible light within the range from wavelength 340 nm to 490 nm has a disinfection effect (Yanagida 1981). Additionally, UV-LED inactivation experiments were conducted at room temperature, but the reactivation experiments were assayed at 4˚C (an extreme temperature) which might cause a temperature shock to the bacteria and changing the reactivation process. This situation might be also result in lowering the activity in DNA repairing enzyme, i.e., the activity of photolyase effective in photoreactivation process might decrease at low temperatures. Thus, photoreactivation at 4˚C could be a result of the combined effect of reactivation and temperature shock (Salcedo et al. 2007).

In summary, the higher the temperature the greater the maximum photoreactivation observed although the intensity of illumination used in this study was rather weak compared to sunlight. *E. coli* K12 in CAJ and AJ showed a more or less enhanced recovery after illumination at 22˚C. The percent recovery was the highest in AJ samples. However, the incubation of the cells in CAJ and AJ under light at 4ºC did not cause any significant increase in the survival number of cells. Gayán et al. (2011) and Salcedo et al. (2007) also reported strong effect of temperature on photo and dark reactivation of *E. coli* following exposure to UV light. Salcedo et al. (2007) found that

the survival of coliforms, under light incubation conditions following the UV-C radiation, increased as the temperature increased up to 30ºC. Similarly, when *E. coli* strain exposed to UV irradiation by means of a medium pressure mercury lamp, it was observed that photoreactivation level was higher near the optimum growth temperatures (23–37ºC) compared to incubation conditions of too high (50ºC) or too low (4ºC) temperatures (Quek and Hu, 2007).

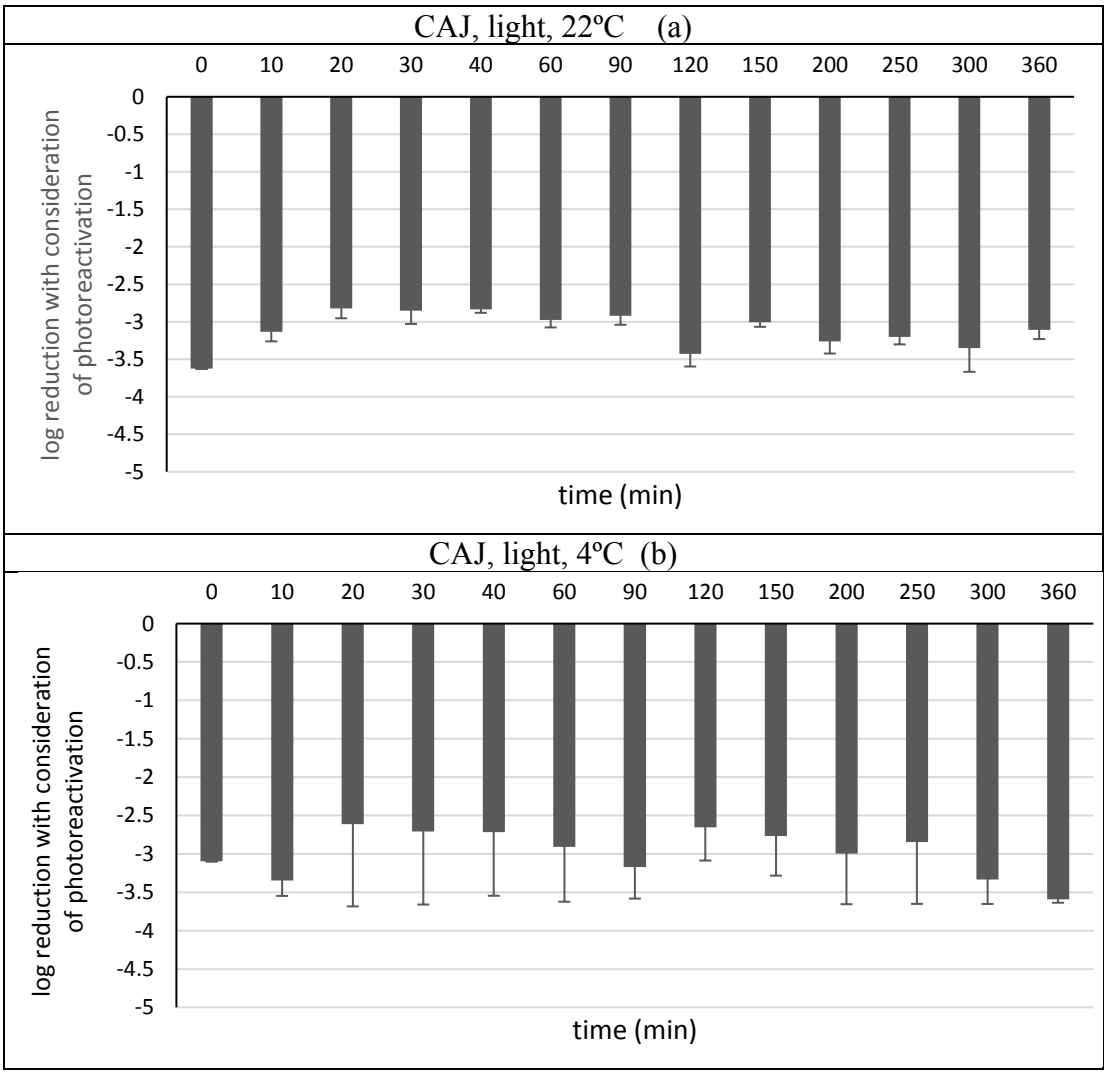


Figure 3.6. Photoreactivation of *E. coli* K12 in cloudy apple juice (CAJ) and clear apple juice (AJ) after 280/265 nm UV-LEDs irradiation as a function of incubation time at different reactivation temperatures (22 ˚C and 4 ˚C)

(cont. on next page)

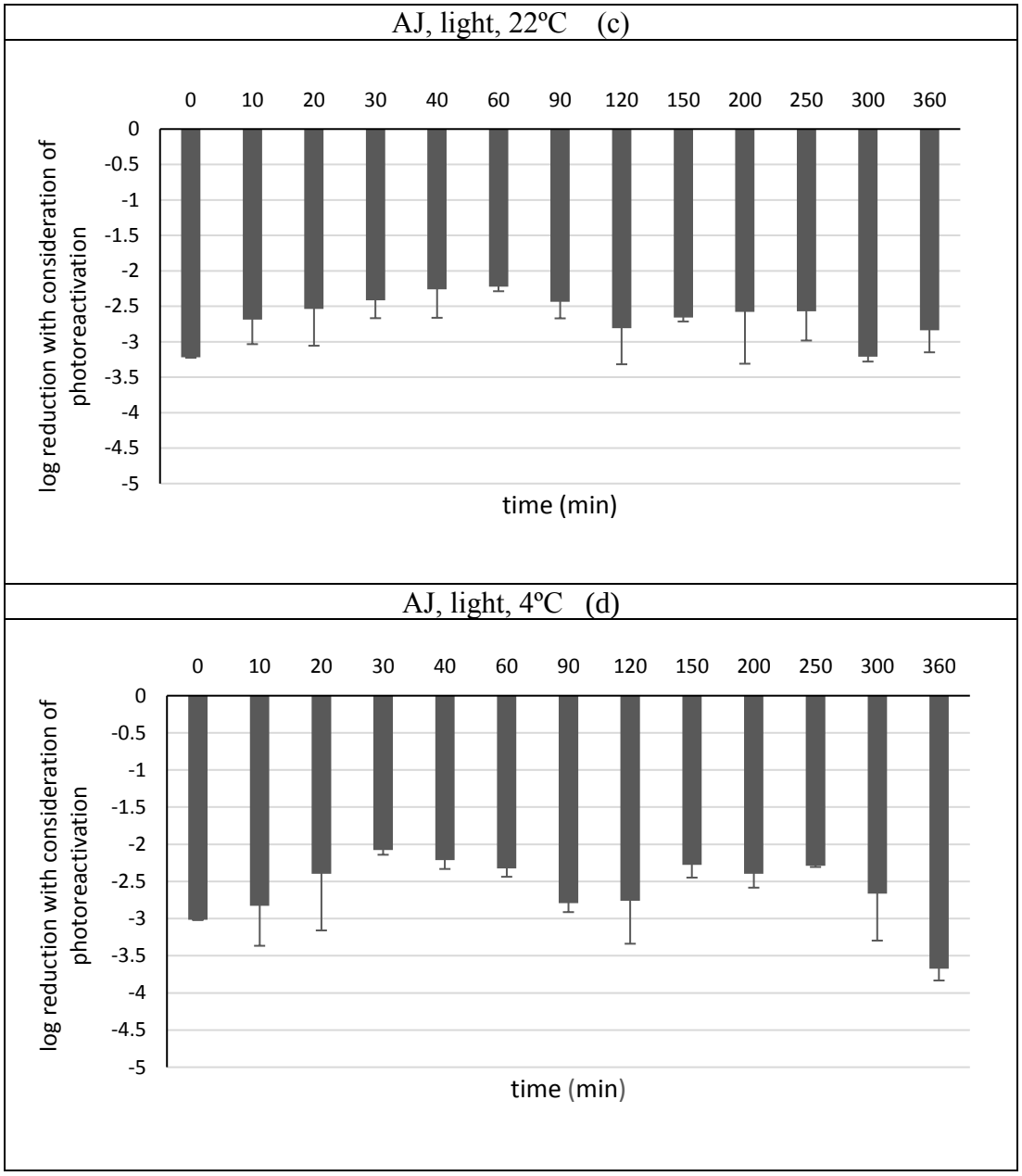


Figure 3.6 (cont.)

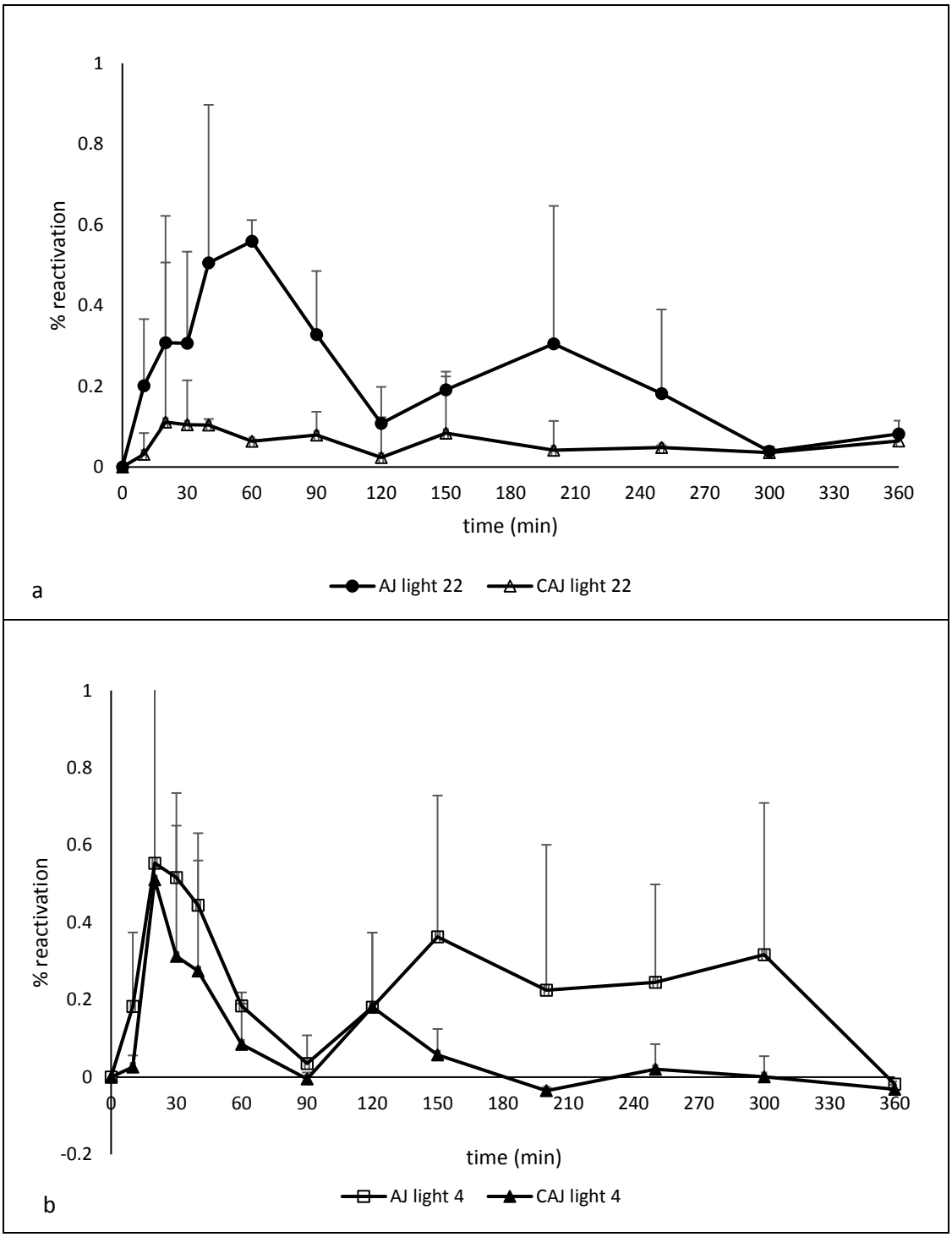


Figure 3.7 Photoreactivation percentages of *E. coli* K12 in cloudy apple juice (CAJ) and clear apple juice (AJ) after 280/365 nm UV-LEDs irradiation as a function of time at 22 ˚C and 4 ˚C reactivation temperatures.

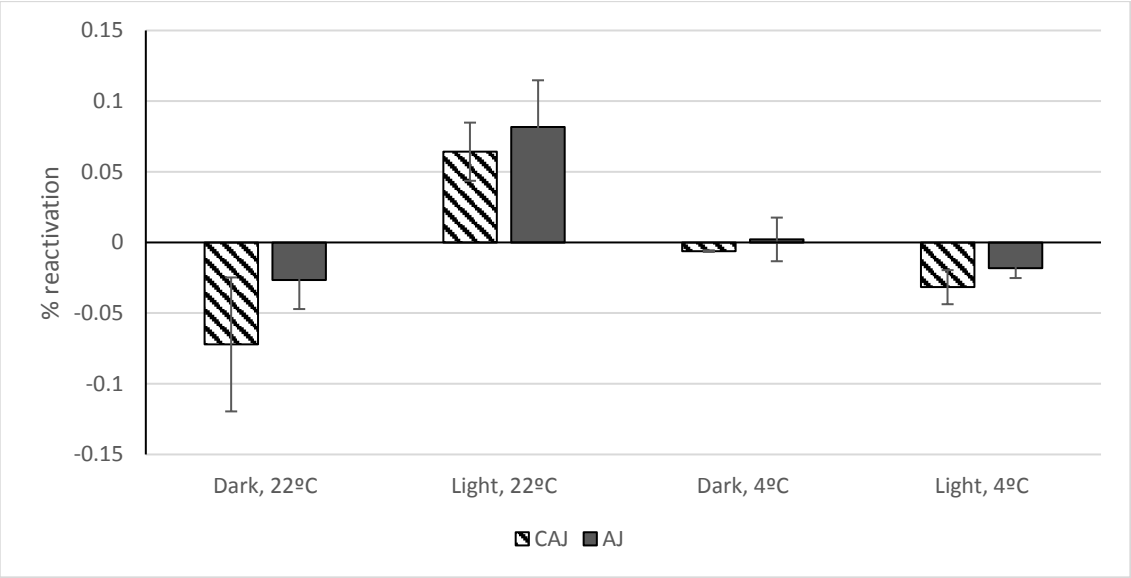


Figure 3.8. Percent reactivation of *E. coli* K12 in cloudy apple juice (CAJ) after 280/365 nm UV-LEDs irradiation for 22ºC and 4˚C reactivation temperatures at the end of 6 h incubation period

3.2.5.2. Dark Repair

Dark repair of *E. coli* K12 in cloudy apple juice (CAJ) and clear apple juice (AJ) after 280/265 nm UV-LEDs irradiation as a function of incubation time at different reactivation temperatures (22ºC and 4˚C) was demonstrated in Figure 3.9, 3.10 and 3.11.

Incubation of CAJ after 280/365 nm UV-LEDs irradiation at 22ºC in dark condition did not lead to an increase in the number of *E. coli* K12 (Fig. 3.8a). Whereas, the recovery of *E. coli* K12 cells in CAJ in dark condition was slightly increased during incubation periods of 60 min at 4º C and reached to approx. 0.6 log (Fig. 3.8b). At the end of dark incubation period (6 h), the recovery was low regardless of the incubation temperature (Fig. 3.7). The percent reactivation of *E. coli* K12 in CAJ incubated in dark condition at 22ºC was not statistically different from that of 4ºC ($p>0.05$) indicating that the repair mechanism in *E. coli* was not effective under dark conditions (Fig. 3.9).

The maximum recovery was 1.05 log obtained at 20 min in the clear apple juice (AJ) incubated in dark at 22 ˚C (Fig. 3.8c). For 1.05 log recovery, the percentage dark reactivation was 0.4% and it was far below 1% (Fig. 3.9a). The number of UV treated cells dramatically decreased reaching to negative values at the end of 6h dark incubation period (Fig 3.7). In the case of incubation under dark at 4ºC, the cells were tried to repair themselves but this repairment attempt was not resulted in permanent recovery of the

cells. Similarly, Salcedo et al. (2007) also observed a decay in survival ratio of different microbial indicators during incubation under dark at different temperature (5-30ºC) (Salcedo et al. 2007). Moreover, the negative reactivation ratio of *E. coli* O157:H7 cells was observed in apple juice after exposure to UV light at 282 nm during dark incubation period (Yin et al. 2015). In addition, such observation was also reported for *E. coli* O157:H7 following exposure to the UV light at 254 nm in buffer (saline) incubated for 48 h under dark phase (Sommer et al. 2000).

It was concluded that the cells in AJ and CAJ could not keep their viability at the end of 6 h of incubation period in dark at 4 ºC and 22ºC (Fig. 3.7). Thus, dark repair mechanism in the UV treated cells was not active in any incubation temperature. This finding was in consistent with those reported previously (Oguma et al. 2002, Quek, Hu, and biotechnology 2008, Zimmer, Slawson, and microbiology 2002). Furthermore, a decrease was observed in the survival numbers of *E. coli* K12 cells during incubation period in dark conditions. This can be explained by the damage occurred in nucleotide excision repair system during 280/365 nm UV-LED irradiation. Proteins coordinating the removal of DNA damage and enrolled in nucleotide excision repair system were denatured (Kashimada et al. 1996, Salcedo et al. 2007, Yin et al. 2015, Zimmer, Slawson, and microbiology 2002, Zimmer-Thomas et al. 2007). Especially, UV-A irradiation causes damage in the proteins of cell membrane and affects the metabolic process that is critical to survival of microorganism. The damage to membrane would result in penetration of apple juice into the cells and changing the balance of the buffer system of intracellular fluid affecting the metabolic activity of the cells (Yin et al. 2015). Thus, the combined 280/365 nm UV-LED irradiation may have induced some delayed mutagenic effects in the cells which continued to kill the cells after irradiation (Queck and Hu, 2008). Still, little is known on DNA damage and inactivation mechanism in the cells subjected to UV irradiation when combinations of different UV wavelengths are used (Nakahashi et al. 2014).

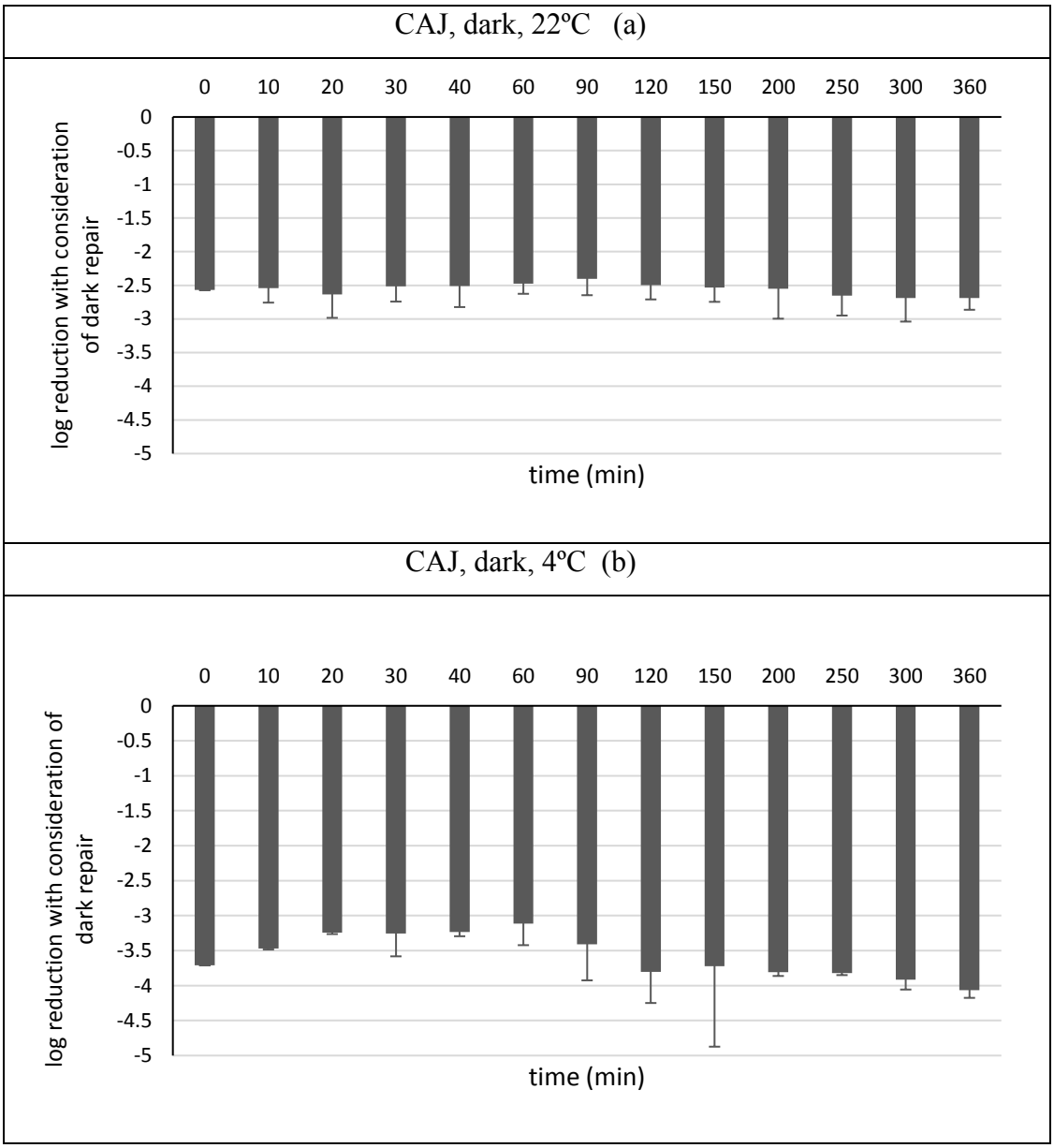
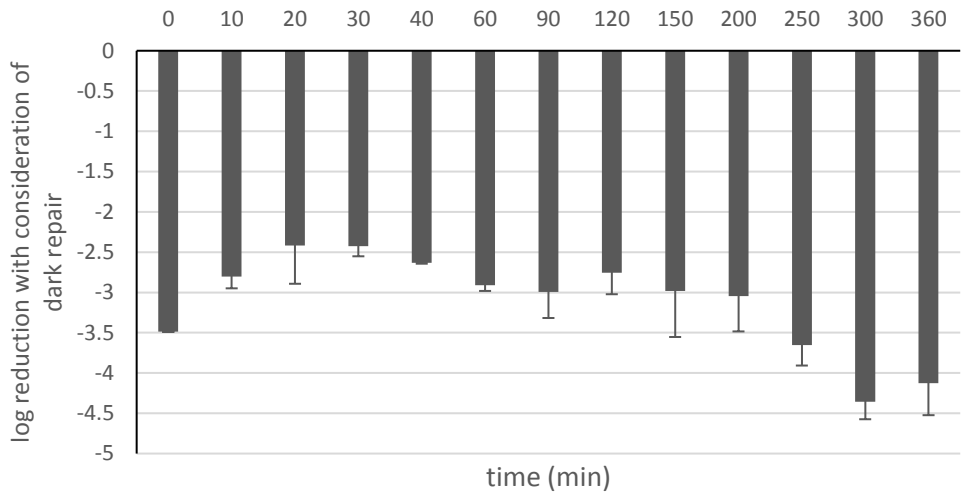


Figure 3.8. Dark repair of *E. coli* K12 in cloudy apple juice (CAJ) and clear apple juice (AJ) after 280/265 nm UV-LEDs irradiation as a function of incubation time at different reactivation temperatures (22 and 4 ˚C)

(cont. on next page)

AJ, dark, 22ºC (c)



AJ, dark, 4ºC (d)

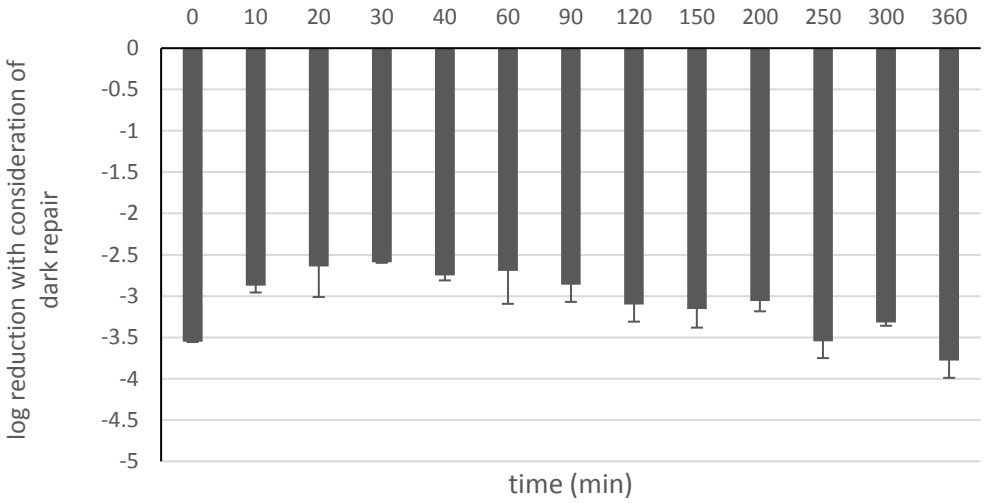


Figure 3.9 (cont.)

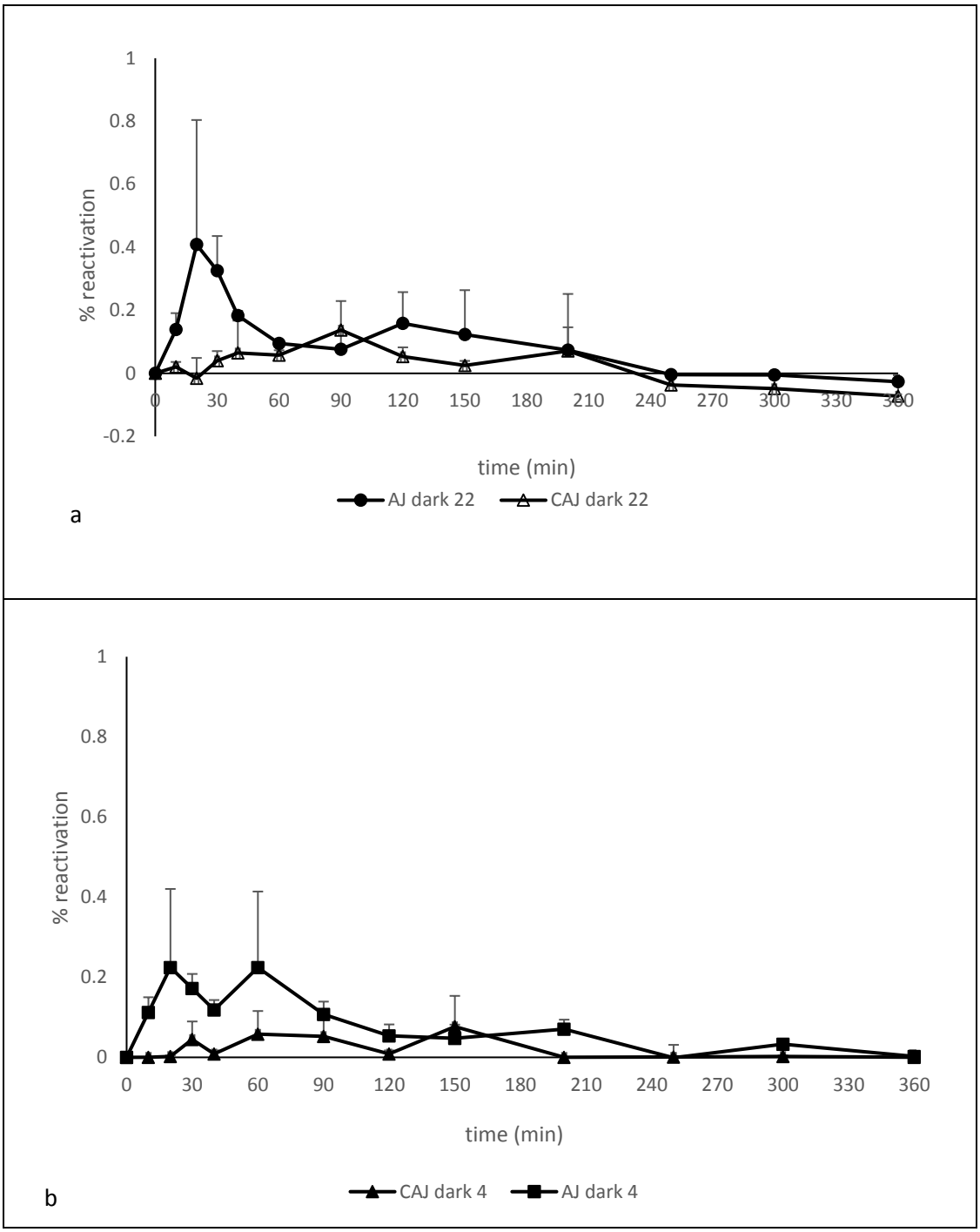


Figure 3.9. Dark repair percentages of *E. coli* K12 in cloudy apple juice (CAJ) and clear apple juice (AJ) after 280/365 nm UV-LEDs irradiation as a function of time at 22ºC and 4˚C reactivation temperatures

3.3. Conclusion

In this chapter of this thesis, the inactivation efficiency of UV-LED treatment on *E. coli* K12 in CAJ and AJ was investigated. For this purpose, the fresh apple juice

extracted from Starking Delicious variety of apples, firstly pasteurized at 70°C for 120 s and inoculated with *E. coli* K12 at a level of 6-7 log CFU/mL was subjected to different UV-LED treatments. In the first treatment, the apple juice having a liquid depth of 0.153 cm was exposed to UV-LED emitting light at 254 nm wavelength for different time periods (5 to 50 min) to optimize the exposure time of treatments. The exposure time that was applied longer than 40 minutes did not change the inactivation efficiency of the lamps significantly. Thus, the maximum exposure time for UV-LED treatments was determined as 40 min. In the other treatments, the microbial efficiency of different wavelength combinations, i.e. 254 nm, 280 nm, 280-365 nm, 280-405 nm, 254-365 nm, 254-405 nm, 254-280-365-405 nm were investigated under the same process conditions, i.e. 40 min exposure time. The results showed that using the 4 UV-LEDs were not enough to provide a 5-log reduction of *E. coli* K12 in CAJ and AJ. The highest inactivation on *E. coli* K12 in both juice samples was obtained at 280 nm wavelength using 4 lamps for 40 min exposure time. Although, coupled wavelength 280/365 nm showed slightly lower inactivation efficiency compared to that of 280 nm, it was more influential on inactivation of *E. coli* K12 than that of 254 nm UV- LEDs.

The inactivation efficacy of UV-LEDs' on PPO in freshly squeezed CAJ was also investigated. The maximum PPO inactivation was achieved when the juice samples were subjected to combination of UV-LEDs emitted light at 280 and 365 nm for 40 min exposure time. It was clearly observed that UV-LED irradiation by coupling UV-A and UV-C rays showed a better inactivation efficiency on PPO enzyme than the one used only UV-C rays. Whereas, the heat treatment was slightly more effective than UV-LEDs irradiation.

The potential of photoreactivation and dark repair ability of *E. coli* K12 were also revealed by incubating the UV treated cloudy (CAJ) and clear apple juice (AJ) at ambient (22ºC) and refrigerated (4ºC) temperatures under visible light or in the dark. The sole photoreactivation ability of *E. coli* K12 was observed in both CAJ and AJ following exposure to 280/365 nm UV-LEDs irradiation after 6 h incubation at 22ºC. Contrarily, dark repair was repressed at studied incubation temperatures, furthermore, a decrease in the survival ratio was recorded in both mediums.

Considering the microbial and enzyme inactivation efficacy and reactivation potential of *E. coli* K12 following UV irradiation, UV-LED irradiation at the combined 280/365 nm for 40 min was suggested as the optimum processing condition for apple juice treatment. However, FDA`s 5 log reduction criteria can be satisfied by combining

UV-LED irradiation with mild heating. The results of this study may lead to helpful information for the future design of UV-LED treatment systems that may be used in pasteurization of juice products.

CHAPTER 4

PASTEURIZATION OF APPLE JUICE BY A COMBINATION OF UV-LED IRRADIATION AND MILD HEAT TREATMENT

The lethal effect of UV light is influenced by the optical properties of treatment medium. The penetration of UV light in a medium shows dependency on the absorptivity of the liquid, which is related to color, transparency and the amount of suspended solids (Gayán et al. 2011). In recent years, the researchers have focused on designing the new combined processes in order to increase the antimicrobial efficacy of UV light in liquid medium. For this purpose, the combination of heat at sublethal temperatures and UV-C irradiation has been thoroughly investigated (Gayán et al. 2011, Gayán et al. 2012a, Gayán et al. 2012b, Gayan et al. 2012). However, little data and information are available regarding the germicidal effect of UV light at combined wavelengths and sublethal temperatures. The aims of this part of the thesis were to determine the efficacy of the combined UV-LED irradiation at 280/365 nm with mild heat treatment on the inactivation of *E. coli* K12 and natural microflora of cloudy apple juice (CAJ), and to evaluate the application of UV-LED irradiation assisted by mild heat (MH) as a pasteurization treatment for fruit juices. The effect of simultaneous application of UV-LED irradiation and mild heating (UV-LED+MH) on polyphenol oxidase enzyme (PPO) in CAJ was also investigated.

4.1. Material and Methods

4.1.1. Apple Juice

All the experiments in this chapter were carried out with freshly squeezed and pre-pasteurized CAJ samples. The details of preparation of CAJ samples and pre-pasteurization treatments were given in Chapter 3, section 3.2.1. and 3.3.2.1.

4.1.2. Experimental Procedure

The combined UV-LED irradiation at 280/365 nm for 40 min was selected as the best processing condition for achievement of the highest inactivation of target microorganism i.e., *E. coli* K12, and PPO enzyme in CAJ (see in Chapter 3). Whereas, this processing condition was unable to provide 5 log reductions of target microorganism in CAJ. It was hypothesized that by combining the UV-LED irradiation with mild heating might increase the microbial inactivation efficiency of UV light. This hypothesis was tested and discussed in this chapter. Figure 4.1 shows the flow diagram of the studies conducted in this chapter. Briefly, pre-pasteurized CAJ samples inoculated with *E. coli* K12 cells and freshly squeezed CAJ with natural microflora were exposed to further treatments such as UV-LED irradiation at the combination of 280/365 nm (UV-LED), mild heating (MH), UV-LED combined with mild heat treatment (UV-LED+MH). All treatments were conducted by using static UV-LED system, i.e. UV-LED irradiation was applied without any heating, MH was applied to CAJ by using the same system without turning on lamps, and combined UV+MH was applied at mild temperatures with turning on UV lamps. The effects of treatments on the inactivation of *E. coli* K12 and natural microflora in CAJ were then evaluated based on 5-log reduction of target microorganisms required for pasteurization of CAJ. Additionally, the effect of the treatments on the activity polyphenoloxidase enzyme in CAJ was also investigated by taking the new exposure times into account.

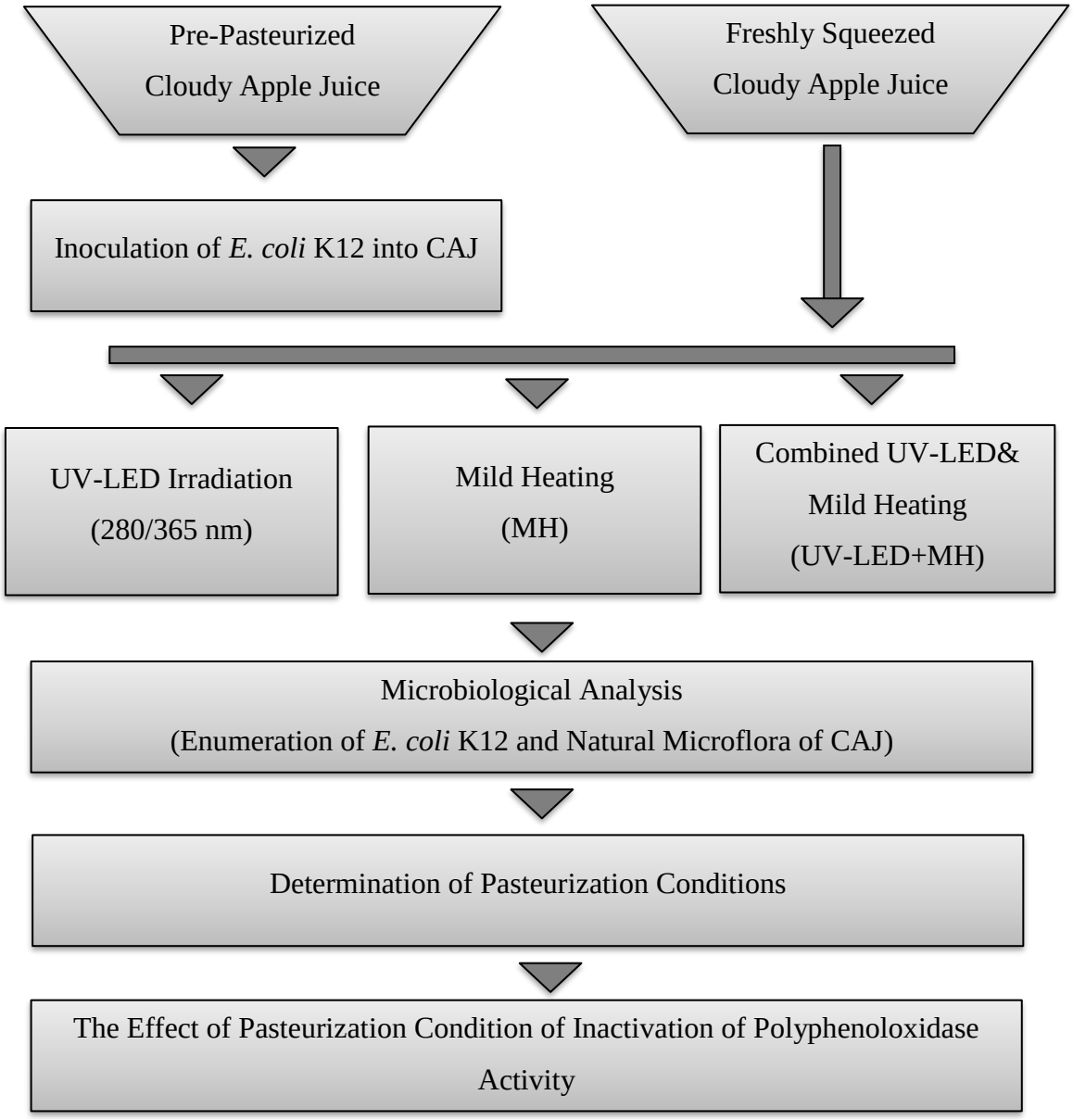


Figure 4.1. The flow diagram of inactivation studies conducted in Chapter 4.

4.1.3. Mild Heat (MH) Assisted UV-LED System

For this purpose, a small glass sample apparatus with a heating jacket was constructed and equipped with the static UV-LED system. A scheme of the experimental device is shown in Figure 4.2. The UV LEDs were fixed facedown, 1 cm above the surface of a sample (3.5 mL) placed in a sterilized glass heating unit. This unit was positioned on a stirrer to provide a good mixing in liquid medium during the process. The hot water at a constant temperature was pumped into the jacket surrounding the glass

apparatus by means of a peristaltic pump. This system allowed the sample to be exposed to UV radiation and heat simultaneously or individually.

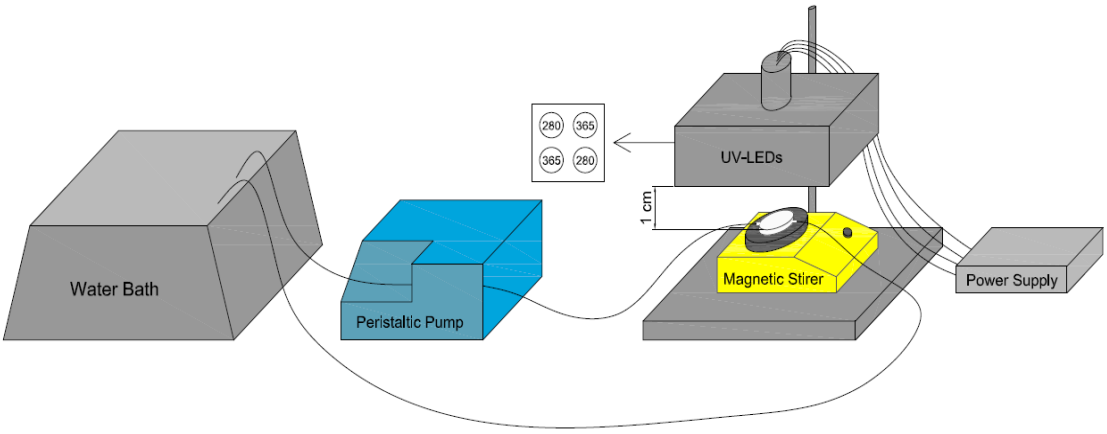


Figure 4.2. The static UV-LED system combined with a small sample apparatus providing heating

4.1.4. Target Microorganism and Inoculation of Juice Samples

It is known that the natural microflora of juice is much more resistant to UV light irradiation and heat than *E. coli* K12 (Unluturk and Atilgan, 2014). Thus, the efficacy of coupled UV-LEDs at 280/365 nm and mild heat treatment on the inactivation of *E. coli* K12 (ATCC 25253) and natural microflora of pre-pasturized and freshly squeezed CAJ were investigated.

The same protocol was carried out for preparation of sub-culture of *E. coli* K12 (see section 3.2.2). Three and half mL of pre-pastuerized CAJ was spiked with 120 µL sub-culture from stationary phase at a concentration of 10^7 CFU/mL. After the treatments, 1 ml juice samples were collected. Appropriate dilutions were made with 0.1% peptone water (Merck, Darmstadt, Germany). The enumeration of *E. coli* K12 was carried out by spread plating on Tryptic Soy Agar (TSA, MERCK, Germany) medium containing 0.1% dihydrostreptomycin. TSA plates were incubated at 37 °C for 24 h. Results were expressed as CFU/mL.

Additionally, the effect of applied treatments on background flora of freshly squeezed CAJ was also investigated. Natural flora of juice samples was analyzed before and after treatments. For this purpose, total aerobic bacteria (TAC), total coliform (TC), yeast and mold (YMC) counts in CAJ were checked by surface plating on plate count

agar (PCA, Merck, Darmstadt, Germany), violet red bile agar (VRBA, Merck, Darmstadt, Germany) and potato dextrose agar (PDA, Difco Laboratories, Detroit, MI), respectively. The PCA plates were incubated at 30 °C for 48 h, the VRBA plates were incubated at 37°C for 24 h and the PDA plates were incubated at 25 °C for 2–5 days.

4.1.5. Determination of UV–LED and Mild Heat Treatment Process Parameters

Since the application of the UV-LEDs irradiation at 280/365 nm for 40 min provided the highest inactivation of target microorganism and PPO enzyme, it was aimed to increase the efficiency of UV light by combining it with mild heating. Prior to application of combined treatment to CAJ, it was necessary to re-adjust the process parameters especially exposure time to eliminate the overtreatment of juice samples

Three different mild heat treatments such as at 40ºC, 50ºC and 55ºC were applied by means of the static UV-LED system (Figure 3.2.). When all the lamps in the system were turned off, the effects of thermal processes on *E. coli* K12 strain and natural microflora in pre-pasteurized and freshly squeezed CAJ samples were determined at three different mild heat temperatures. Then, the lamps were turned on, UV-LED irradiation was combined with three different sublethal temperatures, i.e., 40ºC, 50ºC and 55ºC to study the effect of "hurdle technology" on the microbiological quality of CAJ samples. The aim of the hurdle concept was to increase the logarithmic reduction of *E. coli* K12 and natural microflora in CAJ.

For this purpose, the temperature in water bath which was connected to static UV-LED system was adjusted 45ºC, 57ºC and 62ºC, respectively. Hot water was circulated around the glass sample apparatus to keep the juice temperature at a selected level such as 40ºC, 50ºC and 55ºC. The preliminary experiments showed that heating of juice samples for long time e.g. 40 min at these temperatures resulted in evaporation problem. In order to eliminate this problem, exposure time of the treatment was reduced. The range of exposure times was selected as 5, 10 and 15 min. The temperature of the CAJ within the sample tank was checked by K-type thermocouple (CEMDT-8891E, Shenzhen, China) during processes. The other process conditions, i.e., wavelengths, medium depth, lamp positions and distance between light sources and sample were kept the same e.g., 280/365 nm, 0.153 cm, parallel position and 1 cm as previously indicated in chapter 3.

Additionally, the effect of simultaneous application of UV-LED irradiation and mild heating on activity polyphenol oxidase enzyme in CAJ was investigated. The method details of measurement of PPO activity were given in Chapter 3, section 3.2.4.

4.1.6. Data Analysis

All of the processes were repeated three times. The results of microbiological analyses of apple juice samples were expressed as their means and standard deviations. Measurements were done in triplicated. Regression analysis was applied by using a commercial spreadsheet (Microsoft Excel, Redmond, WA, USA). One-way analysis of variance (ANOVA) was carried out to determine how significantly the independent variables (UV-LED, Mild Heating and UV-LED+MH) affect the dependent variables (microbiological, residual activity of PPO of apple juice) by using the Minitab 16 software program (Minitab Inc., State College, PA, USA). The means were evaluated in terms of Tukey comparison test with a 95% confident interval. Differences of data were significant for p-value is equal or less than 0.05 ($p \leq 0.05$).

4.2. Results and Discussions

4.2.1. Inactivation of *E. coli* K12 by UV-LED irradiation, mild heat (MH) and combined UV-LED+MH Treatments

Figure 4.3 depicts the logarithmic reductions of *E. coli* K12 in CAJ exposed to UV-LED irradiation, mild heat and combined UV-LED+MH treatments at 40ºC, 50 ºC and 55 ºC for different exposure times, i.e., 5, 10 and 15 min. The absorption coefficient of pre-pasteurized CAJ was 20.11cm^{-1} and 4.32 cm^{-1} at 280 nm and 365 nm, respectively. The treatment of CAJ with 280/365 nm UV-LED irradiation for 15 min resulted in 1.20±0.07 log CFU/mL reductions of *E. coli* K12. This low level of reduction was due to the high UV absorption coefficient of apple juice. Different works in the literature have been conducted to analyze the effect of UV light on *E. coli* population in apple juice and proposed that optical properties such as turbidity and absorption coefficient of the liquid media play a significant role in germicidal efficacy of UV light (Basaran et al. 2004, Franz et al. 2009, Müller et al. 2014). On the other hand, the maximum reduction

of *E. coli* K12 in CAJ was 4.44±0.27 log CFU/mL obtained after 15 min mild heat treatment of juice samples at 55ºC. Whereas, 2.11 and 4.31 log CFU/mL reductions of *E. coli* K12 were achieved at 40ºC and 50 ºC after 15 min. Gayán et al. (2012b) observed the same trend for *E. coli* STCC 4201 in commercial apple juice. They treated juice samples at sub-lethal temperatures ranged from 40ºC to 55ºC.

It was clearly observed that individual treatments such as UV-LED irradiation at 280/365 nm and mild heating at 40ºC, 50 ºC, 55ºC applied for 15 min were insufficient for pasteurization of CAJ. In other words, FDA requirement (5 log reduction) for juice pasteurization was not met. On the other hand, when the UV-LED irradiation at 280/365 nm was combined with mild heating, the inactivation of *E. coli* K12 in CAJ significantly increased (Fig. 4.3). For example, 5.36±0.21, 6.08±0.01 and 6.08±0.04 log CFU/mL reductions were observed when the CAJ samples treated with 280/365 nm UV-LED irradiation for 5 min at 40, 50, and 55ºC, respectively. The results showed that a 5-minute application of UV-LED assisted by mild heat provided >5 log reduction in target microorganism in CAJ and met the pasteurization requirement recommended by the FDA. The combination of UV light and mild heating for juice processing has been proposed by some authors. The sub-lethal heat effect of UV+ MH treatment was reported in those studies. For example, Ukuku and Geveke (2010) combined UV light and radio frequency electric field (RFEF) treatments for inactivation of *E. coli* K12 in apple juice at non-lethal temperatures up to 40 °C. The authors found that a combination of UV and RFEF (40ºC) provided 5.8 log CFU/mL reductions at 40°C. Our results were in agreement with these data. Also, Walkling-Ribeiro et al. (2008) found an additive effect in apple juice processed by a hurdle sequence of UV light, pre-heating (46°C), and pulsed electrical field (PEF). In other studies, the lethal effect of mild heat treatment combined with UV-C light was reported for temperatures above 55°C (Gayán, Álvarez, and Condón 2013, Gayán et al. 2011, Gayán et al. 2012a, Gayán et al. 2012b, Ross, Zhang, and McQuestin 2008).

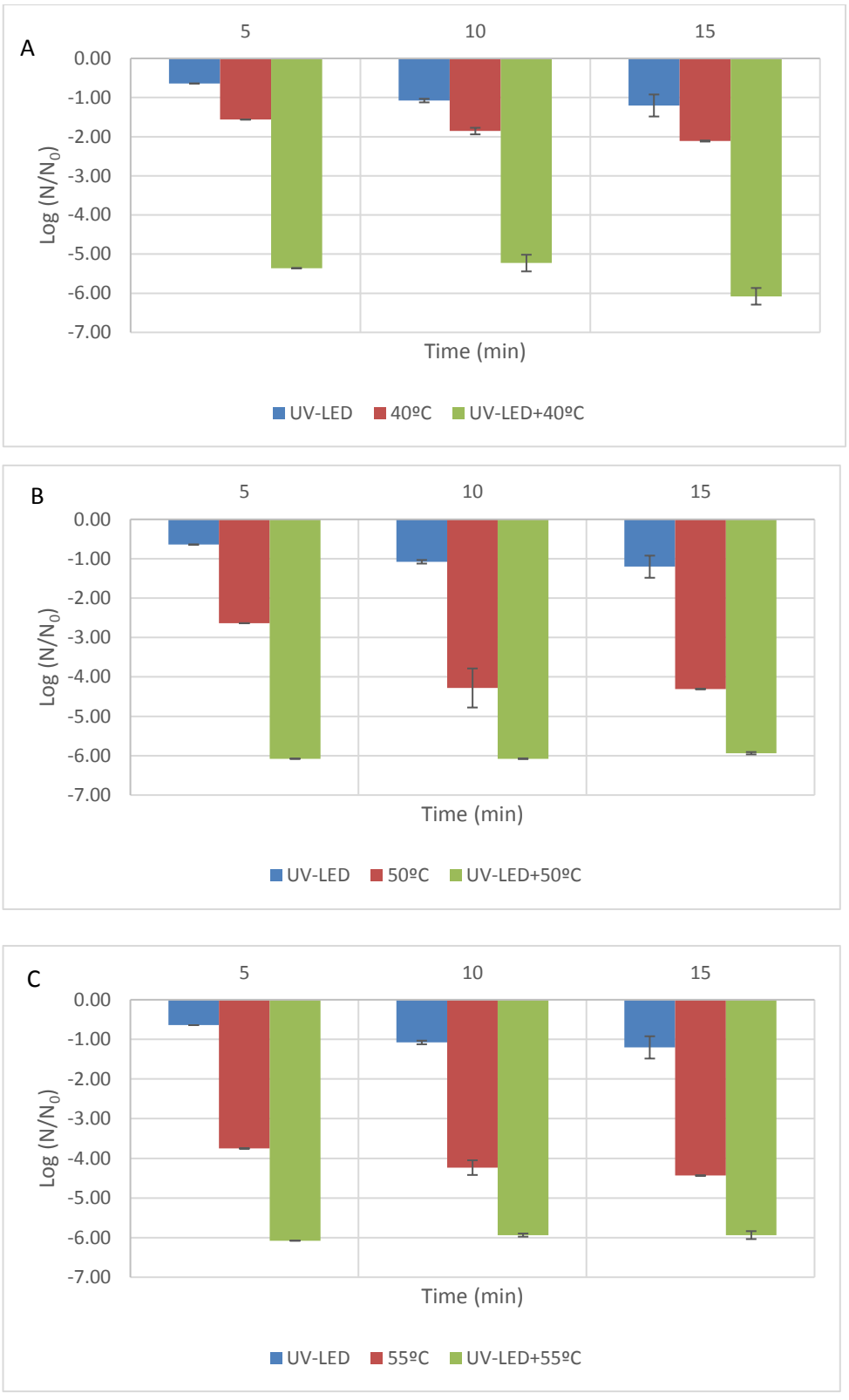


Figure 4.3. Logarithmic reductions of *E. coli* K12 in CAJ exposed to UV-LED irradiation, Mild Heat and the combined UV-LED+MH treatment at 40ºC (A), 50 ºC (B) and 55 ºC (C).

Figure 4.4. shows the logarithmic reductions (log CFU/mL) of *E. coli* K12 in CAJ treated with UV-LED irradiation and mild heat at 40ºC, 50ºC and 55ºC. The results were compared with the case where CAJ treated simultaneously with UV-LED irradiation and mild heat. The additive (blue and red color bars) and synergistic effect (orange color bars) of these treatments were compared in Figure 4.4. In order to investigate whether there is a synergistic effect of the combined UV-LED irradiation and mild heat treatment on target microorganism, the logarithmic reductions obtained after individual treatments were summed up. It was demonstrated that the lethality of the combined process was the result of a synergistic effect of both technologies applied simultaneously. For example, when the logarithmic reductions of *E. coli* K12 in CAJ after applications of individual treatments for 5 min, i.e, UV-LED and mild heat, were summed up, > 2 log CF/mL reductions were obtained (Fig. 4.4). In contrast, when these treatments were simultaneously applied to CAJ, the resultant log reductions was higher than 5 log. The results also demonstrated that *E. coli* K12 was more sensitive to heat treatment than UV irradiation process. The lethality was high at high temperatures (50, 55 ˚C). Thermal dependence of microbial inactivation by UV light is lower than that of heat inactivation. In other words, the synergism between UV light and mild temperature process increased up to a threshold temperature value but above this value the bacterial inactivation occurred by the lethal effect of heat only.

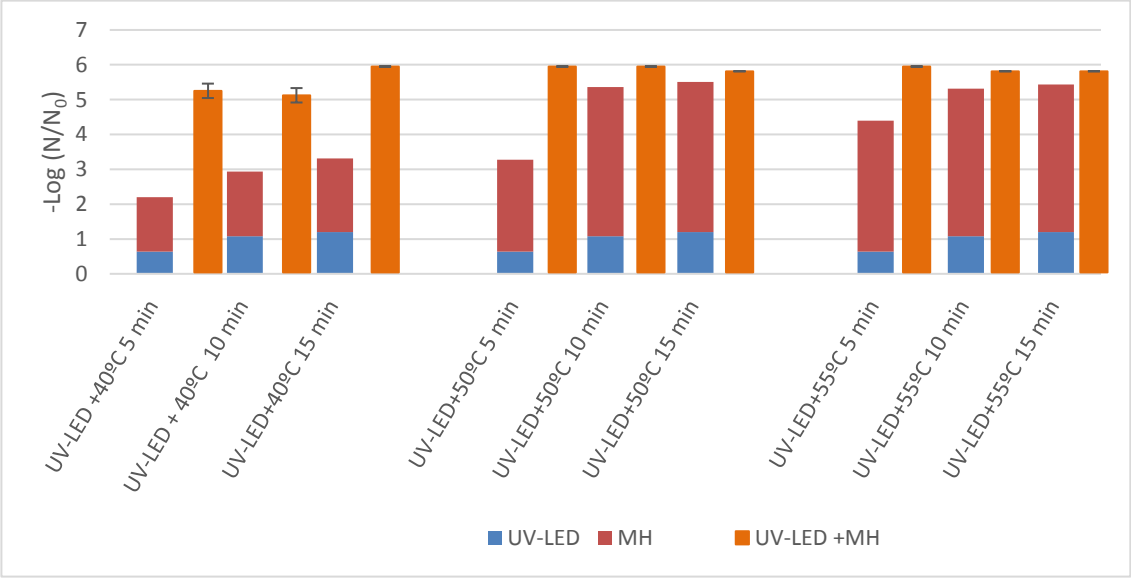


Figure 4.4. The logarithmic reductions (log CFU/mL) of *E. coli* K12 in CAJ treated with individually and simultaneously by UV-LED irradiation and mild heat at 40ºC, 50ºC and 55ºC

4.2.2. Effect of the UV-LED and Mild Heat Treatment on Inactivation of Natural Microflora in Freshly Squeezed Cloudy Apple Juice

It was hypothesized that the hurdle treatment condition (280/365 nm UV-LED + MH at 40 ˚C) which provided a 5-log reduction on *E. coli* K12 would not be sufficient to eliminate natural flora of cloudy apple juice (CAJ). In the first part of this study, freshly squeezed CAJ (24.60_{280nm} cm^{-1}; 6.85_{365nm} cm^{-1}, 829 NTU) samples with 4.04 log CFU/mL of total mesophilic aerobic bacteria count (TAC) and 4.27 log CFU/mL of yeast and mold count (YMC), no initial load of total coliform (TC), were subjected to individual and simultaneous treatments of UV-LED irradiation and mild heat at 50 ºC and 55 ºC for 5, 10 and 15 min.

The logarithmic reductions of TAC and YMC in CAJ samples subjected to different processes i.e. UV-LED, Mild Heating (MH) and UV-LED+MH at 50ºC were depicted in Figure 4.5. The results showed that UV-LED (280/365 nm) irradiation did not have a significant lethal effect on microbial load of freshly squeezed CAJ (Figure 4.5.A). TAC was reduced by 0.02±0.09, 0.05±0.13 and 0.04±0.02 log CFU/mL when CAJ exposed to 280/365 nm UV-LED for 5, 10 and 15 min, respectively. Also, the application of mild heating at 50ºC for 5, 10 and 15 min on freshly squeezed CAJ was resulted in 0.16±0.03, 0.46±0.11 and 0.29±0.24 log CFU/mL reductions in the total mesophilic aerobic count (TAC). When comparing the lethal effect of UV-LED irradiation and mild heat, separately, it was observed that inactivation of natural microflora in CAJ using UV-LED (280/365 nm, 15 min) irradiation and mild heat (50ºC, 5 to 15 min) were negligible (<0.3 log CFU/mL). It could be as a result that the high absorptive and turbid CAJ blocked the UV light and did not induce sufficiently cytoplasmic fluidification, respectively. However, simultaneous application of UV-LED+MH at 50ºC was resulted in higher TAC reduction (0.40±0.04, 0.89±0.08 and 0.42±0.03 log CFU/mL) in CAJ. Similarly, UV-LED irradiation alone was not sufficient to inactivate yeast and molds in freshly squeezed CAJ (Figure 4.5.B). On the other hand, simultaneous processing of CAJ with UV-LED and MH was more effective on yeast and molds. Logarithmic reductions achieved for YMC of CAJ after combined mild heat and UV-LED irradiation were recorded as 0.72±0.13 log CFU/mL, 0.63±0.13 log CFU/mL, 0.86±0.03 log CFU/mL, respectively.

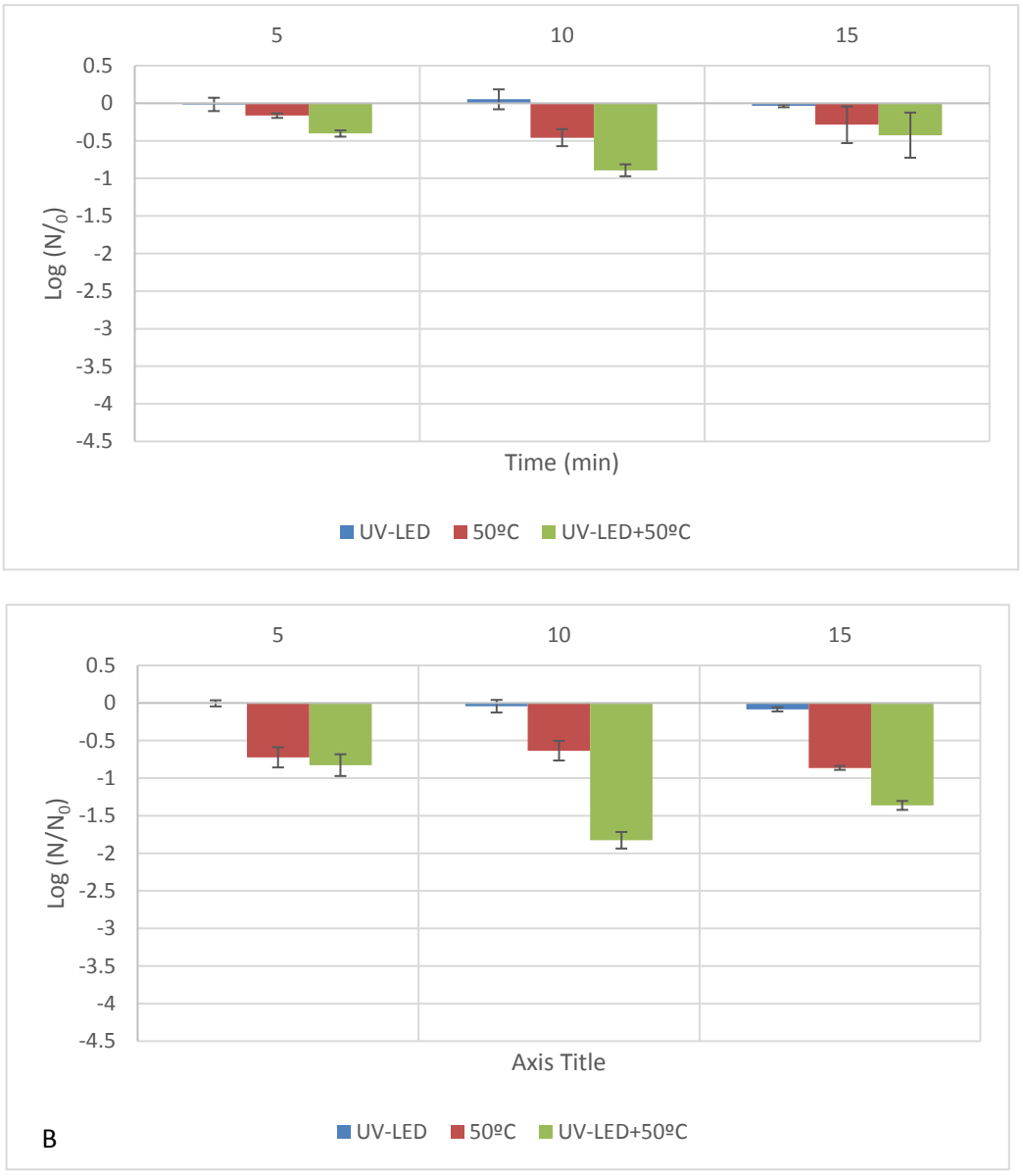
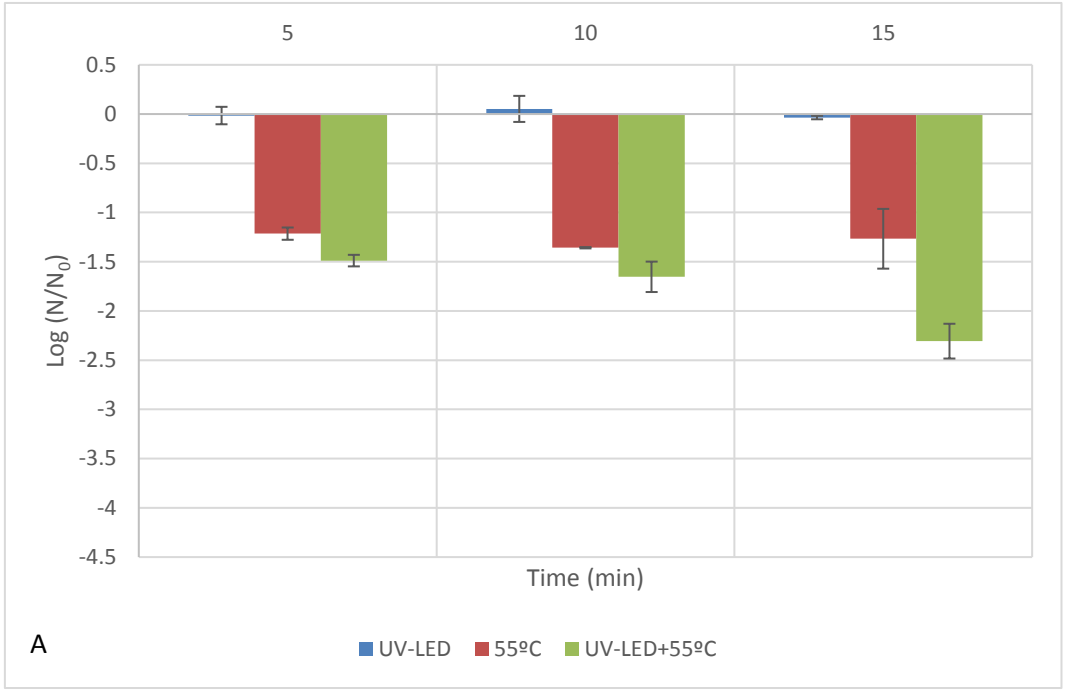


Figure 4.5. Inactivation of mesophilic aerobic bacteria (A) and yeast and molds (B) in CAJ subjected to UV-LED (280/365 nm), MH (50°C) and combination of UV-LED+MH treatments for 5, 10 and 15 min.

Similarly, Gayan et al. (2012) investigated the lethal effect of combined mild heat, and individual mild heat process before and after UV-C irradiation on *Salmonella typhimurium* in McIlvaine buffer adjusted to a pH of 7.0. They observed that an application of heat treatment at 55ºC before the UV irradiation did not increase the efficiency of UV process. The additive effect was observed when UV light treatment was applied after the heat treatment, but the efficiency of individual process was lower than that observed for the case where a combination of these technologies was applied. In many studies, it has been claimed that the synergistic lethal effect may exist in the

temperature range of 50 °C to 60 °C depending on the type of microorganism and the optical properties of the fruit juice (Gayán et al. 2011, Gayán et al. 2012a, Gayán et al. 2012b, Gouma et al. 2015, Ross, Zhang, and McQuestin 2008).



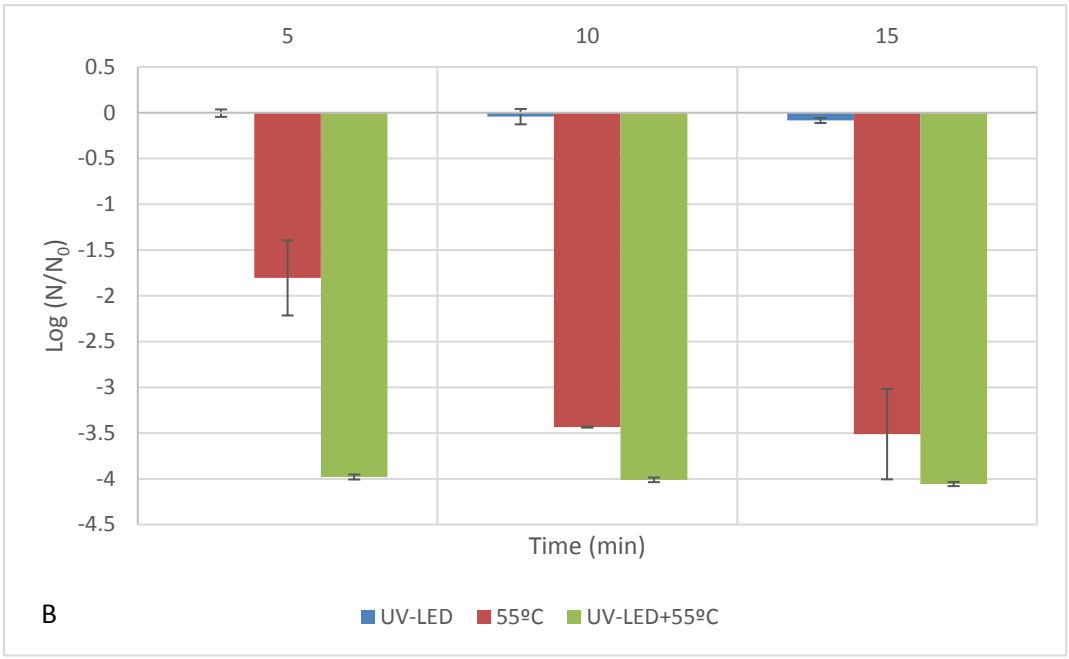


Figure 4.6. Inactivation of total mesophilic count (A) and yeast and molds count (B) in CAJ subjected to UV-LED (280/365 nm), MH (55°C) and combination of UV-LED+MH treatments for 5, 10 and 15 min

In the second part of this study, the process conditions were modified, i.e., the mild heat temperature was increased to 55ºC. Exposure times were kept constant as 5, 10 and 15 min. Freshly squeezed CAJ had 3.82 log CFU/mL of TAC and 4.26 log CFU/mL of YMC initial microbial load. Figure 4.6 demonstrates logarithmic reductions achieved for TAC and YMC of CAJ treated with mild heat (55˚C), UV-LED (280/365 nm) and UV-LED (280/365 nm) + Mild Heat (MH) (55ºC) at different exposure times ranging from 5 min to 15 min. Individual UV-LED (280/365 nm) treatment applied to CAJ had negligible lethal effects on TAC and YMC. Mild heating (55ºC) for 5, 10 and 15 min decreased the TAC and YMC by 1.21±0.62, 1.36±0.01, 1.27±0.30; and 1.81±0.71, 3.44±0.01, 3.51±0.79 log CFU/mL, respectively. The results showed that individual mild heat application at 55ºC had a lethal effect on natural microflora of CAJ. Additionally, the lethal effect of heating on YMC was more pronounced than that of TAC. It is known that yeast and molds are highly sensitive to heat (Gouma et al. 2015). Besides, it was found that a combination of UV-LED irradiation with mild heat at (55ºC) increased the inactivation efficiency of UV-LEDs. TAC was reduced by 1.49±0.06, 1.65±0.15 and 2.31±0.18 log CFU/mL, respectively.

Synergism:

The synergistic effect of the simultaneous application of UV-LED irradiation and mild heating on inactivation of natural microflora was also evaluated by comparing the inactivation data obtained by sum of the individual application of UV-LED irradiation and mild heating with simultaneous application of them. Synergistic effect on TAC and YMC raised by increasing temperature from 50 to 55ºC (Figure 4.7 and 4.8). It was clearly observed from Figure 4.7 that rising exposure time allowed to increase synergistic lethal effect on TAC when combined UV-LED irradiation with MH at 55 ºC. UV-LED+MH application for 15 min exposure time on TAC (2.31 log CFU/mL) had significantly higher inactivation efficiency, approximately 1 log CFU/mL, than both hurdle treatment applied to other exposure times and the sum of UV-LED irradiation and MH, separately. Inactivation level of YMC by combined treatment was higher than sum of the reductions obtained by application of the individual UV-LED irradiation and mild heating. Thus, the synergistic lethal effect was existing when UV-LED and MH (50ºC) treatments were combined and applied simultaneously (Figure 4.8).

Similar lethal effects have been described in bacteria and yeasts in fruit juices (Gayán et al. 2011, Gayán et al. 2012a, Gayán et al. 2012b, Gouma et al. 2015, Ross, Zhang, and McQuestin 2008). Gayan et al. (2013) reported that *E. coli* and *L. monocytogenes* inactivation by UV-MH (27.10 J/mL UV doses) was increased at 55ºC indicating a synergistic effect. The inactivation mechanisms of UV irradiation combined with mild heat could be related to the inhibition of DNA repair systems and inducing of the cytoplasmic membrane fluidification occurred simultaneously. Although, in our study, the inactivation level of YMC seemed to be higher than TAC, combined UV-LED+MH treatment at 55 °C had pronounced synergistic effect on the inactivation of TAC. The synergism was increased by increasing exposure time. It is known that the yeast and molds are more resistant to UV irradiation than bacteria due to their larger sizes, their thicker cell wall and lower content of thymine or cytosine basis in their genomes (Tran and Farid, 2004). Whereas, the combination of UV-LED (280/365 nm) and mild heat (55 ºC) treatment was found very effective on inactivation of yeast and mold in CAJ.

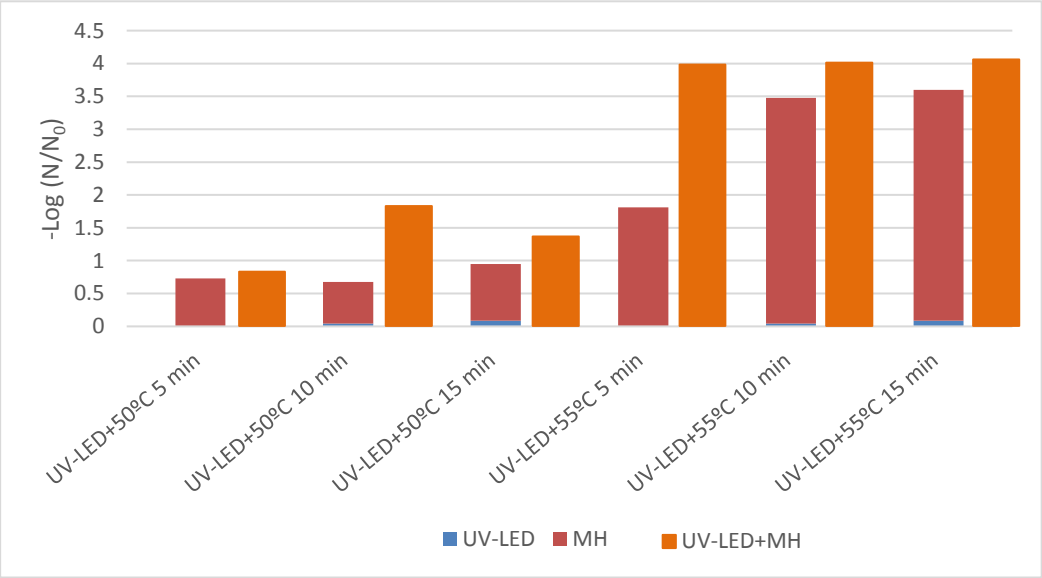


Figure 4.7. The logarithmic reductions (log CFU/mL) of TAC in CAJ treated with individually and simultaneously by UV-LED irradiation and mild heat at 50ºC and 55ºC

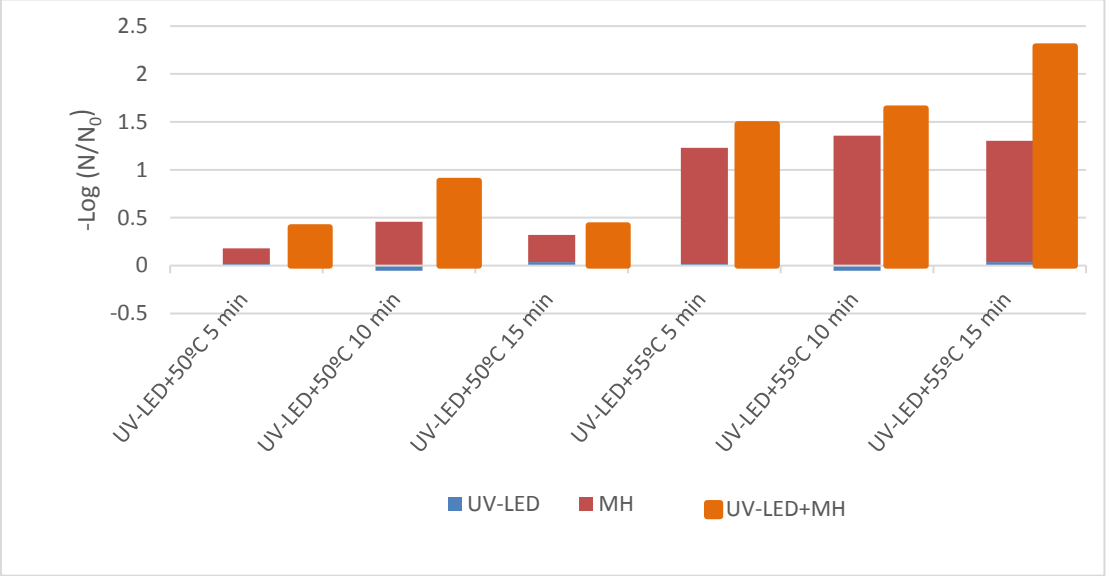


Figure 4.8. The logarithmic reductions (log CFU/mL) of YMC in CAJ treated with individually and simultaneously by UV-LED irradiation and mild heat at 50ºC and 55ºC

In conclusion, a combination of the UV-LED (280/365 nm) and MH (55ºC) treatment applied to CAJ for 15 min achieved maximum reductions in TAC and YMC, i.e. > 4 log CFU/mL. Thus, these processing conditions were selected as the best conditions for pasteurization of CAJ. According to the microbiological criteria of Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21-1997) and Institute of Food Science and Technology (1999) recommended for juice products, TAC and YMC allowable limits in pasteurized juice are required to be maximum 4 and 3 log CFU/mL, respectively (IFST, 1999). Thus, a simultaneous UV-LED (280/365 nm) and MH (55ºC) application to CAJ for 15 min was able to meet the criteria established for pasteurized fruit juice.

4.2.3. Effect of the Combined UV-LED and Mild Heat Treatment on PPO Activity in Freshly Squeezed Cloudy Apple Juice (CAJ)

One of the aims of this study was to inactivate PPO enzyme in CAJ by means of UV-LED+MH treatment. This enzyme is responsible for the browning and causing the loss of juice quality. Therefore, freshly squeezed CAJ was treated with the hurdle approach for 15 min by combining UV-LED (280/365 nm) and mild heating (55ºC). The

treated samples were analyzed in terms of their PPO activity. Table 4.1. depicts the results of PPO enzyme residual activity of untreated samples and treated samples with combined UV-LED (280/365 nm) and mild heat (55ºC). Data related to the mild heat treatment and UV-LED treatment were also given for comparison of their individual effects on PPO. Significant statistical differences were observed in between the treatments. The individual MH and UV-LED, and combined UV-LED+MH treatments were resulted in 65.02, 34.87 and 24.57 % residual PPO activity in CAJ. The residual PPO activity achieved in 15 min treatment of UV-LED (280/365 nm) was found higher when compared with data obtained in the previous chapter. We previously found that UV-LED (280/365 nm) irradiation of CAJ for 40 min resulted in %33 PPO residual activity. Comparing these findings, it could be speculated that the application of UV-LED treatment for longer time may result in higher inactivation of PPO enzyme in CAJ. This might be due to the additional effect caused by UVA light. UV-A light can damage lipids, proteins and DNA via the photooxidation of O_2. When we combined the UV-LED and MH, inactivation rate of PPO was dramatically increased. Overall, our results demonstrated that the combined UV-LED+MH process was more efficient for the PPO inactivation than individual UV-LED irradiation applied at room temperature.

Similarly, Gayan et al. (2013) observed significant inactivation effect on PPO enzyme when UV-C and MH was combined. They observed that the combination of UV-C and mild heating at 55.0 °C for 3.58 min reduced the initial PPO activity of the apple juice by 39.39 %. Other authors (Falguera et al. 2012, Falguera, Pagán, and Ibarz 2011, Manzocco, Quarta, and Dri 2009) have demonstrated the ability of UV light to reduce the activity of the PPO enzyme. Contrarily, Tran and Farid (2004) and Noci et al. (2008) found that UV-C light treatment had no noticeable effect on the enzymes such as pectinmethylesterase (PME), polyphenoloxidase (PPO) and peroxidase (POD). Müller et al. (2014) investigated the effect of UV-C and UV-B irradiation on PPO inactivation in apple (52.4 cm^{-1}) and grape (43.5 cm^{-1}) juice by means of UV-C processing equipped with a Dean flow reactor. Even though, they did not observe any significant effect of UV-C light on PPO enzyme activity, a reduction of PPO activity of more than 20% and 40% was reported in apple and grape juices subjected to UV-B (290-315 nm) irradiation, at UV dose of 100.48 kJ/L. Falguera, Pagán, and Ibarz (2011) irradiated fresh apple juices for 120 min using a high-pressure mercury UV lamp (400 W, emitting light between 250 and 740 nm), They were able to inactivate PPO in 100 min, while peroxidase was completely destroyed in only 15 min.

Table 4.1. PPO residual activity in CAJ treated with UV-LED (280/365 nm) mild heating (55ºC) and combination of UV-LED+MH for 15 min

		PPO (Unit)	% PPO residual activity
UV-LED (280/365)	Untreated	19374±787.53	
	Treated	12180±593	65.02±4.84[a]
MH (55ºC)	Untreated	19374±787.53	
	Treated	6756±334.42	34.87±0.72[b]
UV-LED + MH (280/365 nm/55°C)	Untreated	19374±787.53	
	Treated	4761±215.63	24.57±0.15[c]

Tukey least significant difference test was applied. Different letters for each process show the differences in PPO residual activity ($p<0.05$).

4.3. Conclusion

In this chapter of this thesis, it was aimed to increase the microbial efficiency of UV-LEDs irradiation at 280/365 nm by combining with mild heat at 40ºC, 50ºC, and 55ºC. It was clearly observed that individual UV-LED irradiation at 280/365 nm and mild heat treatment at different temperatures (40ºC, 50ºC, and 55ºC) were insufficient for pasteurization of CAJ. Simultaneous application of UV-LED irradiation and mild heat treatment significantly increased the inactivation of *E. coli* K12 in CAJ. The results showed that an application of UV-LED+MH at different temperatures for 5 min met the pasteurization requirement recommended by the FDA (5 log reduction). In addition to these, the combination of UV-LED irradiation and mild heat treatment on target microorganism showed a synergistic effect.

Moreover, the efficacy of simultaneous application of UV-LED and mild heat treatment on the inactivation of natural microflora of freshly squeezed CAJ was also investigated. The fresh juice was subjected to UV-LED irradiation, mild heating at 50 ºC and 55 ºC and a combined UV-LED+MH treatment for 5, 10 and 15 min. The results showed that UV-LED (280/365 nm) irradiation, and mild heating at 50ºC did not have a pronounced lethal effect on total mesophilic aerobic bacteria of freshly squeezed CAJ. Moreover, a combination of UV-LED irradiation with mild heat at 50ºC for 15 min was inadequate to inactivate the natural microflora in CAJ. Whereas, when the mild heat temperature was increased to 55ºC and combined with the UV-LED irradiation, the maximum reductions of TAC and YMC in freshly squeezed CAJ were achieved. Thus, 280/365 nm UV-LED irradiation assisted by mild heat at 55ºC for 15 min was selected

as the best conditions for inactivation of natural microflora in CAJ. Under this condition, the residual activity of PPO was also reduced to 24.57%.

Additionally, the existence of cell damages induced by the combination of the UV-LED and mild heat stresses should be evaluated. This would contribute to a better understanding of the microbial response to the proposed combined treatments.

CHAPTER 5

SHELF LIFE OF CLOUDY APPLE JUICE (CAJ) PASTUERIZED BY COMBINED UV-LED AND MILD HEAT TREATMENT

The aim of this section is to monitor and evaluate the various quality characteristics of CAJ processed by combined UV-LED irradiation and mild heat during storage period. The process conditions were extensively studied and determined in the chapter 4. According to the selected best conditions, the CAJ samples were treated with 280/365 nm UV-LED irradiation assisted by mild heat at 55 ˚C for 15 min. Then, they were stored for 60 days under refrigerated conditions. Untreated freshly squeezed CAJ and thermally pasteurized CAJ at 70˚C for 120 s were used as negative and positive control. The microbial stability of the samples was evaluated by counting of their total aerobic mesophilic, yeast and molds and total coliform populations. Moreover, the changes in some physicochemical, optical and phytochemical properties such as pH, color, ascorbic acid content, polyphenol oxidase activity and total phenolic content of CAJ were also monitored during the storage.

5.1. Material and Methods

5.1.1. Preparation of Apple Juice Samples

The production of cloudy apple juice (CAJ) was described in section 3.2.1 in detail.

5.1.2. Thermal Pasteurization of Apple Juice

Thermally pasteurized cloudy apple juice (CAJ) (70°C/120 s) was used as positive control in the shelf life study. Freshly squeezed CAJ were processed using the continuous flow thermal pasteurization system (Figure 5.1). Five hundred milliliter of CAJ was initially heated up to 70 °C on the hot plate by stirring in a beaker and transferred to the sample tank of the heat pasteurization system (1). Then, CAJ in the sample tank was

immediately pumped by a peristaltic pump (2) to the helical stainless-steel tube of the system. Temperature of the helical tube was adjusted to 71ºC by using a water bath (3). CAJ sample was passed throughout the helical region of the system with a flow rate of 0.72 mL/s for 120 s, i.e., the pump frequency was adjusted to 13 rpm, and then cooled immediately within an ice bath (4) before analyses and storage. After thermal process, continuous heat pasteurization system was disinfected by passing boiling water several times throughout the system.

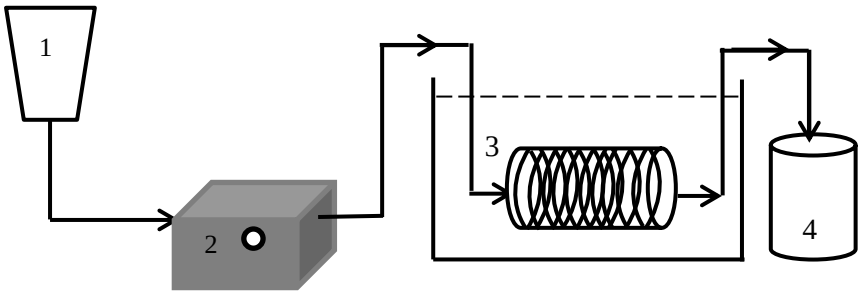


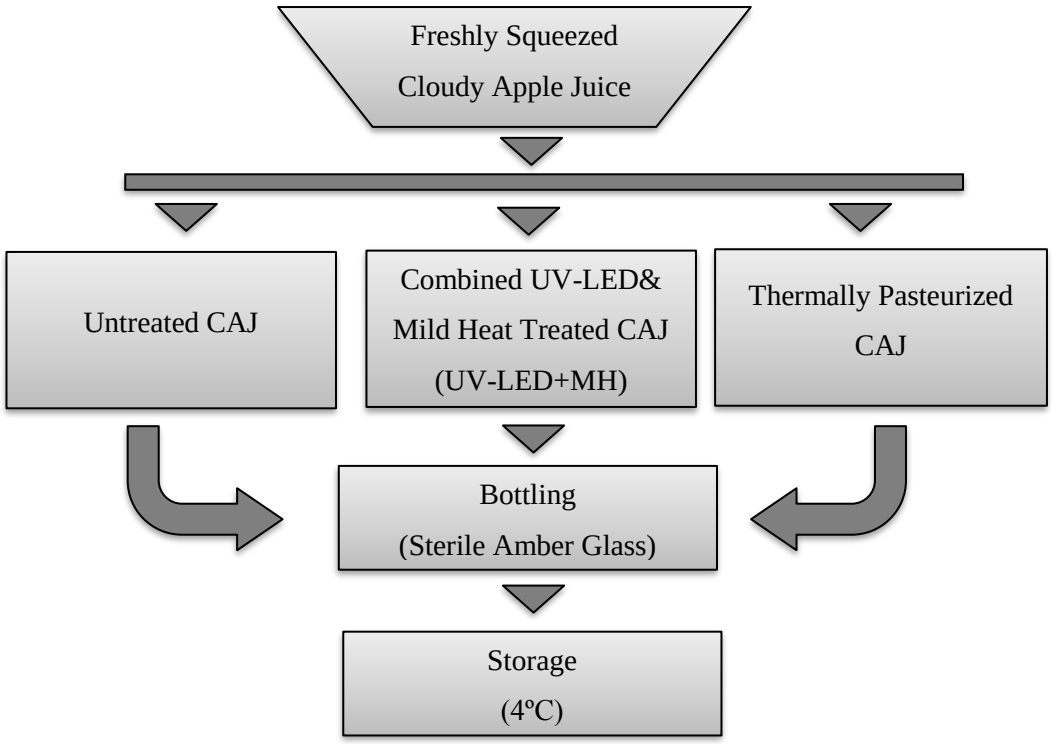
Figure 5.1. Schematic diagram of thermal pasteurization system
(1: sample tank; 2: peristaltic pump; 3: heating coil; 4: cooler)

5.1.3. Pasteurization with Combined UV-LED and Mild Heat Treatment

Cloudy apple juice (CAJ) was pasteurized by using a combined UV-LED and mild heat treatment. CAJ initially subjected to 280/365 nm UV-LED irradiation assisted by mild heat at 55˚C for 15 min. These process conditions were selected according to the results depicted in Chapter 4. For the simultaneous application UV-LED and mild heat treatment, three milliliter CAJ poured into the glass sample apparatus. The apparatus was connected the water bath and juice sample temperature was adjusted to 55ºC. Then the four UV-LED emitted light at 280 nm (2L) and 365 nm (2L) turned on. CAJ was subjected to simultaneously UV-LED and mild heat at 55ºC during 15 min. After the treatment, pasteurized juice sample (3 mL) was collected in a sterile amber glass bottle and stored at the refrigeration condition up to appropriate volume (300 mL) for shelf life study.

5.1.4. Storage and Analysis of CAJ during Shelf Life Study

An approximate volume of 15 mL of the untreated, thermally pasteurized and UV-LED+MH treated CAJ samples were transferred into sterile amber glass bottles and stored at refrigerated condition (4ºC) for 60 days, with periodic sampling 0, 3, 9, 20, 30, 40, 50 and 60 days. The experiments were done in duplicates. The main steps involved in the shelf life study depicted in Figure 5.2.



Microbiological Analysis	
Total aerobic mesophilic count (TAC)	
Yeast and molds count (YMC)	
Coliform bacteria count (TC)	

Physico-chemical and Optical Analysis	
pH	Polyphenoloxidase activity (%)
Color (L*, a*, b*, ΔE)	Total phenolic content (%)
Ascorbic acid content	

Figure 5.2. The diagram of shelf life study of CAJ

The samples microbial stabilities were analyzed in terms of aerobic mesophilic plate count (TAC), yeast and molds count (YMC) and coliform bacteria count (TC) by spread plating on PCA (Merck, Darmstadt, Germany), PDA (Merck, Darmstadt, Germany) and VRB-A (Merck, Darmstadt, Germany), respectively during shelf life.

5.1.4.1. Physical, Chemical and Optical Properties of Apple Juice

The changes in some physicochemical and phytochemical properties including color and pH, ascorbic acid content, polyphenol oxidase enzyme and total phenolic content of juice samples were monitored during shelf life period according to the procedures outlined in chapter 3, section 3.1.2.

5.1.4.2. Total Phenolic Content

Total phenolic contents of CAJ samples were determined by using Folin-Ciocalteu method (Tezcan et al. 2009). CAJ samples were firstly diluted in the ratio of 1:20 by using MeOH:H_2O (3:2). 300 μL of diluted CAJ mixed with 1.5 mL of Folin–Ciocalteu reagent (diluted 10-fold with distilled water) in a glass tube followed by the addition of 1.2 mL of sodium carbonate (75 g/L) considering a time interval up to 5 min. The mixture was thoroughly mixed by using vortex mixer (ZX3, VELP Scientifica S.r.l., Usmate, Italy) and allowed to stand for a further 90 min in dark and the absorbance was measured at 760 nm using a spectrophotometer (Agilent Technologies, Palo Alto, USA). The solvent MeOH:H_2O (3:2) was used to set to read zero absorbance prior to measurements. A control sample tube (blank) was prepared by using 300 μL of MeOH:H_2O (3:2) with the absence of CAJ. The absorbance of control sample was then subtracted from the absorbance of juice samples. Calibration was performed using gallic acid standard solutions prepared at different concentrations using a stock solution (1000 ppm). The same procedure was also applied to gallic acid standard solutions at varying concentrations up to 150 ppm. The concentration of total phenolic compounds in CAJ samples was expressed as mg gallic acid equivalents (GAE) per mL according to the equation obtained from the calibration curve demonstrated in Appendix A.

5.1.5. Data Analysis

All of the pasteurization processes were repeated two times. The results of microbiological, physicochemical and optical analyses of apple juice samples were expressed as their means and standard deviations. Measurements were done in duplicate. Regression analysis was applied by using a commercial spreadsheet (Microsoft Excel, Redmond, WA, USA). One-way analysis of variance (ANOVA) was carried out to determine how significantly the independent variables (UV-LED, UV-LED+MH and thermal processes) affect the dependent variables (microbiological, physicochemical and optical properties of apple juice) by using the Minitab 16 software program (Minitab Inc., State College, PA, USA). The means were evaluated in terms of Tukey comparison test with a 95% confident interval. Differences of data were significant for p-value is equal or less than 0.05 ($p \leq 0.05$).

5.2. Results and Discussions

5.2.1. The Effect of Combined UV-LED+MH Treatment on the Microbial Quality of CAJ During Storage

The fruit juice and nectars sold have to obey microbiological criteria mandated by Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21-1997) and Institute of Food Science and Technology (1999). The accepted maximum limits for total aerobic counts (TAC) and yeast and molds (YM) counts in fruit juice and nectars must be 4 and 3 log CFU/mL, respectively (IFST, 1999). In that regard, the microbial spoilage of CAJ was evaluated based on these criteria during the shelf life period.

Logarithmic changes in total aerobic bacteria (TAC), yeast and molds (YMC) and total coliform (TC) of untreated (control) samples, thermally pasteurized CAJ (70°C 120 sec.) samples and samples subjected to combined treatment of UV-LED (280/365 nm) and MH at 55ºC for 15 min were monitored during 60 days of refrigerated storage (+4.0±1.2°C) (Figure 5.3).

Initially, Untreated (control) CAJ (22.60_{280nm} cm^{-1}; 4.72_{365nm} cm^{-1}, 820 NTU) had 4.48±0.02 log CFU/mL of TAC, 4.22±0.01 log CFU/mL of YMC, and 2.70±0.01 log

CFU/mL of TC, respectively. Consequently, untreated juice had total mesophilic aerobic bacteria (TA) and yeast and molds (YM) counts higher than the maximum microbial accepted limits mandated by Institute of Food Science and Technology (1999). The TA and YM counts of untreated (control) samples increased to 6.05±0.02 log CFU/mL and 4.79±0.10 log CFU/mL within the first 3 days. On the other hand, UV-LED+MH and thermal pasteurization processes were able to inactivate the natural flora of CAJ prior to storage. No significant growth (under the detection limits <30 CFU/mL) of TAC and YMC was reported in CAJ subjected to either UV-LED+MH or thermal pasteurization up to 30 days of refrigerated storage (Fig. 5.3). Whereas, TAC and YMC of the UV-LED+MH treated samples increased to 5.29±0.02 log CFU/mL and 5.23±0.01 log CFU/mL, respectively after 40 days. Thus, it was observed that the shelf life of the samples treated with UV-LED+MH had ended between 30 and 40 days. The shelf life of UV-LED+MH treated samples was shorter than that of thermally pasteurized samples. The difference in the shelf life periods could be attributed to the recovery of cells after the UV-LED+MH treatment. The cells might be exposed to sub-lethal damages and recovered after the treatment during long storage period (Ferrario et al. 2018).

In the literature, there is not enough data about the storage of fruit juices treated with the technology where the UV-LED rays are combined with mild heat. On the other hand, there are many studies on fruit juices processed with UV-C irradiation assisted by mild heat. For example, Ferrario et al. (2018) investigated the changes of total aerobic, coliform and yeast and molds count in cloudy carrot-orange juice blend subjected to the single UV-C irradiation (10.6 kJ/m^2) and its combination with mild heat (50ºC) treatment for 15 min, during the 24 days of storage under refrigerated condition. They observed that untreated sample spoiled with in first 3 days, whereas shelf life of individual UV-C treated sample extended to 13-days. The aerobic mesophilic and total coliform populations in the sample subjected to combination of UV-C and MH remained constant and almost non-detectable throughout the 24-day storage. Whereas, yeasts and molds began to grow by the 17^{th} day, reaching to 3.1–3.2 log CFU/mL by the end of storage. Thus, the combination UV-C/H was successful in extending the shelf life of juice blend.

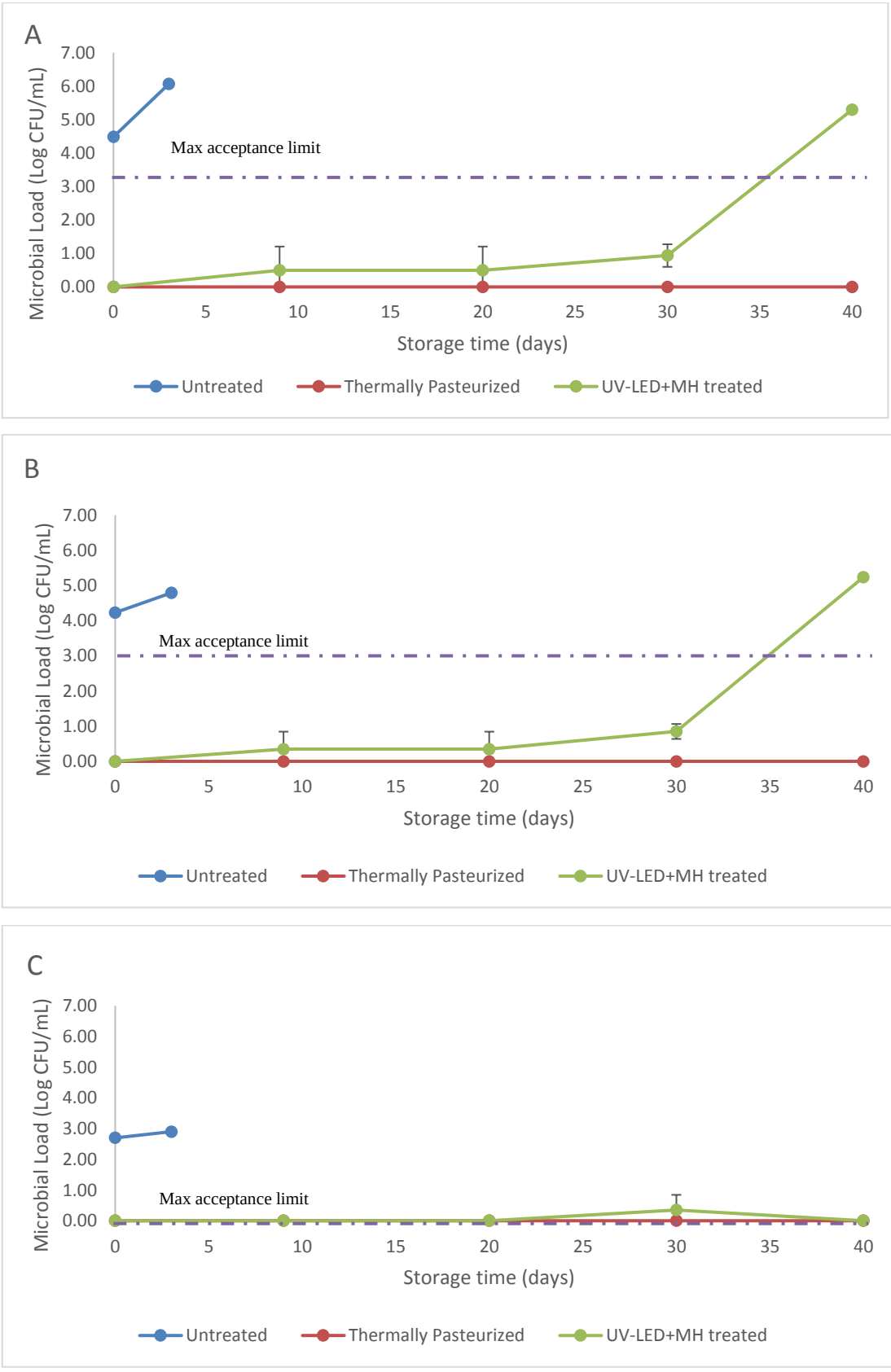


Figure 5.3. Changes in microbial quality of CAJ during storage. Microbial counts refer to total aerobic count (TAC) (A), yeast and molds count (YMC) (B), total coliform count (TCC) (C).

Additionally, Palgan et al. (2011) examined the shelf life of the blend of apple and cranberry juice treated by combined UV-C irradiation (5.3 J/cm^2) and pulse electrical field (1.213 kV/cm). No growth of yeast and molds was observed in UV+PEF treated samples up to day 14. On the other hand, the total aerobic count was recorded as 1 log CFU/mL in day 14 and increased to 5.96 log CFU/mL in day 21. They were able to extent the shelf life of apple and cranberry blend up to 15 days by treating it with combination of UV irradiation and pulse electrical field. Similarly, the microbial shelf life of lemon-melon juice blend treated by UV-C irradiation with 2.462 J/mL applied UV dose was able to extend the juice shelf life by more than 30 days under refrigeration conditions (Kaya, Yıldız, and Ünlütürk 2015). Muller et al. (2014) applied 100 kJ/L UV dose to apple and grape juice and monitored their microbial stability at 4°C storage conditions. Seven log microbial counts in untreated apple and grape juices were recorded after 15 and 18 days, respectively. On the other hand, the microbial load of UV-C treated juices was counted as near around 5 log CFU/mL after 18 days. Santhirasegaram et al. (2015) investigated the shelf life of UV-C treated mango juice at refrigerated temperature during 5 weeks. In their study, the shelf life of mango juice treated with 15, 30 and 60 min of UV-C irradiation (3.525 J/m^2) was determined as 2, 3-4 and 5 weeks respectively, while untreated juice was immediately spoiled within less than 1 week based on the microbiological criteria recommended by IFST (1999).

Consequently, UV-LED+MH treated CAJ was microbiologically safe up to 30 days of storage under refrigerated (4°C) conditions. On the other hand, no microbial growth was detected in thermally pasteurized CAJ by the end of 40 days.

5.2.2. Effect of combined UV-LED+MH treatment on the Physico-Chemical and Optical Properties of Cloudy Apple Juice During Storage

The changes in physicochemical, optical and phytochemical properties of untreated and thermally pasteurized and UV-LED+MH treated samples right after the treatments and during the refrigerated storage were evaluated.

pH value of untreated samples was 4.28±0.01 and increased to 4.32±0.00 and 4.38±0.00 in UV-LED+MH treated and thermally pasteurized samples, respectively (Figure 5.4 and Table 5.1). The pH of thermally pasteurized samples was not significantly changed during the 40 days of refrigerated storage ($p>0.05$). In contrast, pH of UV-

LED+MH treated samples was decreased from 4.32±0.00 to 4.01±0.01 after the 30th day. The change in pH was due to the increase of acidity caused by the metabolic activity of microorganisms during the storage period. The change of pH in untreated juice samples was more pronounced in comparison to irradiated samples (Figure 5.4 and Table 5.2). Similarly, Unluturk and Atilgan (2015) observed a marked decrease in pH of UV-C treated grape juice during storage period due to the increase in the number of microorganisms. Nualkaekul and Charalampopoulos (2011) observed a decrease in pH values of orange, grapefruit, blackcurrant, pomegranate, cranberry, and lemon juice during storage period, in parallel to an increase in the lactic acid and acetic acid counts, indicating that microorganisms were metabolizing the available energy sources.

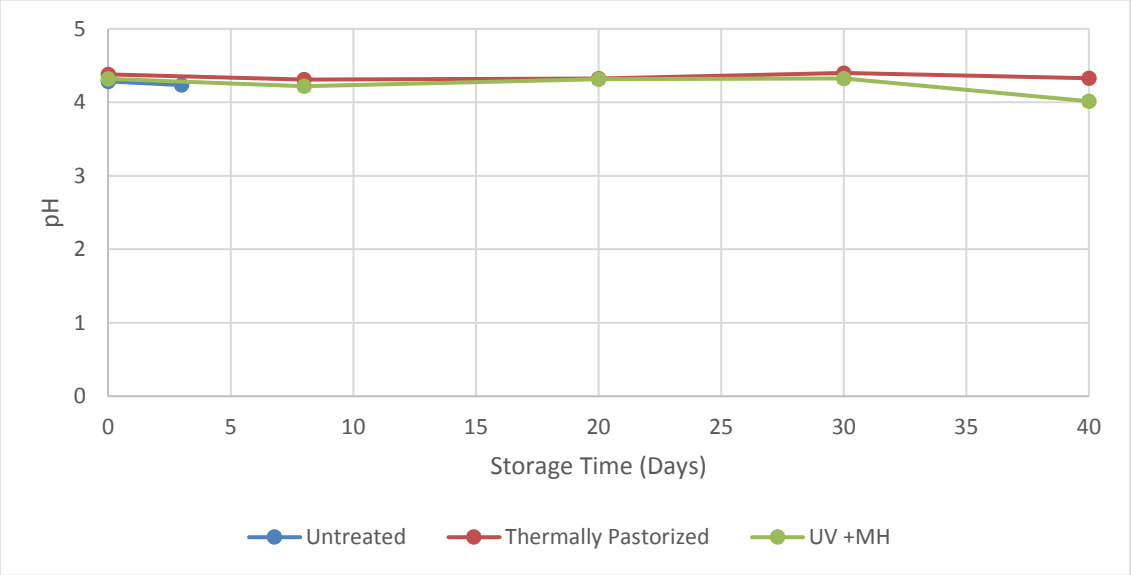


Figure 5.4. Influence of storage duration on pH of CAJ subjected to Thermal Pasteurization and UV+MH processes.

Since the ascorbic acid in fruit juice is an important nutrient value, many studies reported the effect of UV treatment on ascorbic acid (Falguera, Pagán, and Ibarz, 2011, Gayán et al. 2012, Koutchma and Technology 2009, Pala and Toklucu 2013, Unluturk and Atilgan, 2015). In this study, the effect of simultaneous application of UV-LED (280/365 nm) and MH (50ºC) on ascorbic acid content was also investigated even though apple juice is not a significant source (Gayán et al. 2012). The changes of ascorbic acid contents of apple juice samples were monitored after processing and during the storage (Figure 5.5). The ascorbic acid of untreated apple juice was 2.81 mg/L. Degradation of ascorbic acid after combined UV-LED+MH treatment (30%) was significantly higher

than that that of thermally pasteurized ones (20%). In other words, ascorbic acid was more sensitive to UV-LED+MH treatment than thermal treatment. It is known that ascorbic acid is natural antioxidant and has high UV absorbance within UV-C range (peak around at 260 nm). The presence of free radicals such as singlet oxygen, superoxide anion and etc. in the media induce the photooxidation of ascorbic acid. In this study, application of UV-LED emitted light at 365 nm wavelength might enhance the generation of free radical which resulted in the degradation of ascorbic acid following the treatment (Forney and Moraru 2009). After 9 days, all ascorbic acid content in samples were totally degraded. Unluturk and Atilgan (2015) reported the residual ascorbic acid content as 1.8% in grape juice treated with UV-C doses of 280 kJ/L. They found no ascorbic acid content by the end of 14-day storage period. Tran and Farid (2004) recorded 18% of loss in ascorbic acid content of orange juice after UV treatment of 73.8 mJ/cm^2 UV dose. They reported that during the storage, degradation of ascorbic acid depended on exposure of the air oxidation and light, presence of hydrogen peroxides, storage temperature, type of processing and packaging materials.

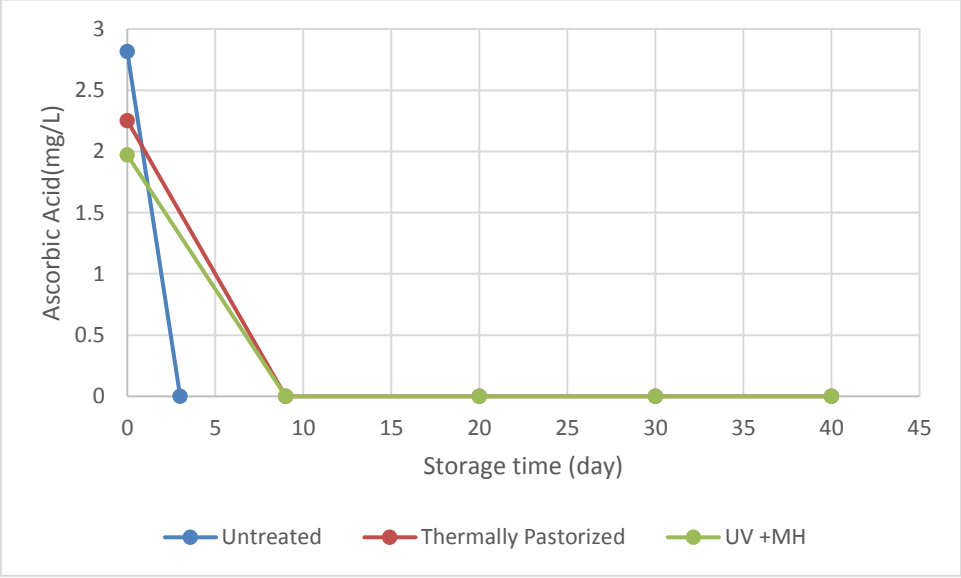


Figure 5.5. Influence of storage duration on ascorbic acid content of CAJ subjected to Thermal Pasteurization and UV+MH processes.

Table 5.1. Changes in pH and ascorbic acid content of CAJ during storage

Days	Treatments	pH	Ascorbic Acid (mg/L)	% Ascorbic Acid Loss
0	Untreated	4.28 ± 0.01^{aC}	2.8140	
	Thermal	4.38 ± 0.0^{aB}	2.2512	20
	UV-LED+MH	4.32 ± 0.00^{aA}	1.9698	30
3	Untreated	4.23 ± 0.01^{a}	0	100
	Thermal			
	UV-LED+MH			
9	Thermal	4.31 ± 0.01^{aA}	0	100
	UV-LED+MH	4.32 ± 0.01^{aB}	0	100
20	Thermal	4.33 ± 0.03^{aA}	0	100
	UV-LED+MH	4.31 ± 0.02^{aA}	0	100
30	Thermal	4.4 ± 0.05^{aA}	0	100
	UV-LED+MH	4.32 ± 0.01^{aA}	0	100
40	Thermal	4.33 ± 0.02^{aA}	0	100
	UV-LED+MH	4.01 ± 0.01^{bB}	0	100

Tukey least significant difference test was applied. Different letters for each process show the differences in properties between days during shelf life study. lower case letters (a,b,c): untreated samples, Italic lower case letters (*a*,*b*,*c*): thermally treated samples, underlined lower case (a,b,c): UV-LED+MH, refers to day differences; CAPITAL LETTER (A,B,C): difference between treatments at the same day.

It has been well-known that color is a crucial quality parameter of a food product which is directly related to consumer acceptance. Furthermore, the measurement of food color allows to determine the changes in the chemical, biochemical, microbial and physical changes which occur during growth, maturation, postharvest handling and processing (Pathare et al. 2013). Thus, the color parameters such as brightness-darkness (L*), redness-greenness (a*) and yellowness-blueness (b*) and total color differences (ΔE) values of untreated, thermally pasteurized and UV-LED+MH treated CAJ were monitored during 40 days of storage and results shown in Table 5.2 and Figure 5.6.

L* values of untreated CAJ increased from 25.63 to 27.52 and 29.34 following the UV-LED+MH treatment and thermal pasteurization. The L* values of untreated and UV-LED+MH treated samples exhibited an increasing trend during the storage period. Similarly, Ibarz et al. (2005) reported the brightening effect of UV-C light by observing an increase in the L* value of UV-C irradiated apple cider for 3 weeks storage period

($p<0.05$). The bleaching of juice could be attributed to the destruction of phytochemical polymeric compounds (Ibarz et al. 2005, Bhat et al. 2011). L* value of thermally pasteurized sample slightly decreased up to day 10 ($p<0.05$), then stayed at this level until the end of the storage period ($p> 0.05$).

Similarly, a* (redness-greenness) values of all the untreated and treated CAJ samples increased significantly ($p<0.05$) during storage period. Thus, CAJ samples lost their green color became redder at the end of shelf life due to the degradation of chlorophyll pigment and generation of the browning reactions. The value of b*, which reflects yellowness, was significantly increased through the end of 40 days of storage in all samples ($p<0.05$). An increase in b* could be attribute to enhancement of carotenoid type compounds during shelf life (Bhat and Stamminger, 2015). Ibarz et al. (2005) observed an increase in yellowness of juice attributing to higher melanoidin formation. Muller et al. (2014) similarly observed higher a* and b* values in grape and apple juice after 12 days of storage. They reported that the increment was due to the enzymatic browning by the residual activity of polyphenoloxidase enzyme retained in the juices after UV-C treatment (100.48 kJ/L). Manzocco et al. (2009) also determined an increment at the of level a* and b* values in UV-C treated apple slices during refrigerated storage and they also attributed this to the enzymatic browning pigment formation caused by the residual polyphenoloxidase enzyme activity.

Total color differences (ΔE), which indicate the magnitude of the color difference between CAJ at initial time and after the storage period, is reported in Table 5.2. Cserhalmi et al. (2006) classified the color difference (ΔE) between the treated and untreated samples as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5 –3.0), well visible (3.0–6.0) and great (6.0–12.0). According to this classification, ΔE value of untreated and treated samples increased with storage period and became "well visible" ($3.0<\Delta E< 6.0$) at the end of storage period (40 days). The changes in ΔE of samples right after UV-LED+MH process was slightly lower than that of thermally treated ones. At the end of storage period, the total color change in the UV-LED+MH treated CAJ samples reached to 3.52 and showed well visible difference. Whereas, the thermally pasteurized CAJ presented higher color differences than that of UV-LED+MH treated samples. Likewise, the change in the color of fruit juices subjected to UV-C light and thermal treatment and during storage has been reported by several studies. This change was attributed to the deterioration of color compounds by photodegradation and formation of dark color components from enzymatic and nonenzymatic browning

reactions (Chia et al. 2012, Falguera, Pagan and Ibarz, 2011, Ibarz et al. 2005, Kaya et al. 2015, Santhirasegaram et al. 2015, Unluturk & Atilgan, 2015, Cortes et al. 2008).

Table 5.2. Influence of storage duration on color parameters of CAJ subjected to Thermal Pasteurization and UV+MH processes.

Days	Treatments	Color			
		L*	a*	b*	ΔE
0	Control	25.63±0.01^{bC}	0.63±0.04^{bA}	7.97±0.01^{bB}	
	Thermal	29.34±0.00^{aA}	0.55±0.00^{cC}	4.07±0.02^{cC}	5.50
	UV-LED+MH	27.52±0.07$^{\underline{d}B}$	0.13±0.04$^{\underline{d}B}$	10.55±0.01$^{\underline{d}A}$	3.23
3	Control	26.65±0.05^{a}	2.19±0.04^{a}	10.25±0.06^{a}	2.9
	Thermal	-	-	-	
	UV-LED+MH	-	-	-	
9	Thermal	28.20±0.03^{aA}	0.62±0.01^{aB}	5.08±0.04^{bB}	3.84
	UV-LED+MH	27.98±0.03$^{\underline{c}B}$	1.07±0.09$^{\underline{bc}A}$	10.98±0.04$^{\underline{c}A}$	3.86
20	Thermal	28.19±0.09^{aA}	0.60±0.19^{aA}	5.30±0.05^{abB}	4.41
	UV-LED+MH	28.32±0.14 $^{\underline{bc}A}$	0.88±0.07$^{\underline{c}A}$	11.45±0.16$^{\underline{b}A}$	3.70
30	Thermal	28.33±0.44^{aA}	0.29±0.07^{bB}	5.60±0.45^{abB}	4.8
	UV-LED+MH	28.62±0.12 $^{\underline{ab}A}$	1.35±0.02$^{\underline{ab}A}$	11.67±0.06$^{\underline{ab}A}$	3.61
40	Thermal	28.48±0.78^{aA}	0.76±0.01^{aB}	5.93±0.01^{aB}	5.05
	UV-LED+MH	28.77±0.05$^{\underline{a}A}$	1.55±0.12$^{\underline{a}A}$	11.81±0.03$^{\underline{a}A}$	3.52

Tukey least significant difference test was applied. Different letters for each process show the differences in properties between days during shelf life study. lower case letters (a,b,c): untreated samples, Italic lower case letters (*a*,*b*,*c*): thermally treated samples, underlined lower case (a,b,c): UV-LED+MH, refers to day differences;· CAPITAL LETTER (A,B,C): difference between treatments at the same day

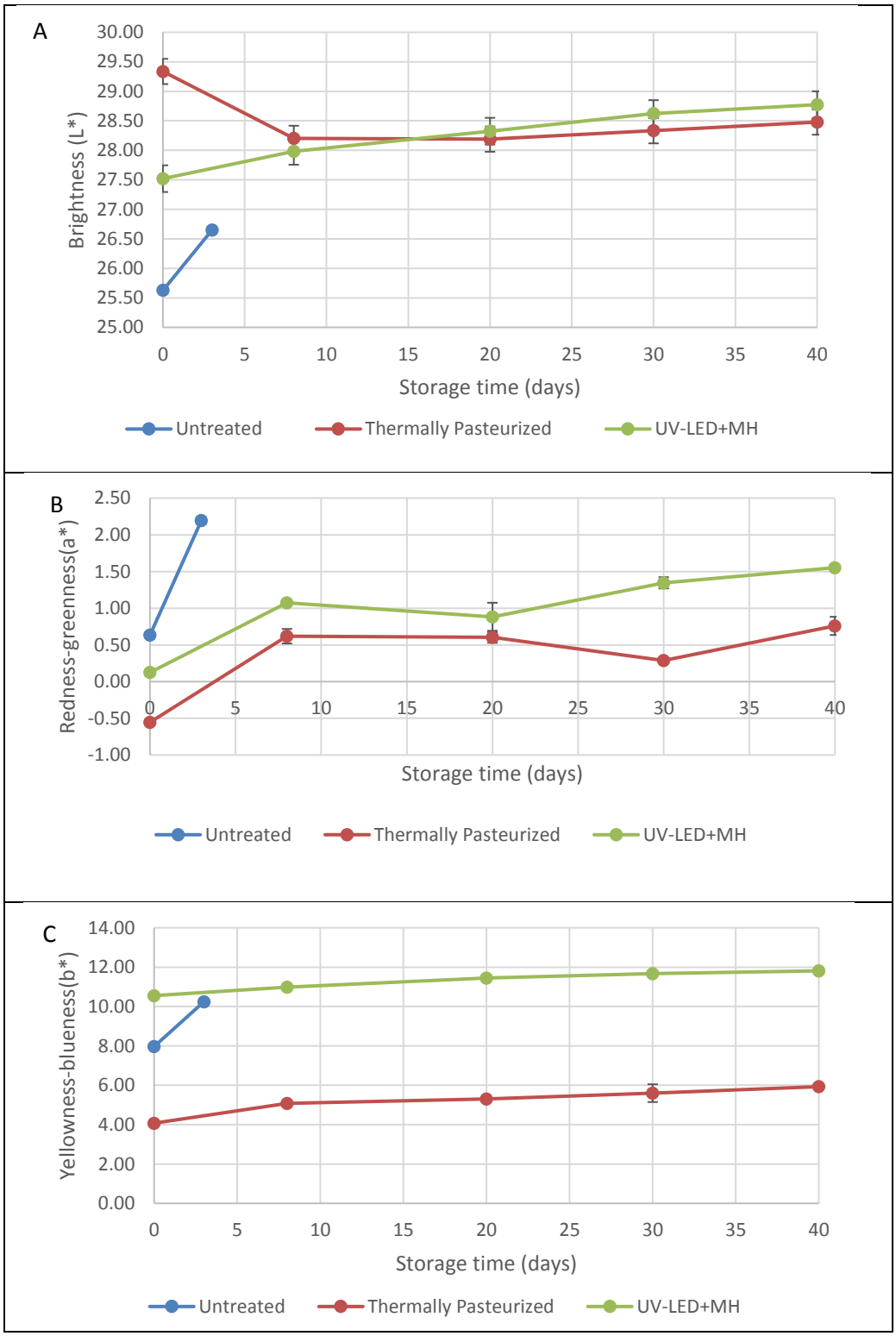


Figure 5.6. Influence of storage duration on color parameters such as L* (A), a* (B), b* (C) and total color changes ΔE (D) of CAJ subjected to Thermal Pasteurization and UV+MH processes.

(cont. on next page)

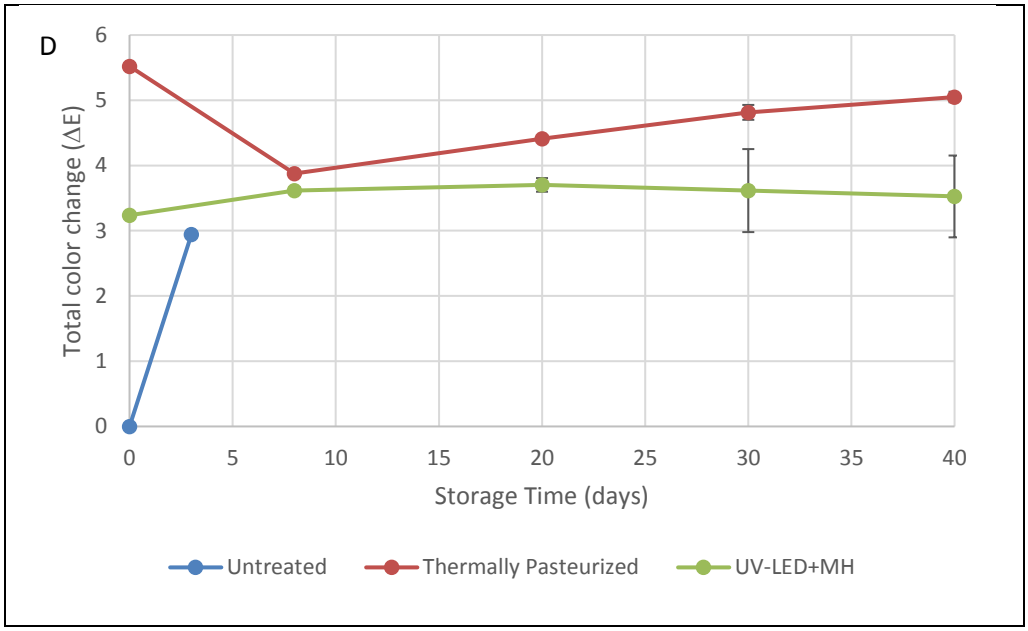


Figure 5.6 (cont.)

5.2.3. The Effect of UV-LED+MH Treatment on Polyphenoloxidase Activity of Apple Juice During Storage

After the treatments, the residual activity of PPO in thermally pasteurized and UV-LED+MH treated CAJ was recorded as 30.20±3.38% and 22.88±2.02%, respectively (Figure 5.7). However, there was small fluctuations in TPC of the samples stored at room temperature. Increasing or decreasing of the phenolic compounds throughout the storage period may be related to formation of intermediate dark colored products. The residual activity of PPO in UV-LED+MH treated CAJ remained constant around 21-22% for 40 days of storage. ($p>0.05$). During the storage period, increase of residual activity of PPO in treated CAJ was not significant ($p>0.05$). Similarly, Luo et al. (2008) reported that during 28 days of storage, residual relative PPO activity of PEF treated juice remained constant around 49% while residual relative activity of thermally treated sample remained around 7% at 4ºC.

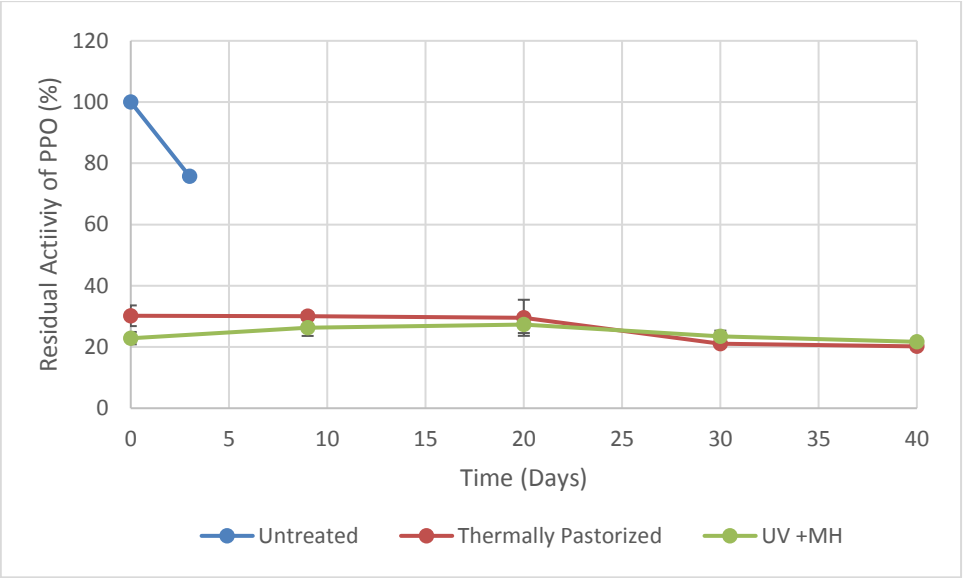


Figure 5.7. Influence of storage duration on polyphenol oxidase activity of CAJ subjected to Thermal Pasteurization and UV+MH processes.

5.2.4. The Effect of UV-LED+MH Treatment on Total Phenolic Content (TPC) of Apple Juice During Storage

TPC of untreated (control) samples, thermally pasteurized CAJ (70°C, 120 sec.) samples and samples subjected to combined treatment of UV-LED (280/365 nm) and MH at 55ºC for 15 min were monitored during 40 days of refrigerated storage (+4.0±0.50°C). The results are shown in Figure 5.8. Total phenolic content of untreated freshly squeezed CAJ was initially determined as 103.08±0.16 mg GAE/100 mL. Right after thermal pasteurization and UV-LED+MH treatments, TPC of CAJ samples increased to 138.48±4.65 and 159.97±5.12 mg GAE/100 mL, respectively. The increase in TPC of CAJ was statistically significant after processes ($p<0.05$). This increment was attributed to breakdown of complex phenolic polymers that led to release of simpler phenolic compounds. In the analysis of total phenolic content, it was thought that each one of these simpler molecules reacted with Folin-Ciocalteau reagent giving rise to higher TPC level. Additionally, the increase could be also attributed to the enhancement of phenylalanine ammonia-lyase in CAJ which might contribute to activation of phenolic biosynthesis pathway leading to increase of phenolic compounds (Alothman et al. 2009). Furthermore, the polyphenoloxidase enzyme may prevent further loss of polyphenols (Oms-Oliu, Odriozola-Serrano, and Martín-Belloso 2012) since following the processes, PPO in CAJ could not be completely inactivated. This finding is consistent with the study conducted

by Bhat et al. (2011). They treated starfruit juice with UV-C and observed a significant increase in polyphenol content. Similarly, Santhirasegaram et al. (2015) reported 31% of inducement in polyphenols in Chokanan mango juice after exposure to UV-C light. Moreover, Falguera et al. (2013) detected a slight increase in phenolic content of UV-vis-irradiated pear juice.

During 40 days of storage period, the phenolic compounds in untreated samples decreased sharply to 34.66±1.29 GAE/100 mL after day 3, whereas, TPC of thermally pasteurized and UV+MH treated samples decreased to 87.30±2.71 and 90.72±8.48 mg GAE/100 mL, respectively, after day 9. TPC of thermally pasteurized and UV+MH treated samples at the end of the storage was recorded as 66.63±7.13 and 58.90±2.04 GAE/100 mL with insignificant difference. The results indicated that total phenolic content of either thermally pasteurized or UV+MH treated samples were still higher than that of untreated ones. The decrease in TPC of apple juices during storage could be due to the insufficient inactivation of enzymes responsible for the degradation of phenolic compounds such as polyphenoloxidase, peroxidase and β-glucosidase.

There are many studies on the effect of non-thermal technologies on TPC of juice products. For example, blueberry juice was processed by high pressure (HP) (600 MPa/42°C/5 min) and pulsed electric fields (PEF) (36 kV/cm, 100 µs). During 56 days of storage of unprocessed and processed juices at 4 °C, TPC were fluctuated around the initial value (Barba et al. 2012).

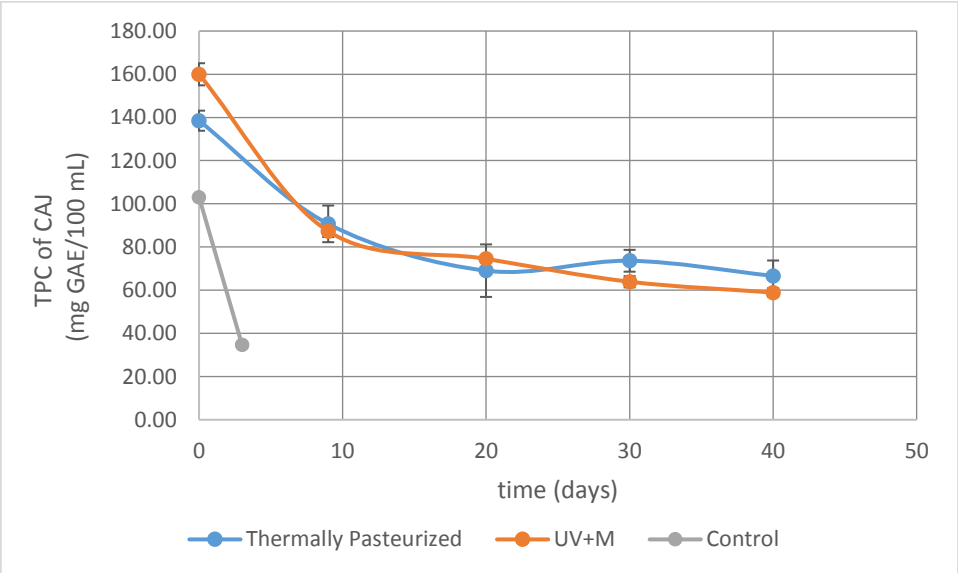


Figure 5.8. Influence of storage duration on TPC of CAJ subjected to Thermal Pasteurization and UV+MH processes.

5.3. Conclusion

In this chapter, the effect of 280/365 nm UV-LED irradiation assisted by mild heat at 55ºC on the microbiological, some physicochemical, optical and phytochemical properties such as pH, color, ascorbic acid content, polyphenol oxidase activity and total phenolic content of CAJ were monitored during 40 days under refrigerated storage conditions. Thermally pasteurized (70ºC, 120 sec) and untreated juice samples were used as the positive and negative controls. UV-LED+MH and thermal pasteurization processes were able to inactivate the natural flora of CAJ prior to storage. While, untreated sample was spoiled completely within the 3 days, no significant growth (under the detection limits <30 CFU/mL) of TA and YM was detected in either UV-LED+MH or thermal pasteurized juice samples up to 30 days of refrigeration storage. Although the pH value of UV-LED+MH treated CAJ samples did not change markedly during storage period, the optical properties were slightly affected. Ascorbic acid content of the samples was fully degraded within the first days of the storage. The residual activity of PPO in CAJ treated with UV-LED+MH process remained constant around the 21-22% by the end of storage. Thermal pasteurization and UV-LED+MH treatments increased the total phenolic content of CAJ samples. TPC values of treated samples were still higher than that of untreated ones during the storage period.

In conclusion, the simultaneous application of UV-LED and MH extended the shelf life of CAJ from 3 to 30 days by maintaining the quality losses to a minimum level. Additionally, polyphenol oxidase enzyme activity in CAJ was substantially degraded by the applied process. Thus, simultaneous application of UV-LED and mild heat can be an effective method for fruit juice pasteurization, if an appropriate equipment can be designed by considering optimal process parameters.

CHAPTER 6

INACTIVATION MECHANISM OF *E. coli* K12 by UV-LEDs

Antimicrobial effect of UV-C light is very well known, and this technique is used for disinfection of fruits surfaces, hospital equipment, water resources etc. (Begum et al. 2009, Pan et al. 2004, Bintsis et al. 2000, Nigro et al. 1998). Inactivation mechanism of UV-C irradiation is based on the absorption of UV photons by the genetic material and the formation of dimers which inhibit the transcription and replication of the cell (Oguma et al. 2002, Bolton et al. 2003, Koutchma, 2009). On the other hand, many microorganisms are able to repair damages on their DNA caused by UV-C irradiation by means of two different mechanisms such as photoreactivation and dark-repair (Chevremont et al. 2012a, Oguma et al. 2002).

There are limited numbers of studies related to the use of UV-LEDs for water disinfection. Chatterley and Linden (2010) reported that most of those data were available for LEDs emitting light at UV-A range. However, UV-C LEDs were also indicated to be preferred for this purpose (Li et al. 2010, Chevremont et al. 2012a, Chevremont et al. 2012b, Bowker et al. 2011, Würtele et al. 2011; Hamamoto et al. 2007). Moreover, combination of UV-A and UV-C LEDs was used in some studies (Aoyagi et al. 2011, Chevremont et al. 2012a). Bowker et al (2011) indicated that emitted UV light at 275 nm resulted in much higher microbial inactivation. This is due to the fact that protein absorption spectrum reaches the maximum near 280 nm and thus enzymes become more sensitive to inactivation at these wavelengths. Moreover, at a wavelength range of 200 to 280 nm (UVC) and 280 to 315 nm (UV-B), it has a damaging effect on DNA replication and transcription. Direct exposure to UVC or UV-B results in dipyrimidine dimers, pyrimidine hydrates, or cross-links between proteins and DNA. Hence, it is capable of inactivating a variety of pathogens such as bacteria, viruses, fungi, protozoa, and other pathogenic organisms (Lui et al. 2014). Furthermore, it is known that UV-A radiation mechanism is based on the inactivation of microorganisms by damaging proteins and producing hydroxyl and oxygen radicals which destroy cell membrane and other cellular components (Chevremont et al. 2012a). Although DNA damage caused by UV-C

radiation can be repaired by the enzyme photolyase, there is no possibility to repair the damage to bacterial membranes by UV-A radiation. Chevremont et al (2012a) showed that coupling UV-A and UV-C could be paired by using the germicidal effect of UV-C and greater penetrating ability of UV-A. They also found that use of coupled wavelengths 280/365 nm and 280/405 nm caused total disappearance of fecal enterococci, total coliforms and fecal coliforms in the effluent. Besides lack of possibility to repair the damage in the bacterial membranes occurred after UV-A exposure increased the efficiency of microbial inactivation (Chevremont et al. 2012a).

One of the aims of this thesis is to investigate the effect of different wavelengths and their combinations (UV-A and UV-C) on the mechanism of microbial inactivation. In this respect, the goals of this chapter were to:

i) investigate to inactivation performance of UV-LEDs emitting at 254 nm, 280 nm and combined 280 nm and 365 nm wavelengths on *E. coli* K12 in Phosphate-buffered saline (PBS) solution.

ii) reveal the sub-lethal injury caused by UV-LED irradiation in *E. coli* K12

iii) test the photoreactivation and dark repair mechanism of *E. coli* K12 in PBS during the 6 h incubation period at refrigerated (4ºC) temperatures.

iv) investigate of the formation of DNA breakages, protein and lipid oxidation, ROS generation in cell.

v) monitor the structural changes in the cell surfaces by scanning electron microscopy (SEM).

6.1. Material and Methods

6.1.1. Microbial Inactivation Studies

The inactivation performances of UV-LEDs were determined using following steps. Three milliliters of PBS media (pH 7.2) spiked with *E. coli* K12 at a concentration of 10^6 CFU /mL and transferred in a sterilized Petri dish (55 mm diameter). The same protocol was carried out for preparation of sub-culture of *E. coli* K12 (see section 3.2.2) without acid adaptation. The inoculated PBS samples were placed on a stirrer and exposed to different UV-LEDs wavelengths using different numbers of lamps at 254 nm (4 lamps), 280 nm (4 lamps), combination of 280 nm and 365 nm (2 lamp/2 lamp), 254 nm (only 2

lamps), 280 nm (only 2 lamps) and 365 nm (only 2 lamps), for different exposure times ranging from 10 min to 40 min. The same UV-LED batch top system was used in this study, described in detail in section 3.1.3.1. Each UV-LED irradiation treatments were conducted in duplicate. After treatments completed, the viable population of *E. coli* K12 in the UV-LED treated samples were enumerated by spread plating onto tryptic soy agar (TSA, Merck, Darmstadt, Germany) containing 0.1% dihydrostreptomycin with appropriate dilutions. All the plates were incubated at 37 °C for 24 h and then counted.

6.1.2. Sub-lethal Injury Test

The sub-lethal injury caused by UV-LED irradiation in *E. coli* K12 cells was determined via two different methods such as flow cytometric measurement and selective medium plating technique.

6.1.2.1. Staining Procedure and Flow Cytometric Measurement

After UV irradiation, cell membrane damage was determined by flow cytometric measurement. Not only the number of dead and living cells, but also the number of cells that continue metabolic activity, but cannot form colonies in the plate counting method, have been tried to be determined with this technique. Application of double staining with fluorescein diacetate (FDA) (Sigma Aldrich St Louis, MO, USA) and propidium iodide (PI) (Sigma Aldrich St Louis, MO, USA) was used for flow cytometry according to Schenk et al. (2011) with some modifications). While the FDA fluorescent dye indicated in the method can penetrate all (dead, live, semi-dead) cells, PI can only penetrate dead cells. Non-irradiated stained cells and cells thermally pasteurized at 90ºC for 15 min and subsequently stained with PI were used as controls of PI negative and positive histogram regions, respectively. Control and UV irradiated cells were initially incubated for 30 min at 37°C with 20 µL of FDA (5 mg/ml acetone) added. The cells were then centrifuged (5000 g, 5 min, 4°C) in 500 µl PBS buffer with containing 1 mM EDTA, 0.2% Pluronic^{TM} F-68 and 0.1% sodium azide (pH 7.0). This step was followed by the addition of 2 µL of PI (1 mg / ml sterile water) and incubation for 10 minutes at room temperature in the dark. Following incubation with PI, the samples were placed on ice in the dark until analysis (maximum 15 minutes). Following the staining of the cells, analysis of the cells was

performed on a flow cytometer (FACSCANTO, BD) equipped with an air cooled 488 nm argon laser. Scatter or fluorescence signals of individual cells passing through the laser zone were collected as logarithmic signals. Green fluorescence of cells stained with FDA was collected in the FL1 channel (525±15 nm), whereas red fluorescence of cells labeled with PI was collected in the FL2 Channel (585± 21 nm). Flow rate and cell concentration of the samples were adjusted to keep acquisition at 100 microorganism cells per second. A total of 10.000 events were registered per sample.

6.1.2.2. Sublethal Injury Test with NaCl Sensitivity

The sub-lethal injury caused by UV-LED irradiation in *E. coli* K12 cells was determined via selective medium plating technique. The sensitivities of illuminated and non-illuminated cells to NaCl were compared in order to investigate a loss of bacterial cytoplasmic or outer membrane functionality by LED illumination. For this purpose, stationary-phase cells grown at 37 ºC for 24 h were plated onto both tryptic soy agar (TSA, Merck, Darmstadt, Germany) and TSA containing various concentrations of NaCl (1-5%). The sensitivity was measured by comparing the difference in the number of colonies grown on TSA (non-selective agar) and TSA supplemented with NaCl. The increase in bacterial sensitivity to NaCl indicates the loss of outer membrane function as a permeability barrier to (*i*) hydrophobic compounds and (*ii*) osmotic functionality of cytoplasmic membrane, respectively (Ghate et al. 2013, Kim et al. 2016).

The inoculated samples were initially irradiated at different UV-LEDs, i.e., (254 nm (4 lamps), 280 nm (4 lamps), a combination of 280 nm and 365 nm (2 lamp/2 lamp), 254 nm (2 lamps), 280 nm (only 2 lamps) and 365 nm (only 2 lamps) for different exposure times (10, 20, 30 and 40 min). After incubation at 37ºC for 24 h, the number of colonies was counted, and sensitivity was calculated with the following equation (Ghate et al. 2013):

$$Sublethal\ injury\ (sensitivity)(\%) = \left[\left(1 - \frac{Colonies\ on\ TSA+NaCl}{Colonies\ on\ TSA}\right) \times 100\right] \quad (6.1)$$

The maximum levels of NaCl used for *E. coli* K12 was required to be determined prior to UV-LEDs illumination. This was the maximum non-inhibitory concentration for stationary-phase cells. Kim et al. (2016) previously defined the maximum non-inhibitory

concentration as the maximum concentration of NaCl that does not inhibit the growth of healthy and intact cells. To determine this concentration level, stationary-phase growth cells at 37ºC for 24 h were plated onto TSA and TSA containing various concentrations of NaCl (1-5%) and the sensitivity was compared as described above.

6.1.3. Photoreactivation and Dark Repair of *E. coli* K12

The photoreactivation and dark repair mechanisms of *E. coli* K12 in PBS solution were investigated after the PBS samples were exposed to UV-LEDs at 254 nm (4 lamps), 280 nm (4 lamps), a combination of 280 nm and 365 nm wavelengths (2 lamp/2 lamps), for 40 min. Same experimental steps described detailly in session 3.1.5 were applied to reveal the potential of photoreactivation and dark repair ability of *E. coli* K12 in PBS under 6 hour incubation at refrigeration condition (4ºC). The experiments replicated three times. The reactivation ratio of *E. coli* K12 in juice was calculated according to Equation 3.10 (Li et al., 2017).

6.1.4. Inactivation Mechanism Analysis

6.1.4.1. Determination of DNA Strand Breakages

UV-induced DNA damages of *E. coli* K12 was assessed by quantification of DNA strand breakages (DSB). For this purpose, *E. coli* K12 cells in PBS were subjected to different UV-LEDs wavelengths using different numbers of lamps at 254 nm (4 lamps), 280 nm (4 lamps), combination of 280 nm and 365 nm (2 lamp/2 lamp), 254 nm (only 2 lamps), 280 nm (only 2 lamps) and 365 nm (only 2 lamps) for different exposure times ranging from 10 min to 40 min. DSB were determined by fluorometric analysis of DNA unwinding (FADU) modified from a method described by He and Hader (2002).

Immediately after irradiation, untreated and irradiated cells were harvested by centrifugation (3000 x g for 10 min at 4ºC), then 20 μL of 0.5 M EDTA was added to the pellet. Subsequently, the pellet was resuspended in 164 μL of TE buffer (100 mM Tris-10mM EDTA). Then 16 μL of lysozyme (50 mg/mL) was added to the suspension, and the mixture was incubated at 37ºC for 60 min to destroy the cell walls completely. A 15 μL of 10% SDS, 10 μL of 4 M NaCl, 15 μL of Proteinase K (6 mg/ mL) and 60 μL TE

buffer were added to a final volume of 300 μL and incubated at 60ºC for 30 min to lyse the cells. According to method requirement, in addition to test samples, the two set of untreated control samples needed: (1) samples which was not subjected to the alkaline unwinding and remained complete double strand DNA (dsDNA) (T), and (2) sample which was subjected to complete alkaline unwinding and showed lowest level of fluorescence (B). Thus, three different sample groups were subjected to different unwinding protocols.

The first reference set was used for estimating the total fluorescence (T-fluorescence). The contribution of fluorescence of T sample come from the double strand DNA of cell and debris such as unbound dye. The cell suspension was sonicated under neutral condition in order to prevent the unwinding of the DNA.

Procedure. A 300 μL suspended cell of T-sample was lysed by additional of 300 μL of 0.1M NaOH, for neutralization, immediately, a 300 μL of 0.1M HCl was added into the mixture. Then, mixture was incubated at 20ºC for 30 min, afterwards sonicated for 15 s.

The second reference set provided to estimate the contribution of background components other than double strand DNA (including unbound dye) from untreated samples (B-fluorescence). The B-sample cells was sonicated under alkaline conditions to break the all DNA and to bring about complete unwinding of low-molecular-weight DNA fragments.

Procedure. A 300 μL suspended cell of B-sample was lysed by additional of 300 μL of 0.1M NaOH, sonicated for 5 s, then incubated at 20ºC for 30 min. Subsequently the samples were neutralized by adding a 300 μL of 0.1M HCl and sonicated again for 15 s.

The third sample set was used for the estimation the rate of irradiated-damaged DNA (P-fluorescence). The cells were subjected to alkaline conditions that bring about a partial unwinding of DNA. The degree of the unwinding was proportionate to the number of DNA breaks.

Procedure. A 300 μL suspended cell of P-sample was lysed by gentle additional of 300 μL of 0.1M NaOH, without mixing, followed by incubation at 20ºC for 30 min. During this unwinding period the alkali diffused into the viscous lysate to give a final pH of 12.4. For neutralization, a 300 μL of 0.1M HCl (pH 7.1–7.4) was added into the mixture, then sonicated for 15 s.

Fluorescence Measurement of dsDNA. a 300 μL of 20 μM Hoechst 33258 (in 0.6 M phosphate buffer pH 7.6) was added to all samples and centrifuged at 10000xg for 5 min at ambient temperature. Three hundred microliter supernatants of the samples were put into the black, flat bottom 96 well plates. The fluorescence intensities were read from a spectrofluorimeter (VARIO SKAN FLASH, THERMO) operating at 350 nm (excitation) and 480 nm (emission). The fraction of dsDNA (F %) was calculated as from the following equation

$$F = (P - B)/(T - B) \times 100 \quad (6.2.)$$

Where T, B, and P are fluorescence intensities. The difference P minus B (P -B) provides an estimate of the amount of DNA that still remained double-stranded, thus indicating the amount of the induced DNA damage. The difference T minus B (T - B) provides an estimate of the amount of dsDNA in untreated cells.

6.1.4.2. Lipid Oxidation

Lipid peroxidation levels of *E. coli* K12 in PBS subjected to different UV-LEDs wavelengths using different numbers of lamps at 254 nm (4 lamps), 280 nm (4 lamps), combination of 280 nm and 365 nm (2 lamp/2 lamp), 254 nm (only 2 lamps), 280 nm (only 2 lamps) and 365 nm (only 2 lamps) for different exposure times ranging from 10 min to 40 min. were carried out for the determination of the amount of thiobarbituric acid reactive substance (TBARS) in *E. coli* K12 cells according to a previously described method by Semchyshyn et al. (2005) with some modification. For this purpose, the untreated and treated cells were collected by centrifugation (3000 x g for 10 min at 4ºC). Then, the cells were resuspended in 1 mL PBS (pH 7). After then, cell homogenates was precipitated with addition of 1 mL of 20% (w:v) trichloroacetic acid (TCA, Merck, Germany) and centrifuged at 10000xg for 5 min. The supernatant was mixed with 2 mL saturated solution containing 0.5% (w:v) thiobarbituric acid (TBA, Merck, Germany) in 0.1 M HCl and 10 mM butylated hydroxytoluene (BHT, Merck, Germany). The samples were boiled for 60 min at 100ºC in a water bath. The aliquot of 1.5 mL was removed and cooled to room temperature, then mixed with 1.5 mL butanol (Merck, Germany). The mixture was centrifuged at 4000 x g for 10 min at 4ºC. the organic fraction was recovered,

and optical density was measured at 535 nm spectrophotometrically (SHIMADZU Co. Ltd., Japan). Untreated bacteria were used as a negative control. All the experiments were repeated three times. Relative lipid oxidation level of bacteria was calculated using the following equation:

$$\% \text{ lipid oxidation} = \frac{(A_1 - A_0)x100}{A_0} \quad (6.3)$$

A_1 is the absorbance after UV-LED treatment; A_0 is the absorbance of untreated sample.

6.1.4.3. Protein Oxidation

After *E. coli* K12 cells in PBS were subjected to different UV-LEDs wavelengths using different numbers of lamps at 254 nm (4 lamps), 280 nm (4 lamps), combination of 280 nm and 365 nm (2 lamp/2 lamp), 254 nm (only 2 lamps), 280 nm (only 2 lamps) and 365 nm (only 2 lamps) for different exposure times ranging from 10 min to 40 min, protein oxidation levels were assessed from carbonyl level in cellular proteins as described by Semchyshyn et al. (2005). Firstly, the cells were harvested by centrifugation (3000 x g for 10 min at 4ºC). Then, the cells were resuspended in 2 mL PBS (pH 7). Two milliliter cell homogenates were treated with 8 mL of 10 mM dinitrophenylhydrozine (DNPH) in 2 M HCl and incubated at room temperature for 60 min. Proteins were precipitated with 1 mL 20% (w:v) trichloroacetic acid (TCA, Merck, Germany), centrifuged at 14000xg for 10 min at 4 ºC. The pellets were washed three times with 1 mL ethanol-ethylene acetate. The final precipitate was dissolved in 1 mL 6 M guanidine hydrochloride in 2 mM potassium phosphate. The absorbance at 360 nm was measured. Untreated bacteria were used as a negative control. All the experiments were repeated three times. Relative protein oxidation level of bacteria was calculated the following equation:

$$\% \text{ Protein oxidation} = \frac{(A_1 - A_0)x100}{A_0} \quad (6.4)$$

A_1 is the absorbance after UV-LED treatment; A_0 is the absorbance of untreated sample.

6.1.4.4. Intracellular Reactive Oxygen Species (ROS) Generation

Intracellular ROS generation was detected by using the probe 2', 7' -dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is a nonpolar compound which is converted to the polar derivative DCFH by cellular esterases when it is taken up. DCFH is nonfluorescent but highly fluorescent when oxidized to DCF by intracellular ROS and other peroxides (He and Häder, 2002). Therefore, it is so important to maintain consistent incubation conditions including minimal ambient light and temperature. For this purpose, 1 mL sample after irradiation were immediately mixed DCFH-DA dissolved in dimethyl sulfoxide (DMSO) (final concentration 10 μM from stock solution of 10 mM) and stayed incubated at room temperature for 30 min. Then the bacteria were subsequently washed with PBS (pH 7), 100 μL of homogenate were mixed with 1 mL with the same buffer. The fluorescence of the samples was measured with a spectrofluorometer (Thermo, Vario Skan Flash) at room temperature, with an excitation wavelength of 490 nm and emission wavelength of 519 nm. Untreated bacteria were used as a negative control. All the experiments were repeated three times. Relative protein oxidation level of bacteria was calculated the following equation:

$$\% \text{ ROS generation} = \frac{(A_1 - A_0)x100}{A_0} \quad (6.5)$$

A_1 is the absorbance after UV-LED treatment; A_0 is the absorbance of untreated sample.

6.1.4.5. Monitoring of Surface Structural Changes in *E. coli* K12

Structural changes in *E. coli* K12 subjected to different UV-LEDs wavelengths using different numbers of lamps at 254 nm (4 lamps), 280 nm (4 lamps), combination of 280 nm and 365 nm (2 lamp/2 lamp), 254 nm (only 2 lamps), 280 nm (only 2 lamps) and 365 nm (only 2 lamps) for different exposure times ranging from 10 min to 40 min were determined by Scanning Electron Microscopy (SEM) according to the method described in Molva and Baysal (2014). Firstly, the control and treated cells contained in the PBS buffer were collected by centrifugation at 4800 x g for 5 min. Next, the bacterial pellet

was suspended in sterile deionized water and centrifuged again. This washing step was repeated three times. After that, the pellet was re-suspended in sterile deionized water. Finally, the resultant suspension (10 μL) was fixed onto clean glass slides and air–dried overnight. For SEM imaging, samples were coated with gold at a vacuum of 0.09 mbar for 90 s and examined with a scanning electron microscope (Quanta 250 FEG) with 15 kV acceleration voltages.

6.1.5. Statistical Analysis

Statistical analyses, one-way analysis of variance (ANOVA) was carried out to determine how significantly the independent variables (254 nm (4 lamps), 280 nm (4 lamps), combination of 280 nm and 365 nm (2 lamp/2 lamp), 254 nm (only 2 lamps), 280 nm (only 2 lamps) and 365 nm (only 2 lamps)) affect the dependent variables (microbiological, sublethal injury and reactivation ratio, double strand breakages of DNA, lipid and protein oxidation, ROS generation) by using the Minitab 16 software program (Minitab Inc., State College, PA, USA). The means were evaluated in terms of Tukey comparison test with a 95% confident interval. Differences of data were significant for p-value is equal or less than 0.05 ($p \leq 0.05$).

6.2. Results and Discussion

6.2.1. Microbial Inactivation Studies

The inactivation efficiency of UV-LEDs at 254 nm (4 lamps), 280 nm (4 lamps), 254 nm (2 lamps), 280 nm (2 lamps) and 365 nm (2 lamps) and the combination of 280 nm and 365 nm on *E. coli* K12 in PBS were investigated for different exposure times from 10 to 40 min. Absorption coefficients of PBS were measured as 0.0185 cm^{-1} at 254 nm, 0.065 cm^{-1} at 280 nm, and 0.034 cm^{-1} at 365 nm, respectively. Logarithmic reductions (log CFU/mL) of *E. coli* K12 in PBS exposed to UV-LED irradiation at different wavelengths and exposure times are presented in Fig. 6.1.

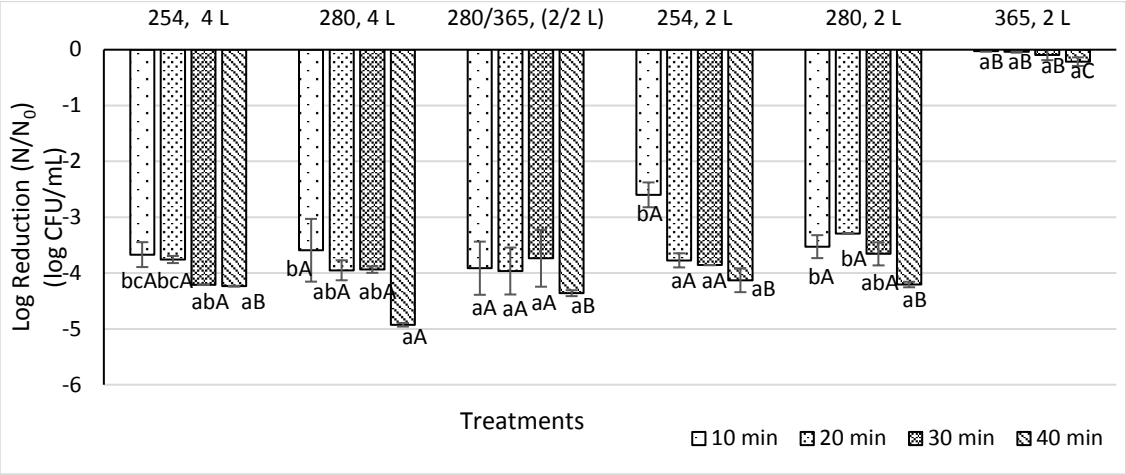


Figure 6.1. Logarithmic reductions (log CFU/mL) of *E. coli* K12 in PBS exposed to UV-LED irradiation at different wavelengths and exposure times.

Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. A–B:Values within each exposure time at same wavelength or combination followed by the same letter are not significantly different (P > 0.05). a–c: Values within each wavelength and their combinations at the same exposure time followed by the same letter are not significantly different (P > 0.05).

It was found that the log reductions of *E. coli* K12 in PBS solutions exposed to four UV-LEDs (4 lamps) at 280 nm and 254 nm were slightly higher than two UV-LEDs (2 lamps) used at the same wavelengths. Samples subjected to two UV-LEDs at 254 and 280 nm for 10 and 40 min showed 2.60±0.22 and 4.12±0.21; 3.57±0.21 and 4.25±0.05 log CFU/mL reductions in logarithmic count of *E. coli* K12. Increasing exposure time from 10 min to 40 min resulted in an increment in logarithmic reductions of *E. coli* K12 in PBS. UV doses applied at 254 nm and 280 nm UV treatments were in the range of 13.66 (2 lamps) and 99.56 mJ/cm^2 (4 lamps), and 15.70 (2 lamps) and 108.44 mJ/cm^2 (4 lamps), respectively (Table 6.1). Exposure of samples to four UV-LEDs slightly increased the log reductions. 3.66±0.22 (24.89 mJ/cm^2) and 4.23±0.01 (99.56 mJ/cm^2) log CFU/mL, and 3.59±0.56 (27.11 mJ/cm^2) and 4.92±0.03 (108.44 mJ/cm^2) log CFU/mL reductions were observed following 254 nm; and 280 nm UV treatments. In other words, the application of high UV doses to PBS solutions inoculated with *E. coli* K12 slightly affected the inactivation efficiency of UV-LEDs (Figure 6.2). This could be a result of tailing effect of UV-LED irradiation. Tailing may have arisen from the aggregation of microorganisms (Koutchma, 2009, Oguma et al. 2013, Li et al. 2017). The longer treatment time in UV-LED system provided the higher possibility for microbial aggregation. The tailing phase was also reported in the other studies using UV-LEDs irradiation. For example, Oguma et al. (2013) investigated the inactivation efficiency of *E. coli* exposed to combined UV treatment with wavelength of 265/280, 265/310, 280/310

and 265/280/310 nm using a flow-through reactor. The tailing was observed at high fluence rates, regardless of the emission wavelengths. Additionally, it was clearly observed that the germicidal effect of UV-LED lamps emitting light at 280 nm was higher than that of 254 nm. One of the reasons of this was the higher UV dose applied at 280 nm (Table 6.1). The other reason was the protein absorption spectrum which was reported to reach the maximum near 280 nm. Thus, cellular enzymes become more sensitive to UV irradiation at these wavelengths. Recent studies on water disinfection by UV-LEDs also showed that wavelengths near 280 nm, e.g. 275 nm, provided more efficient disinfection than 254 nm (Bowker et al., 2011; Würtüle et al., 2011). The authors indicated that UV irradiation at 280-275 nm wavelengths caused a damage on cellular proteins rather than DNA of the cells. Furthermore, the 2 UV-LED lamps emitted light at 365 nm did not show a significant lethal effect on *E. coli* K12 despite the applied high UV dose of 121.60 mJ/cm^2 compared to other wavelengths.

Table 6.1. Time-dependent UV Dosages

Time (min)	UV Dose (mJ/cm^2)				
	254 nm (4 Lamps)	**280 nm (4 Lamps)**	**254 nm (2 Lamps)**	**280 nm (2 Lamps)**	**365 nm (2 Lamps)**
10	24.89	27.11	13.66	15.70	30.40
20	49.78	54.22	27.34	31.40	60.80
30	74.65	81.33	41.01	47.09	91.20
40	99.56	108.44	54.68	62.78	121.60

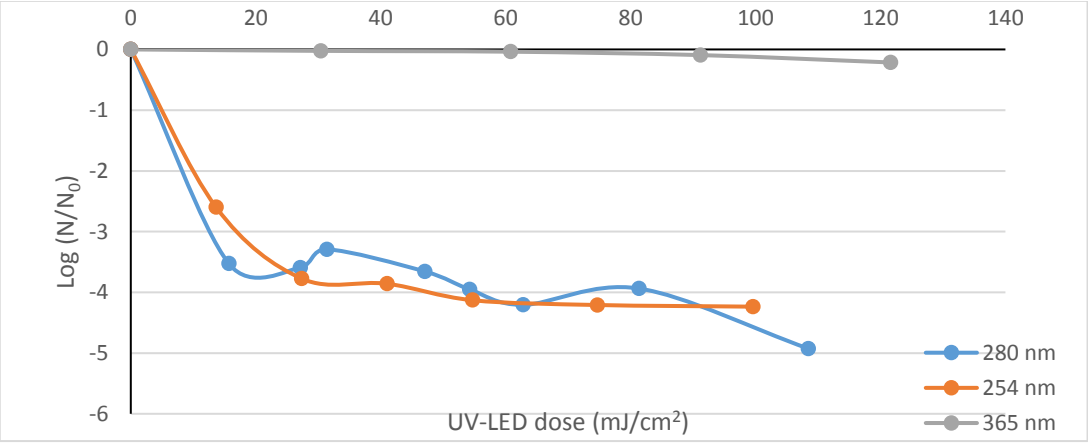


Figure 6.2. Logarithmic reductions (log CFU/mL) of *E. coli* K12 in PBS exposed to UV-LED irradiation at different wavelengths and UV-LED dosages

Figure 6.3. demonstrates the inactivation performance of the combined UV treatment with wavelength of 280/365 nm (2 lamps/2 lamps) to determine whether a synergistic inactivation effect could be achieved. The additive effect was calculated by using sum of the individual log count reduction of *E. coli* K12 in PBS, obtained at 280 nm (2 Lamps) and 365 nm (2 Lamps). The results were compared to UV inactivation rate achieved by using 280 nm (4 Lamps) wavelength.

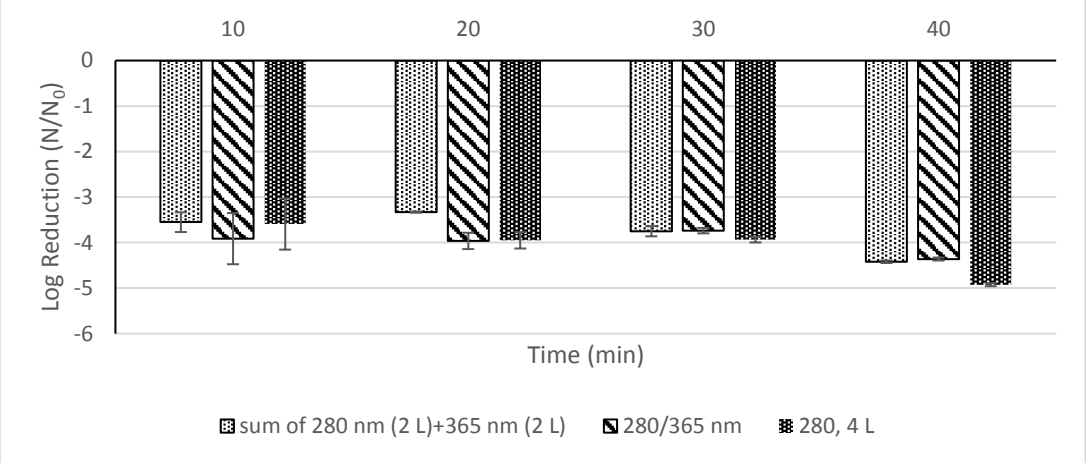


Figure 6.3. Logarithmic reductions (log CFU/mL) of *E. coli* K12 in PBS exposed to UV-LED irradiation at 280 nm (4 L)(⬚) and 280/365 nm (▨), and the sum of individual logarithmic reductions of *E. coli* K12 at 280 nm and 365 nm (▩).

In chapter 3, the inactivation efficiency of UV-LEDs at 280 nm (4 Lamps) and the combination of 280 nm and 365 nm (2/2 Lamps) on *E. coli* K12 in clear and cloudy apple juices was investigated. Even so, these treatments showed approximately same microbial inactivation efficiency. Additionally, the combination of 280 nm and 365 nm (2/2 L) wavelengths provided higher PPO enzyme inactivation than that of 280 nm used alone. Thus, the optimum treatment condition was selected as the UV-LEDs at combined wavelengths of 280/365 nm for 40 min exposure time.

In contrast, in this part of the study, 4 UV-LED lamps at 280 nm used alone with 4.92±0.03 log CFU/mL was more effective on inactivation of *E. coli* K12 in PBS than that the combination of 280 and 365 nm (4.36± 0.05 log CFU/mL) for 40 min exposure time. Additionally, the synergistic inactivation effect was observed after following UV-LED irradiation with the combination of 280 nm and 365 nm (2/2 lamp) wavelengths. Combined UV-LEDs illumination for 10 and 20 minutes provided a higher reduction rate

compared to the individual reduction obtained at 280 nm (2 lamps) and 365 nm (2 lamps) wavelengths. On the other hand, as exposure time increased to 30 or 40 min., the synergistic effect decreased, and additive effect became dominant. So, the results demonstrated that when 365 nm combined with 280 nm, 365 nm had a lethal synergistic effect on the microorganism up to 20 min only. Longer UV exposure time than 20 min at 365 nm did not have a lethal effect but rather it was speculated that it played a role in repair mechanism of cell. In the literature, most of studies related to investigation of photoreactivation of bacteria, used fluorescent lamps irradiated at UV-A range (310 – 480 nm). Since, it is known that the photolyase enzyme which reverses the damages by specifically binding to cyclobutene pyrimidine dimers, absorption the light in these ranges, additionally, gives a major peak near around at 375 nm which is close to 365 nm (Kim et al., 1994). Similarly, Nakahashi et al. (2014) observed synergistic effect when the UV-A LED and UV-C was used simultaneously. They suggested that simultaneous irradiation suppresses one or more recovery systems for DNA damage, especially cyclobutane pyrimidine dimers (CPDs). They also pointed out that UV-A LED irradiation has an energy threshold. If the energy threshold for the bactericidal effect is lower than the energy threshold for suppression of the recovery system, the synergistic bactericidal effect may not be seen. This threshold was reached at 20 min exposure time in this study. Additionally, fluorescent lamps emitting light at UV-A wavelength range (310 – 480 nm) were commonly used in most of the studies related to investigation of photoreactivation of bacteria. Because it is known that the photolyase enzyme reverses the UV damages by specifically binding to cyclobutene pyrimidine dimers. This enzyme absorbs the light in these ranges and gives major peak near around at 375 nm which is close to 365 nm (Kim et al., 1994). Thus, it was speculated that, the photoreactivation of cells during the UV-LED irradiation was stimulated when the 280 and 365 nm wavelengths were combined, and this resulted in reduction of the inactivation efficacy of combined UV-LED treatment. Similarly, Chevremont et al. (2012) stated that a wavelength of 280 nm was more effective than 365 nm for microbial inactivation, while the optical power of 280 nm and 365 nm UV-LEDs were only 0.55 mW and 350 mW, respectively. Moreover, they found that the combination of 280/365 nm was as efficient as 280 nm wavelength for *E. coli* ATCC 11303 inactivation in water. It was clearly observed that by combining the 280 and 365 nm wavelengths, 365 nm irradiation did not provide an additive or synergistic effect on inactivation of the cell

As a result, it could stimulate the photoreactivation of cells during the UV-LED irradiation with the combination of 280 and 365 nm wavelength and reduce the inactivation efficacy of UV-LED treatment. Similar findings were obtained in previous studies and a wavelength of 280 nm was found more effective than 365 nm, while the optical power of LEDs was only 0.55 mW and 350 mW, for the LED emitting at 280 nm and 365 nm, respectively. Moreover, combination of 280/365 nm was as efficient as 280 nm used alone on *E. coli* ATCC 11303. It was clearly observed that in the combination of 280 and 365 nm wavelengths, 365 nm irradiation had no an additive or synergistic effect on inactivation of the cell (Chevremont et al. 2012).

6.2.2. Sublethal Injury Caused by UV-LEDs in *E. coli* K12

6.2.2.1. Sublethal Injury Tested by Flow Cytometry

The sublethal damages in the *E. coli* K12 cells membrane caused by UV light was tried to be revealed by flow cytometry. The number of dead and live cells or the number of the cells which continue their metabolic activity but cannot form colonies in the plate counting method were determined. In this method, the cells in PBS solution not exposed to UV radiation were used as negative control and thermally pasteurized (90ºC, 15 minutes) cells were used as positive control.

Data from flow cytometry presented in Figure 6.4. Figure 6.4 a, b and c show percentage of live, damaged and death *E. coli* K12 cells which were untreated, thermally pasteurized and exposed to UV-LED irradiation at 254 nm for 40 minutes.

In the Figure 6.4. a, b and c, the area of P2 shows *E. coli* K12 cells separated from debris, P3 area represented alive cells, P4 area exhibits damaged bacteria and P5 area referred as the dead cells. 10000 event readings were obtained by flow cytometry. Only 38% of these events was detected as a total countable *E. coli* K12 cells (P2) i.e, live, death and damage ones, while remaining percentages were separated as impurities and/or debris (Fig. 6.4a). The 94 % and 2.5% of these untreated cells were counted as alive and death, respectively. On the other hand, 68% of the thermally pasteurized cells depicted in the area P2 was countable (Fig. 6.4b). The alive pasteurized cells were around 7.2%. Thus, 7.2% of *E. coli* K12 cells after the thermal pasteurization (90ºC for 15 min), kept their viability.

Unlikely, these results did not overlap with the data obtained from colony counting method. The colony forming method did not resulted in any countable colonies after thermal pasteurization. Similarly, 94% of the *E. coli* K12 cells treated at 254 nm for 40 minutes was determined as alive (Fig. 6.4 c). Based on this data, it is concluded that the application of 254-minute UV-LED at 40 minutes has no effect on bacteria. On the other hand, when the results of the colony counting method were compared, it was found that approximately 10^4 KOB / ml of 10^6 KOB / ml *E. coli* K12 bacteria were inactivated. This value corresponds to approximately 99.99%. As a result, it was concluded that there was no correlation and any agreement between the data obtained from flow cytometry and colony counting method. This was attributed to the role of the cell sizes measurable in flow cytometry. As it is known, microorganisms are quite small in size and volume compared to eukaryotic cells. Most bacteria range from 0.2–2.0 μm (micrometers) in diameter. Less than 1 micron (0.001 mm/0.00004 inch) in length. Therefore, precise measurement of small particles in flow cytometry highly depends on the size of the orifice. For example, the fungi can be easily distinguished in flow cytometry because they are larger in size than the bacteria. Additionally, the flow cytometry device that was used in the measurements was an old model and led to erroneous results. The total bacterial count was not correctly determined by this method. Therefore, an alternative method was used in order to determine the damage cells. The sub-lethal injury caused by UV-LED irradiation in *E. coli* K12 cells was determined via selective medium plating technique. The results of sub-lethal damages subjected to UV-LED irradiation are given in the following section.

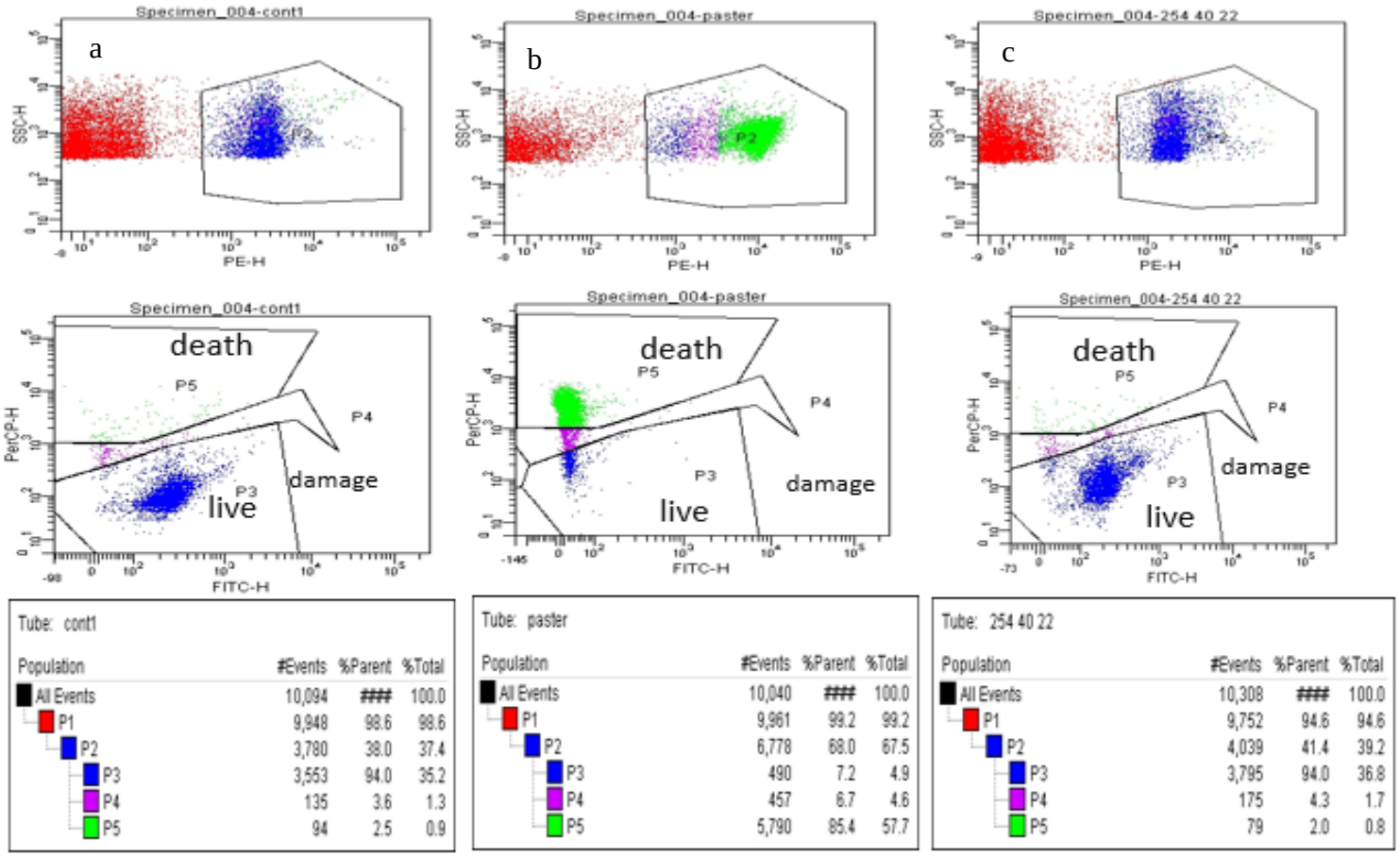


Figure 6.4. The ratio distributions of live, damaged and death *E. coli* K12 cells obtained by flow cytometry in untreated (a), thermally pasteurized (b), and treated with UV-LED light at 254 nm (c).

6.2.2.2. Sub-lethal Injury Tested by NaCl Sensitivity

The sub-lethal injury of *E. coli* K12 was determined by plating TSA and TSA supplemented with NaCl to elucidate whether UV-LED lamps emitted light at 254 nm (4 lamps), 280 nm (4 lamps), 254 nm (2 lamps), 280 nm (2 lamps) and 365 nm (2 lamps) and the combination of 280 nm and 365 nm caused bacterial damage. Several NaCl concentrations (1-5%) were tested for the determination of the maximum concentrations that do not affect the growth of healthy untreated cells. In this preliminary study, 3% NaCl concentrations was selected for sublethal injury study since above these concentrations healthy *E. coli* K12 cells were inhibited (Figure 6.5).

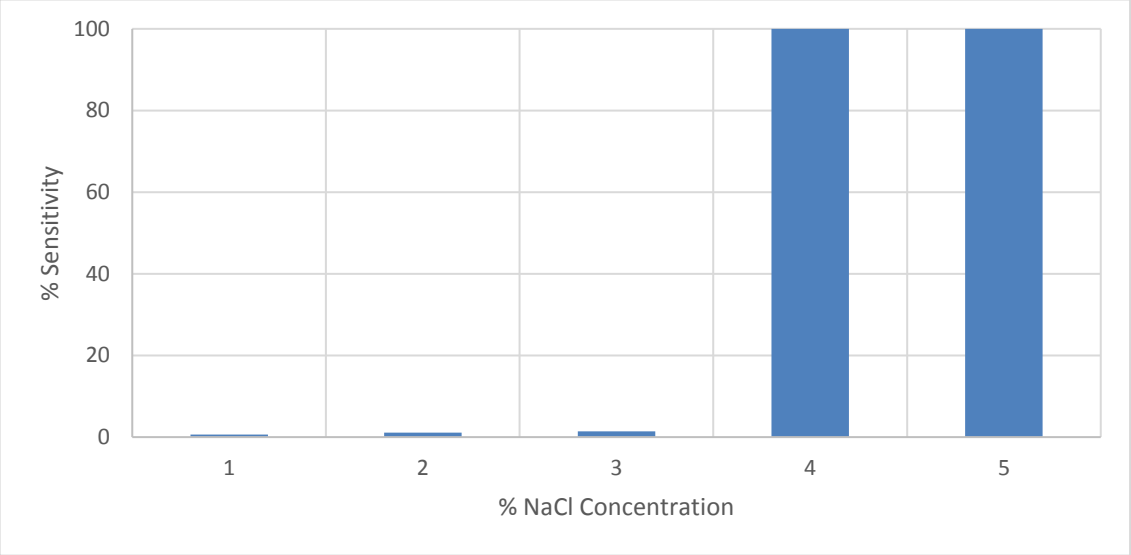


Figure 6.5. The sensitivity of untreated *E. coli* K12 cells to different NaCl concentration

It is known that outer membrane of Gram-negative bacteria such as *E. coli* K12 contain a lipopolysaccharide layer. It allows passage of hydrophobic molecules from the external environmental medium. Additionally, the cytoplasmic membrane is responsible for the regulation of osmotic pressure (Kim et al. 2016). The noticeable sublethal injury was detected in *E. coli* K12 cells irradiated with 254 (4 L), 280 (4 L) and 280/365 nm (2/2 L) LEDs', although the degree of injury varied with the types of UV-LED (Figure 6.6). Overall, the percentage of sub lethally injured cells increased by increasing the applied UV dosage. UV dosages supplied by four lamps emitted light at 254 nm and 280 nm LED lamps were higher than two lamps used at the same wavelengths. On the other hand, an illumination at 365 nm and 254 nm using 2 UV-LED lamps did not cause significant cell injury.

Additionally, upon increasing the number of lamps at 254 nm, prolonging the irradiation time from 10 to 40 min also resulted in an increase in sensitivity percent from 6.3% to 22.70%. The inactivation mechanism of UV-C irradiation at 254 nm is attributed to the absorption of UV light by nucleic acids of microorganisms causing breakage in bonds of their DNA and crosslinking adjacent pyrimidine bases on the same DNA strand. These findings showed that UV-LED lamps emitted at 254 nm at high dosages do not cause only formation of pyrimidine dimers, but also contribute the membrane or cytoplasmic damages (Koutchma, 2009).

When the cells were illuminated at 280 nm using 2 lamps, the number of injured cells was not significant up to 30 min exposure time. But, the sensitivity of the cells was increased to 19% when the cells were illuminated for 40 min. On the other hand, the sensitivity of cells increased from 8.73% to 49.48% with the increase of exposure time when the higher UV doses (4 lamps) were applied at 280 nm. Still, the illumination of cells for 40 min provided the highest injury in the cells.

The degree of sublethal damage on cells by the combination of UV-LEDs' emitted light at 280 nm and 365 nm (2 L/2 L) showed similar trend that was observed in the irradiation with 280 nm lamps. The injury in the cells following UV-LED application up to 30 min were not significant, whereas for 40 min application achieved the sublethal injury around 26.73%. The data indicated that usage of 365 nm (2L) alone was not effective on sublethal injury of *E. coli* K12 regardless of time. When combined with 280 nm, similarly, they did not cause any considerable damage on cells up to 30 min. On the other hand, for 40 min application, they showed a synergistic effect on sublethal injury on cells. But still, the percentage of sublethal injury by combination of 280 and 365 nm was lower than that by 280 nm (4 L).

It was expected more cell injury when a combination of 280 and 365 nm wavelengths was used. Because the different inactivation mechanisms are involved at these wavelengths. The formation of reactive oxygen spices oxidizing the membrane and proteins of microorganisms resulting in a protein damage by 365 nm wavelength, and DNA damage by 280 nm wavelength was expected to occur simultaneously when these two wavelengths were combined. But in our study, the overall results indicated that both outer and cytoplasmic membrane of *E. coli* K12 significantly more damaged or suffer more from the loss of their functionality when the cells were exposed to UV-LED light at 280 nm (4 L) out of the tested wavelengths.

In the literature, there are a few studies revealing the sublethal injury of bacteria exposed to UV irradiation via testing with NaCl sensitivity (Kim et al. 2016, Kim et al. 2015, Ghate et al. 2015). Kim et al. (2016) evaluated the antibacterial effect and mechanism of 405±5 nm light emitting diode (LED) on *Escherichia coli* O157: H7, *Salmonella Typhimurium,* and *Shigella sonnei*. Their results showed that LED illumination increased bacterial sensitivity to bile salts and NaCl. Because of LED illumination, the loss of bacterial membrane integrity was confirmed, whereas no DNA fragmentation was observed. Moreover, in another study, the sublethal injury was observed in some Gram-positive and -negative pathogens during illumination with the 461 and the 521 nm LED and the percentage of injured cells increased as the treatment time increased (Ghate et al. 2015).

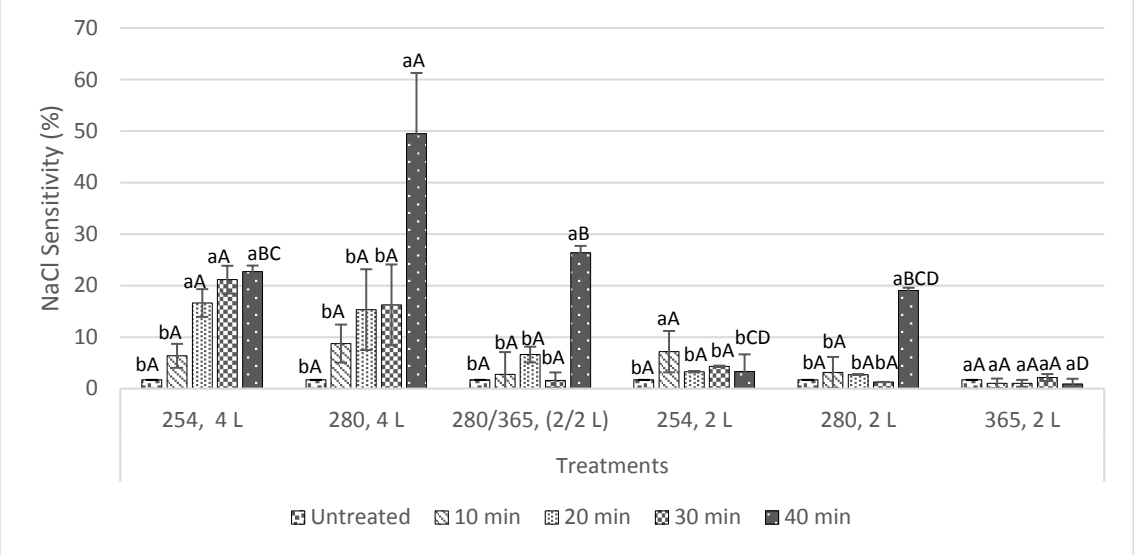


Figure 6.6. Changes in the sensitivity of *E. coli* K12 to 3% NaCl concentration during UV-LED irradiation with different individual and combined wavelengths and exposure time. Results were presented as "means ± standard error". The least significant difference was determined by Tukey pairwise comparison test. $^{A–B}$: Values within each exposure time at same wavelength or combination followed by the same letter are not significantly different ($P > 0.05$). a–c: Values within each wavelength and their combinations at the same exposure time followed by the same letter are not significantly different ($P > 0.05$).

6.2.3. Photoreactivation and Dark Repair Mechanism of *E. coli* K12

The photoreactivation and dark repair behaviors of *E. coli* K12 treated with UV-LED by individual 254 nm (4 lamps) and 280 nm (4 lamps), combining 280 nm and 365

nm wavelengths (2 lamp/2 lamps) for 40 min were investigated during 6 h incubation period at 4ºC.

The effects of different wavelengths and their combinations as a function of incubation time under dark conditions on reactivation mechanism of *E. coli* K12 in PBS were demonstrated in Figure 6.7, 6.8 and 6.11. Incubation of PBS after 254 nm UV-LED irradiation from 0 up to 90 min at 4˚C led to 0.9 log of increase in the recovery of the *E. coli* K12 cells (Fig. 6.7a), i.e., approx. 0.1% (Fig. 6.7), then the number of the cells dramatically decreased and stayed constant around 0.02 % between 90- and 360-min dark incubation period. Whereas, the recovery of cells following the 280 nm UV-LED irradiation tend to an increment at a level of 0.1 % (Fig. 6.8b) up to 90 min, further prolongation of the dark incubation time gave a weak improvement in recovery. At the end of the 6 h dark incubation period, not a reliable recovery was observed in cells subjected to UV-LED irradiation at 280 nm. The recovery of *E. coli* K12 cells in PBS following 280/365 nm UV-LED irradiation was slightly increased during 360 min of dark incubation periods at 4º C and reached to approx. 0.9 log CFU/mL (Fig. 6.8c). At the end of the dark incubation period, the results indicated that the repair mechanism in *E. coli* K12 was not effective under dark conditions (Fig. 6.11). However, it is interesting that the highest repair was observed in cells treated with 280/365 nm combination. It is widely known that the combination of UV-A and UV-C light cause an irreversible damage on DNA. UV-A irradiation helps to increase the inactivation efficiency by the way of damaging proteins and producing hydroxyl and oxygen radicals which can destroy cell membranes and other cellular components (Chatterley and Linden, 2010; Santos et al. 2013). In this respect, it was expected that combination of UV-A and UV-C irradiation inhibit the recovery of cells. In contrast, it was revealed that 365 nm irradiation played a role in repairment of cells which were damaged by 280 nm. Thus, it was speculated that during the dark incubation period, injured cells tended to recover themselves. Besides, percent recovery of cells treated with 280 nm is lower than that of treated with 254 nm. It was concluded that 280 nm was more effective in damaging the nucleotide excision repair system compared to 254 nm. These results were in line with the studies conducted by Salcedo et al. 2007, Yin et al. 2015a, Zimmer and Slawson, 2002, Zimmer-Thomas et al. 2007. They pointed out that illumination at 280 nm wavelength may inhibit the protein activity by causing denaturation of proteins. These proteins play a role in nucleotide excision repair system by coordinating the removal of DNA.

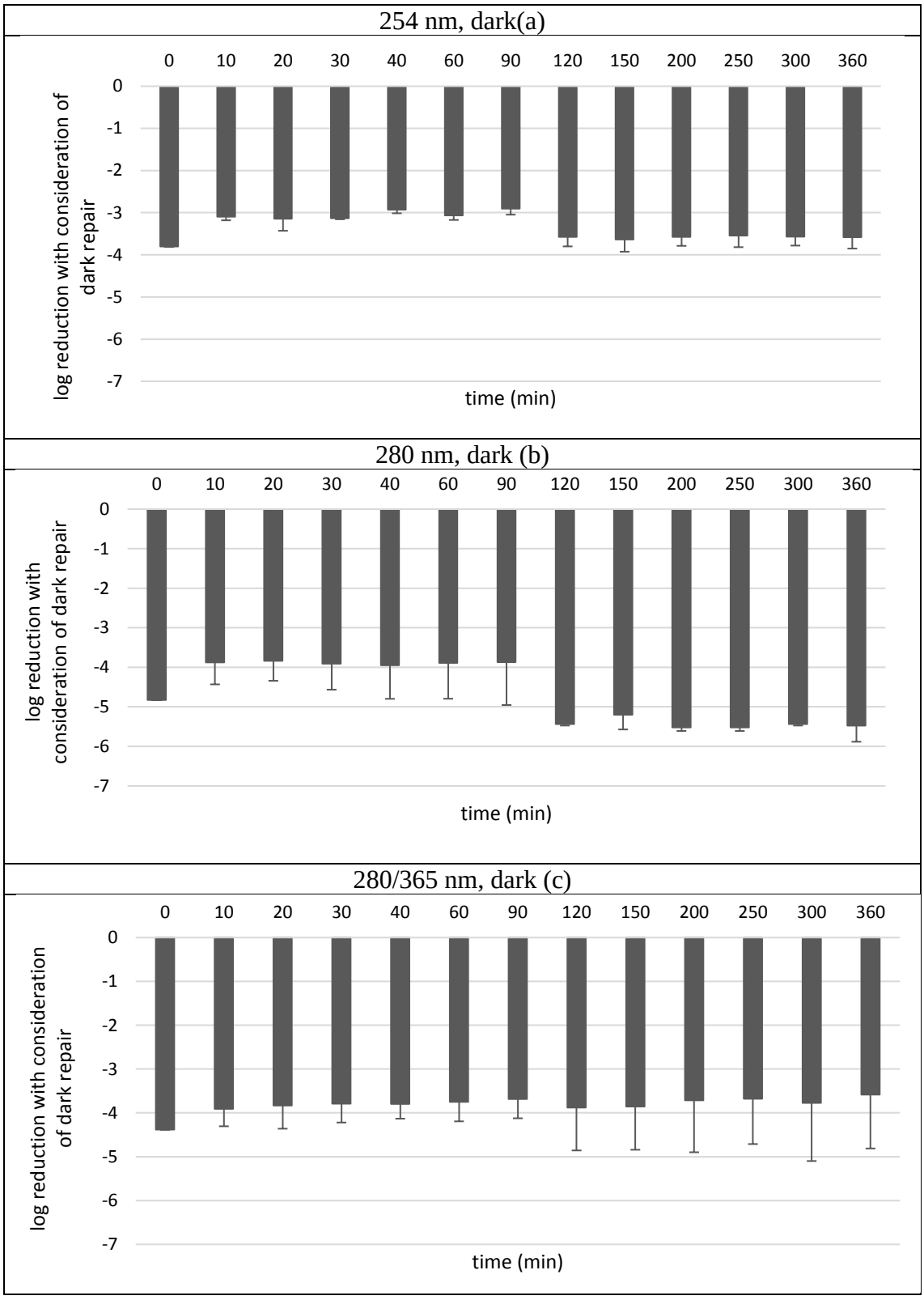


Figure 6.7. Regrowth of *E. coli* K12 in PBS subjected to 254 nm, 280 nm and 280/365 nm UV-LED irradiation during 6 h dark incubation period

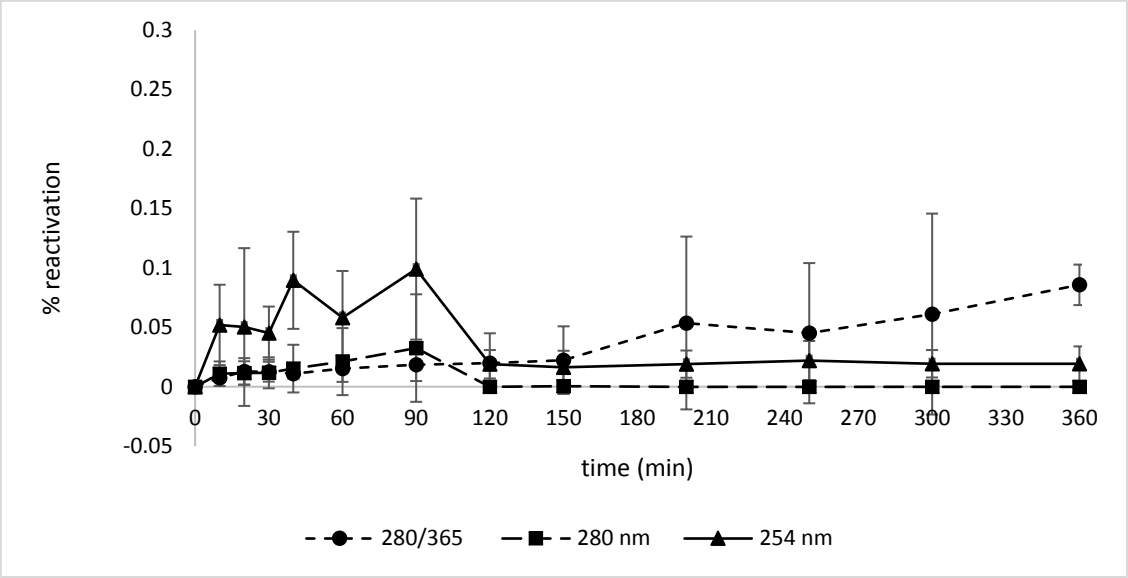


Figure 6.8. Dark repair percentages of *E. coli* K12 in PBS subjected to 254 nm, 280 nm and 280/365 nm UV-LED irradiation as a function of time.

Photoreactivations of *E. coli* K12 in PBS after 254 nm, 280 nm and 280/265 nm UV-LEDs irradiation with 40 min exposure time as a function of incubation time at 4 ˚C for 6 h were demonstrated in Figure 6.9, 6.10 and 6.11. Illumination of CAJ after 254 nm UV-LEDs irradiation up to 90 min led to a distinct increase in the recovery of the cells in CAJ, i.e., approx. 1.2 log (Fig. 6.9a). The photoreactivation of cells repeated for 6 h between the periods of 20-90 min, and 120-360 min. The maximum recovery was 1.1 log obtained at the end of the incubation in 254 nm treated cells. For 1.1 log recovery, the percentage photoreactivation was 0.15% and it did not exceed the 1% (Fig. 6.10). On the other hand, under the same illumination condition, the photoreactivation percentages was the lowest for 280 nm irradiated cells illuminated for 6 h (Fig. 6.8b; Fig. 6.10). Photoreactivation ratio of 280/365 nm irradiated cells led to an increment up to 250 min of illumination period at a percentage of 0.04% (Fig. 6.9c; Fig. 6.10), then the cells kept their viability at a stationary phase until the end of the incubation period. At the end of the dark incubation period, the results demonstrated that 280 and its combination with 365 nm could also effectively inhibit the photoreactivation of cells compared to 254 nm wavelength (Fig. 6.11). They could cause the damage on photolyase enzyme and not allow to repair the cells via reversing the formation of dimers at the same DNA strand.

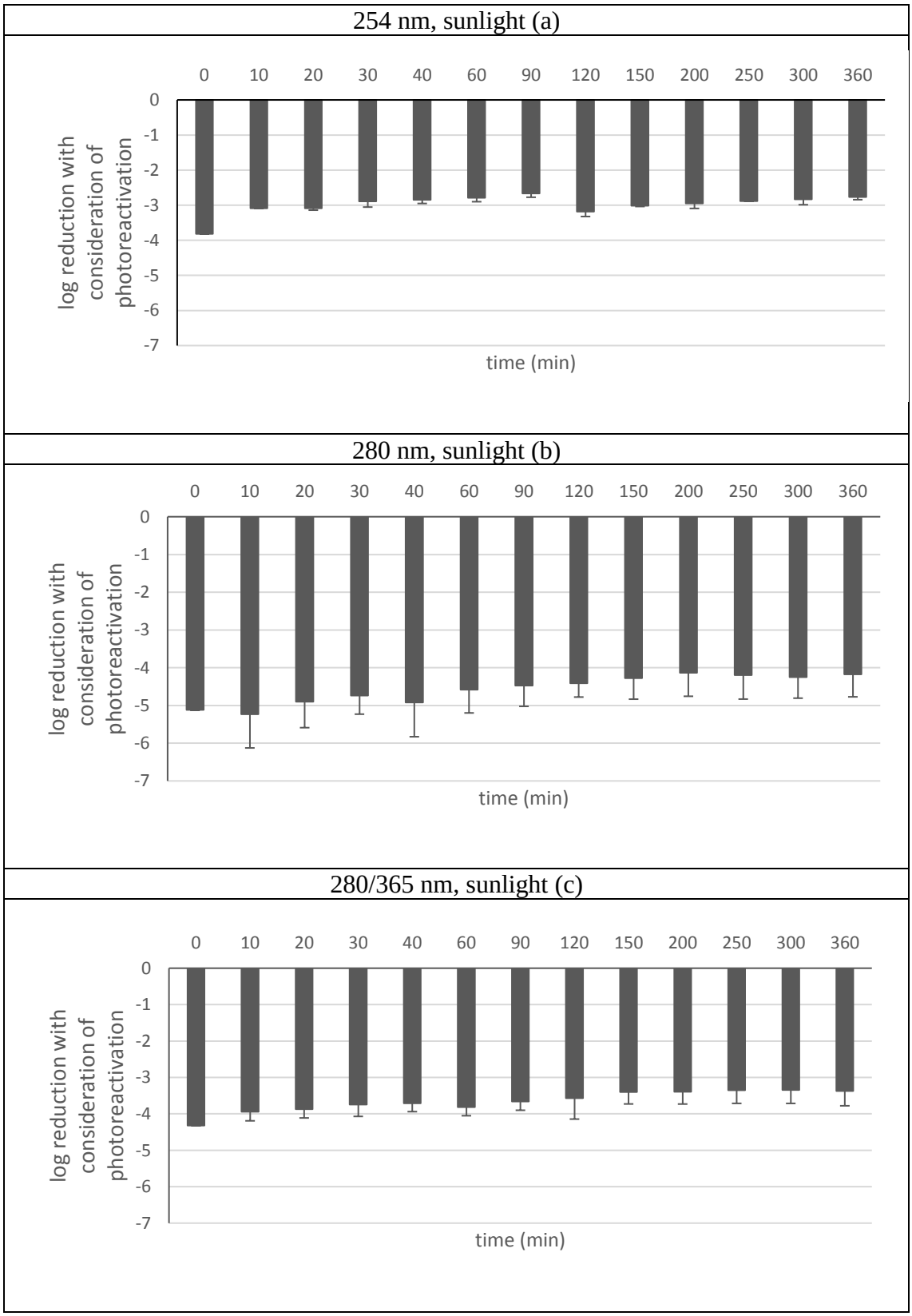


Figure 6.9. Regrowth of *E. coli* K12 in PBS subjected to 254 nm, 280 nm and 280/365 nm UV-LED irradiation during 6 h light incubation period

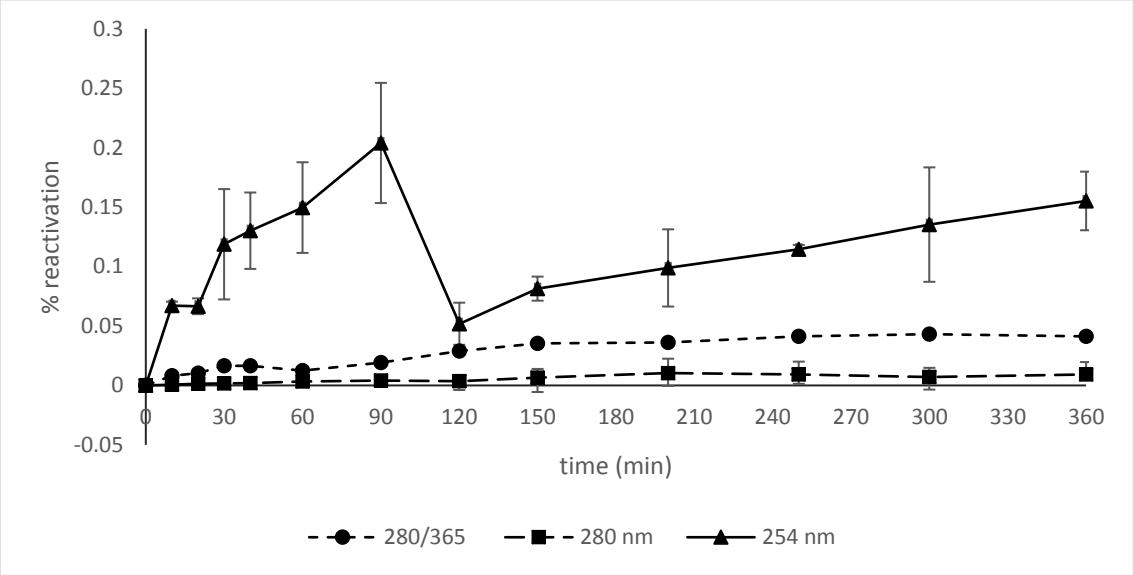


Figure 6.10. Percent photo-reactivation of *E. coli* K12 in PBS subjected to 254 nm, 280 nm and 280/365 nm UV-LED irradiation during 6 h incubation period

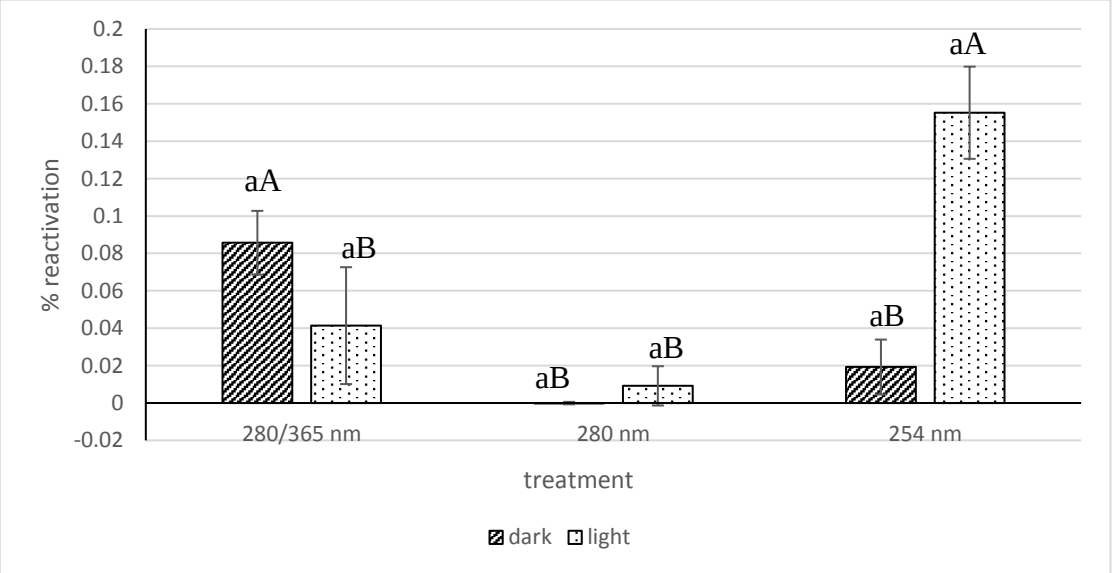


Figure 6.11. Percent reactivation of *E. coli* K12 in PBS subjected to 254 nm, 280 nm and 280/365 nm UV-LED irradiation at the end of 6 h dark and light incubation period. Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. A–B:Values within each exposure time at same wavelength or combination followed by the same letter are not significantly different ($P > 0.05$). a–c: Values within each wavelength and their combinations at the same exposure time followed by the same letter are not significantly different ($P > 0.05$).

In summary, no significant photoreactivation and dark repair occurred after UV-LED irradiations at the end of the 6 h incubation period (Figure 6.11). However, the level of photoreactivation and dark repair after 280 nm LED irradiation were lower than 254 nm and 280/365 nm. Such observation was also reported by the others (Li et al. 2017). The photoreactivation and dark repair behavior of *Escherichia coli* following UV-LEDs at 265 nm, 280 nm, the combination of 265+280 (50%), and 265+ 280 (75%) and low pressure (LP) UV disinfection were tested. While, 280 nm UV-LED irradiation showed lower inactivation performance on *E. coli*, significantly repressed the photoreactivation and dark repair mechanism (Li et al., 2017). Still, little is known on DNA damage and inactivation mechanism in the cells subjected to UV irradiation when combinations of different UV wavelengths are used (Nakahashi et al. 2014).

6.2.4. Inactivation Mechanism Analysis

6.2.4.1. Determination of DNA Strand Breakages

Quantification of DNA strand breakages (DSB) of *E. coli* K12 in PBS followed by 254 nm (4 lamps), 254 nm (2 lamps), 280 nm (4 lamps), 280 nm (2 lamps), 365 nm (2 lamps) and combining 280 nm and 365 nm wavelengths (2 lamp/2 lamps) were determined by fluorometric analysis of DNA unwinding (FADU). The dsDNA content has negative relationship with DNA damage in cells (He and Häder, 2002), and it was found that DNA strand breaks of *E. coli* K12 cells changes with exposure of UV-LED irradiation depending on wavelengths and exposure time, the results shown in Figure 6.12. and 6.13.

For ten minutes application of UV-LED irradiation, the maximum double strand breakages on the DNA of the *E. coli* K12 cells were observed when the cells subjected to 254 nm 4 lamps and combination of 280/365 nm. A 48.68% of double strand breakages was observed on DNA of the cells with exposure of 254 nm 4 lamp, application of the combination of the 280 nm and 365 nm caused 45.82 % breakages. Although, it is widely known that 254 nm is attributed to the absorption of UV light by nucleic acids of microorganisms causing breakage in bonds of their DNA and crosslinking adjacent pyrimidine bases on the same DNA strand, it was clearly observed that 280/365 nm with 10 min exposure time was as effective on the formation of DSB as 254 nm (4 L) with the

same exposure time ($p>0.05$). When the exposure time increase from 10 min to 40 min, 254 nm with 2 L UV-LED irradiations slightly increased the level from 37.12% to 48.71%, with 4 L irradiation increased that from 45.83% to 63.59%, when the exposure time increase from 10 min to 40 min. It was an expected result when considering the inactivation mechanism of the 254 nm. It is widely known that the germicidal effect of UV light at 254 nm is attributed to the absorption of UV light by DNA molecules. DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption at 254 nm. This absorption creates damage in the DNA by altering the nucleotide base pairing, thereby creating new linkages between adjacent nucleotides on the same DNA strand (Abdallah et al, 2012). Two major classes of mutagenic DNA lesions induced by UV irradiation are cyclobutane-primidine dimers (CPD) and 6-4 photoproducts (6-4 PPs). Between them, the CPDs are the most abundant and make up around 75% of the UV-induced DNA damage products (Sinha and Hader, 2002). The formation of CPDs causes DNA strand breakages and prevents the replication of the microorganisms so that the cells ultimately die off (Quek and Hu, 2008).

280 nm 4 lamp application was not effective as 254 nm 4 lamps' on the formation of the DSB for 10 min exposure time. The percentage of DSB on cells was recorded as 35.43% which was lower than that of 254 nm ($p<0.05$) even though they have similar UV dosages (24.98 mJ/cm^2 at 254 nm and 27.11 mJ/cm^2 at 280 nm) and take part in at the same UV region. It could be an evidence that not only DSB formation, but also different mechanisms played the role on the inactivation of *E. coli* K12 subjected to 280 nm UV-LED irradiation (Santos et al. 2012).

The DSB percentage of DNA on cells rise to 20.47% with in 10 minute following 365 nm UV-LED irradiation, then they stayed their level up to 30 min. For 40 min application a slight increase was recorded as 28.71%, but it was not statistically different that obtained from 10 min application.

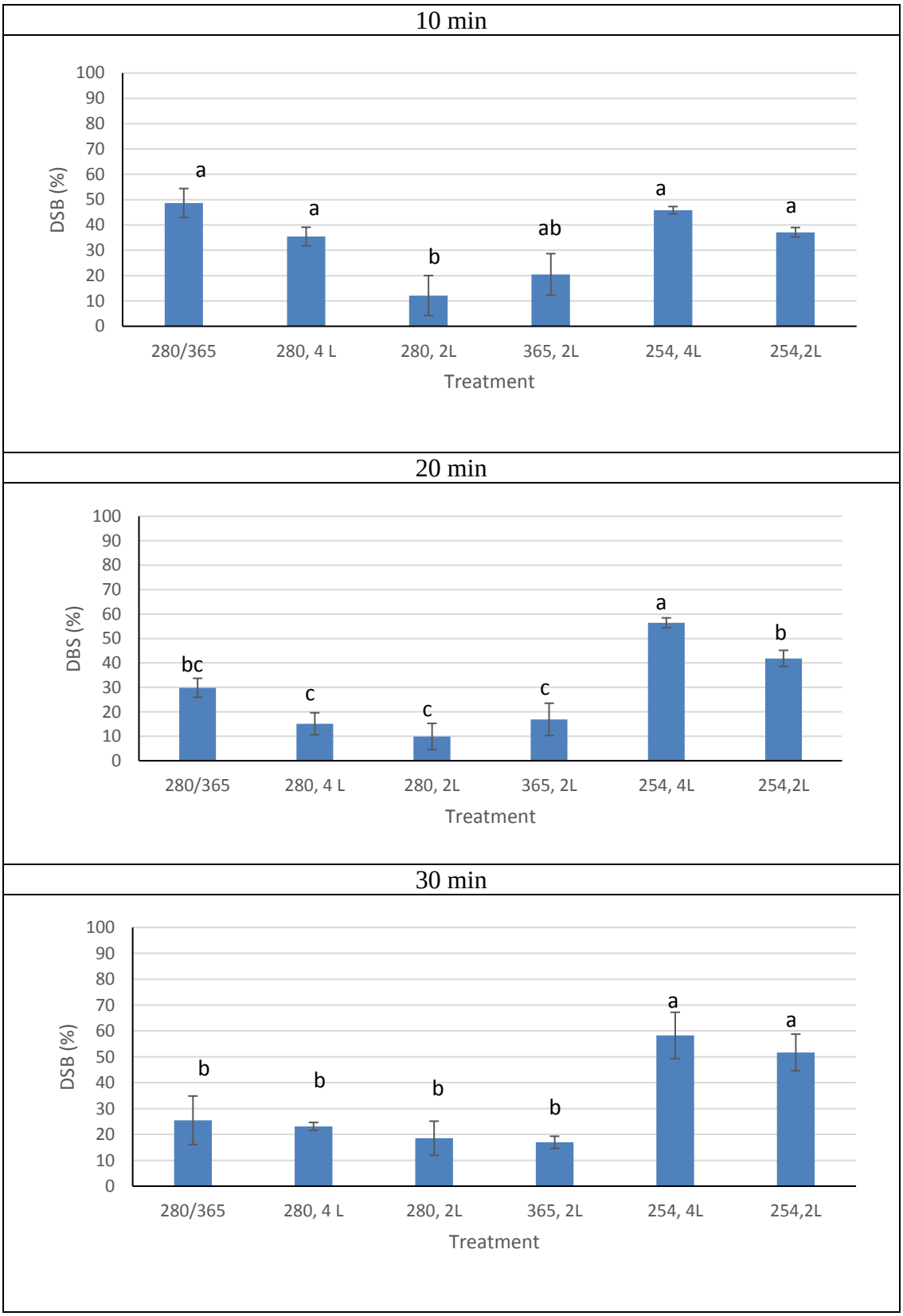


Figure 6.12. Effects of different UV-LED wavelengths on Double Strand Breakage (DSB) of *E. coli* K12 at different exposure times. Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. a–c: Values within each wavelength and their combinations at the same exposure time followed by the same letter are not significantly different (P > 0.05).

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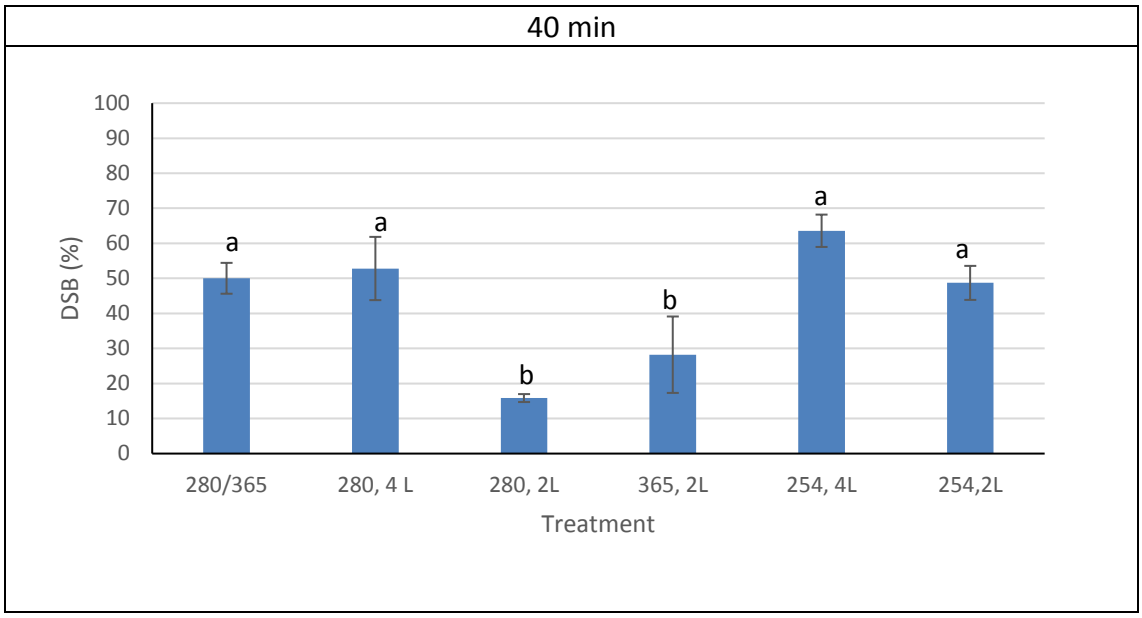


Figure 6.12. (cont.)

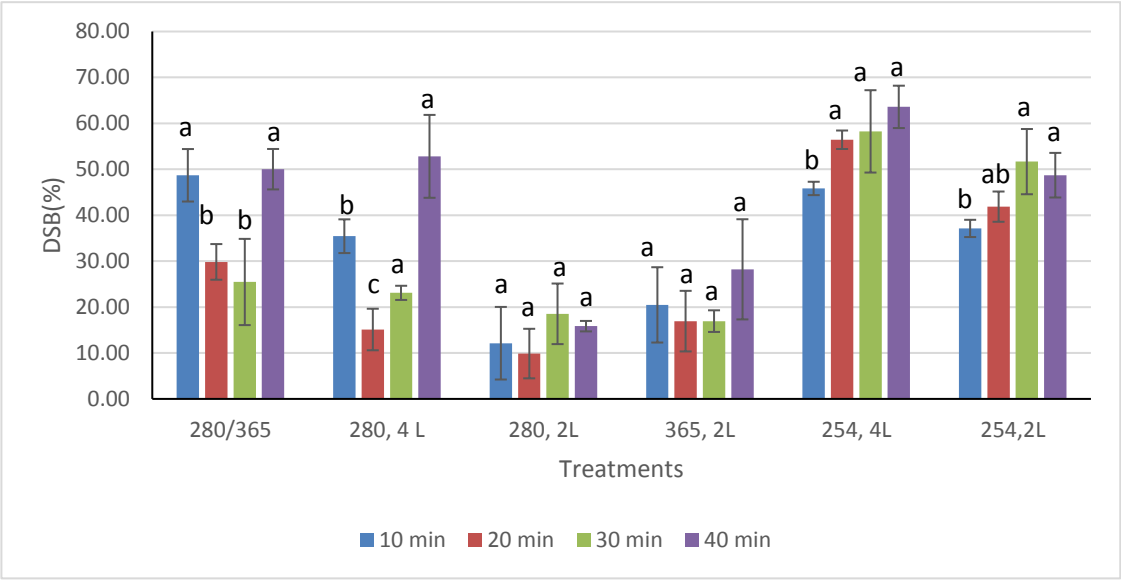


Figure 6.13. Effects of different UV-LED wavelengths on Double Strand Breakage (DSB) of *E. coli* K12 as a function of exposure time. Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. a-c: Values within each wavelength and their combinations at the same exposure time followed by the same letter are not significantly different (P > 0.05).

Figure 6.14 demonstrates the double strand breakage percentage of the combined UV treatment with wavelength of 280/365 nm (2 lamps/2 lamps) to determine whether a synergistic inactivation effect could be achieved. The synergistic effect was calculated that by using sum of the individual double strand breakage percentage of *E. coli* K12 in PBS, obtained at 280 nm (2 Lamps) and 365 nm (2 Lamps) with 10 min exposure time.

280/365 nm UV-LED irradiation with 10 min exposure time caused a 48.69% DSB, that was higher than the sum of the DSB of DNA (32.60%) obtained by individual application of 280 nm (2 L) (12.12%) and 365 nm (2L) (20.47%) UV-LED irradiation and application of 280 nm (4 L) (35.43%). In other words, the combination of 280 nm and 365 nm wavelengths with 10 min exposure time stimulated to formation of DSB on DNA of *E. coli* K12 compared to individual application of them. A meaningful change was not observed the level of DSB following the individual application of 280 nm (2 L) and 365 nm (2 L) between the 10, 20 and 30-min exposure times ($p>0.05$). On the other hand, the DSB formation significantly decreased with simultaneous application of 280 nm and 365 nm UV-LED irradiation with 20- and 30-min exposure time compared with 10 min. The synergistic effect on DSB formation decreased, and additive effect became dominant. It was speculated that 365 nm wavelength played a role in repair DSB on DNA of cell by the way of induced the production of photolyase enzyme (Santos et al. 2013). For 40 min exposure time, the level of DSB obtained by simultaneous application of 280 nm and 365 nm unexpectedly increased to 50% which was higher than sum of the individual application of them. This increment could be a result of the effects of oxidative products on DNA of the cells. Because UV-A wavelength was attributed to enhance production of reactive oxygen species (ROS) resulting in oxidative damage to lipids, proteins and DNA. ROS can lead to strand damages on DNA indirectly by reacting hydroxyl radical, and singlet oxygen with sugars, purines and pyrimidines (He and Häder, 2002). Nakahashi et al. (2014) investigated the bactericidal effect of simultaneous UVC irradiation and UV-A LED illumination on *Vibrio parahaemolyticus*. They found that UV-A LED in this combined system had two functions, one function was the suppression of the recovery of the cells, and second one was the direct bactericidal effect. They also pointed out an energy threshold value for UV-A LEDs. They indicated that if this energy threshold is lower than the energy threshold for suppression of the recovery system, the synergistic bactericidal effect may not be observed.

In summary, wavelengths in the UV-A range can affect membranes and membrane functions, and wavelengths in the UV-B and UV-C ranges have shown to be absorbed by proteins (Zimmer and Slawson, 2002). UVA wavelengths are less efficient in inducing DNA damage because they are not absorbed by native DNA but they can still produce secondary photoreactions of existing DNA photoproducts or damage DNA via indirect photosensitizing reactions. The high energy short-wavelength photons absorbed by chromophoric molecules can lead to the formation of singlet oxygen or free radicals

known to destroy membranes and other cellular components. In fact, photons from UV-A radiation (320-400nm) and visible light up to 670 nm are also able to generate $^{1}O2$ through type II photosensitization reactions (Sinha and Hader, 2002). Inhibition of repair might reflect the specific ability of UV-A to induce irreversible physiological changes, including metabolic inhibition (Zimmer and Slawson, 2002).

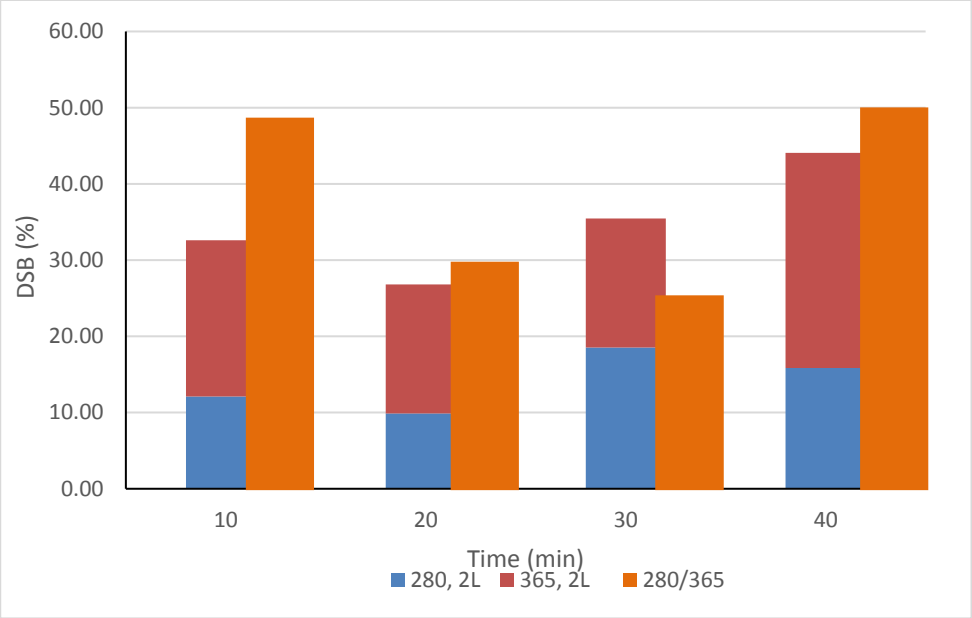


Figure 6.14. The Double Strand Breakage (DSB) percentage in DNA of *E. coli* K12 treated with individually and simultaneously by UV-LED irradiation at 280 nm and 365 nm

6.2.4.2. Lipid Oxidation

In biological membrane systems, lipid peroxidation is frequent consequence of free radical and ROS attack and yields products that often react with DNA and proteins leading to oxidative modification. Peroxidation of lipids generates reactive species such as free radicals, carbonyl compounds and hydroperoxides. These reactive species decompose via chemical degradation and catalytic transition metal ions to give rise to more toxic breakdown products. Malondialdehyde (MDA) is a major lipid peroxidation product which is formed by peroxidation of polyunsaturated phospholipids in lipid membranes (Cho et al. 2010). The lipid peroxidation level of *E. coli* K12 cells subjected

to 254 nm (4 lamps), 254 nm (2 lamps), 280 nm (4 lamps), 280 nm (2 lamps), 365 nm (2 lamps), and combination of 280 nm and 365 nm wavelengths (2 lamp/2 lamps) were determined by measurement of malondialdehyde (MDA) formation. Figure 6.15 shows the effect of UV-LED irradiation emitted light at different wavelengths and exposure times on the MDA level of the *E. coli* K12.

In earlier publications indicated that the bacterial inactivation under UV-C irradiation emitted light at 254 nm was attributed to inhibition of the DNA replication (Yakobson et al. 1989). However, some new studies suggested that the UV-C irradiation was also caused the alteration of the membrane structure (Zhang, Zhou, and Zhang 2014). In this study, it was clearly observed that the application of the 254 nm UV-LED irradiation stimulated the formation of the lipid oxidation. The subjection of the 254 nm UV-LED irradiation on *E. coli* K12 accumulated the oxidative damage on lipids of *E. coli* K12 cells with increasing exposure time for two or four lamps applications. 254 nm with 4 L UV-LED irradiations slightly enhanced the level from 21.65% to 40.72%, with 2 L irradiation increased from 8.94% to 24.57% when the exposure time increase from 10 min to 40 min. Our results showed similarity with previous studies in the literature. Zhang, Zhou, and Zhang (2014) investigated the impact of the UV-C irradiation emitted light at 254 nm on formation of the lipid peroxidation during the inactivation of the *Bacillus subtilis* spores. Approximately 34% rise in the MDA level of the cells was observed after UV-C irradiation. Cho et al. (2010) was also reported an increment on MDA level of the *E. coli* when the UV-C dosage increased.

On the other hand, the treatment of 280 nm UV-LED irradiation had a contrary effect on formation of lipid peroxidation product i.e. MDA in the cells compared with 254 nm applications. The MDA level of cells subjected to 280 nm (4L) UV-LED irradiation with 10 min exposure time was stimulated to a maximum level, reached to 60 %. After 10 min. exposure time, the formation of MDA was tended to decrease. MDA level of the cells reduced to 14.43% at the end of the 40 min. The same trend was observed when the cells subjected to 280 nm (2L) UV-LED irradiation. The MDA level of the cells exposed to 2 L 280 nm UV-LED irradiation enhanced to 41% within a 10 min, then it dramatically decreased to the level of untreated ones at the end of the 40 min although the exposure time increased. On the other hand, the individual application of the 365 nm UV-LED irradiation caused 30% of increment on the level of MDA with 10 min exposure time. The prolongation of the exposure time did not cause any significant change in MDA level, stayed between the level of 35% and 30%. The changes in the MDA level of the

cells that were exposed to simultaneous application of 280 nm and 365 nm showed the similar tendency with ones of the individual 2 L application of 280 nm UV-LED irradiation. The increment of the MDA level similarly reached to 35.91% within 10 min. However, after 10 min, MDA level slightly decreased and become at the same level with untreated ones ($p>0.05$). In this respect, it could be attributed that 365 nm UV-LED irradiation did not make a major contribution on the changes of the MDA percent of the cells during the simultaneous application of the 280 nm and 365 nm UV-LED irradiation. The decrease in the MDA level after prolonged illumination of the 280 nm could be a reason of the participation of the MDA in other chemical reaction. It is widely known that UV irradiation at wavelengths near 280 nm cause the oxidative stress on proteins in cells. It was thought that the accumulation of the MDA products could modify proteins by the carbonylation or form protein-MDA complex (Mannes et al. 1999). Semchyshyn et al. (2005) indicated that the oxidative stress induced by hydrogen peroxide causes damage to both proteins and lipids in *E. coli* cells. Thus, TBARS production was decreased, whereas a significant rise in carbonyl proteins was registered only at the maximum concentration used.

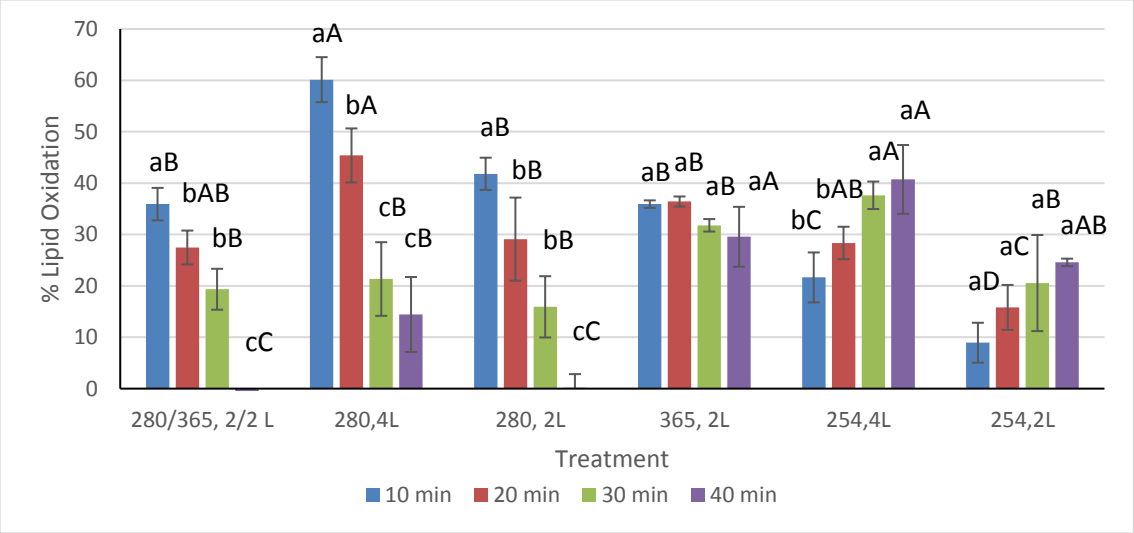


Figure 6.15. Effects of different UV-LED wavelengths on lipid oxidation in *E. coli* K12 cells as a function of exposure time. Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. A–D:Values within each exposure time at same wavelength or combination followed by the same letter are not significantly different ($P > 0.05$). a–c: Values within each wavelength and their combinations at the same exposure time followed by the same letter are not significantly different ($P > 0.05$).

6.2.4.3. Protein Oxidation

The protein oxidation level in *E. coli* K12 cells subjected to 254 nm (4 lamps), 254 nm (2 lamps), 280 nm (4 lamps), 280 nm (2 lamps), 365 nm (2 lamps) and combination of 280 nm and 365 nm wavelengths (2 lamp/2 lamps) were determined by measurement of carbonyl level of the cell. Accumulation of carbonyl level is widely used as an indicator of the intensity of oxidative stress and measurement of the ratio give an idea about the relation between the oxidative stress and power of the protective system (Semchyshyn et al. 2005).

Figure 6.16 depicts the effect of UV-LED irradiation at different wavelength and their combination with different exposure time on the formation of the protein oxidation in *E. coli* K12 cells. It was clearly observed that the formations of the protein oxidation in cells subjected to UV-LED irradiation at different wavelengths was stimulated with prolongation of the illumination time. The subjection of the 254 nm UV-LED irradiation on *E. coli* K12 accumulated the oxidation of protein in cells with increasing exposure time both two and four lamps applications. 254 nm with 4 L UV-LED irradiations slightly enhanced the level from 42.60% to 84.34%, with 2 L irradiation increased that from 20% to 79.13% when the exposure time increase from 10 min to 40 min. Similar oxidation level results were obtained with the application of 254 nm UV-LED irradiation when the cells exposed to 280 nm with 2 L UV-LED irradiations. The level reached at the percent of 32.15% with 10 min exposure time, then this percent slightly increased to 83.93% when the exposure time increased to 40 min. On the other hand, the maximum protein oxidation increment was recorded when the cells subjected to 280 nm with 4 L UV-LED irradiations for each exposure time. The oxidation level increased to 133.05% with in the 10 min illumination. After 40 min irradiation, 221.74% of the enhancement was observed on the protein oxidation level in the cells. As it mentioned in lipid oxidation session, the malondialdehyde level in cells decreased when prolongation of exposure time of 280 nm irradiation. Therefore, it was thought that the MDA products could react with proteins and stimulate protein carbonylation. The excessive increment on the protein oxidation level of cells subjected to 280 nm with 4 L could be an evidence of this phenomena. Besides, 10 min 365 nm UV-LED irradiation did not cause any significant changes on the protein oxidation of the cells. After 20 min exposure time, oxidation slightly increased to the percent of 66.10% at the end of the 40 min. Although it is known that UV-A

irradiation induced the oxidative damage on the membrane lipids and proteins of the microorganism, the application of 365 nm illumination to cells resulted in low level oxidation. Different microorganisms show resistance to UV irradiation according to their cell wall structure, genomic composition, and etc. (Santos et a. 2013). The results demonstrated that *E. coli* K12 resists to 365 nm irradiation and has defense mechanism against the protein carbonylation.

The changes in the protein oxidation level of the cells that exposed to simultaneous application of 280 nm and 365 nm showed the similar tendency with ones that the individual 2 L application of 280 nm UV-LED irradiation. The increment of the protein oxidation percentage similarly reached to 30.91% within 10 min ($p>0.05$), after 20 min, oxidation suddenly increased to 121.42% and 208.92 % and become at the same levels with the ones that exposure to 280 nm 4L for 30- and 40-min application, respectively ($p<0.05$). This effect could be observed in simultaneous application of 280 nm and 365 nm UV-LED irradiation with 10 min exposure time. At this condition, a percent of 30.53 % protein oxidation increment was determined which was lower than the sum of that obtained by individual application of 280 nm (2 L) and 365 nm (2L) UV-LED irradiation. On the other hand, up to 10 min, it was clearly observed that the percentages of protein oxidation obtained by the simultaneous application of 280 and 365 nm were higher than the sum of values obtained by individual application of them, the differences between them increased with prolongation of the illumination time from 20 min to 40 min, respectively. The simultaneous application induced the oxidation at the level of 76.79%, 121.43 and 208.93%, whereas the sum of values was calculated 60.71%, 91.07% and 150% for 20, 30- and 40-min exposure time, respectively. It could be a reason of the synergistic effect of the simultaneous application of 280 nm and 365 nm UV-LED irradiation. But still, the percentage of oxidation values by combination of 280 and 365 nm was lower than that by 280 nm (4 L).

Figure 6.17 demonstrates the protein oxidation level of the *E. coli* K12 cells subjected to combination of 280/365 nm (2 lamps/2 lamps) and sum of oxidation level of the cells subjected to individual application of them to determine whether a synergistic effect could be achieved. It was clearly observed in the Figure 6.15 that the percentages of protein oxidation obtained by the simultaneous application of 280 and 365 nm were higher than the sum of values obtained by individual application of them, the differences between them increased with prolongation of the illumination time from 10 min to 40 min. The simultaneous application induced the oxidation at the level of 76.79%, 121.43

and 208.93%, whereas the sum of values was calculated 60.71%, 91.07% and 150% for 20, 30- and 40-min exposure time, respectively. Thus, it could be concluded that there is a synergistic effect of the simultaneous application of 280 nm and 365 nm UV-LED irradiation on the stimulation of the protein oxidation of the *E. coli* K12 cells.

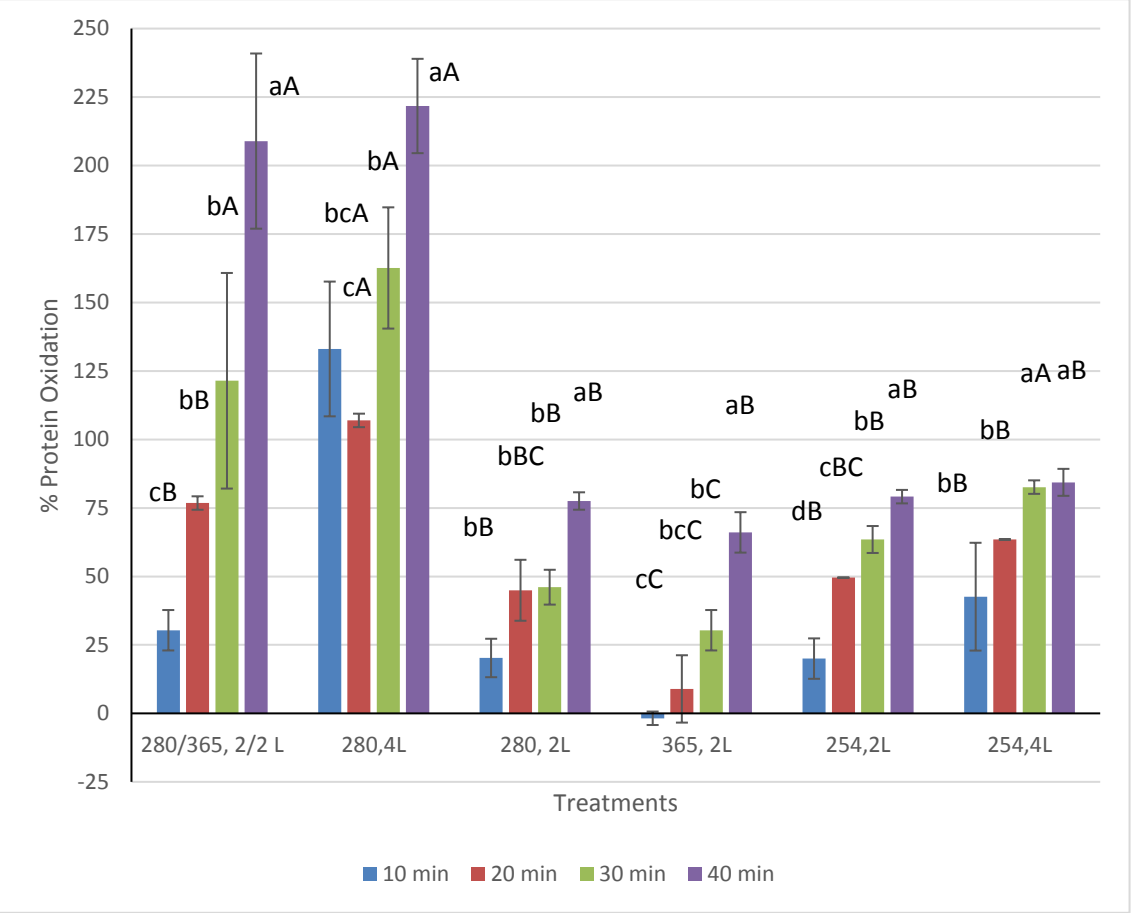


Figure 6.16. Effects of different UV-LED wavelengths on protein oxidation in *E. coli* K12 cells as a function exposure time. Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. A–C:Values within each exposure time at same wavelength or combination followed by the same letter are not significantly different ($P > 0.05$). a–c: Values within each wavelength and their combinations at the same exposure time followed by the same letter are not significantly different ($P > 0.05$).

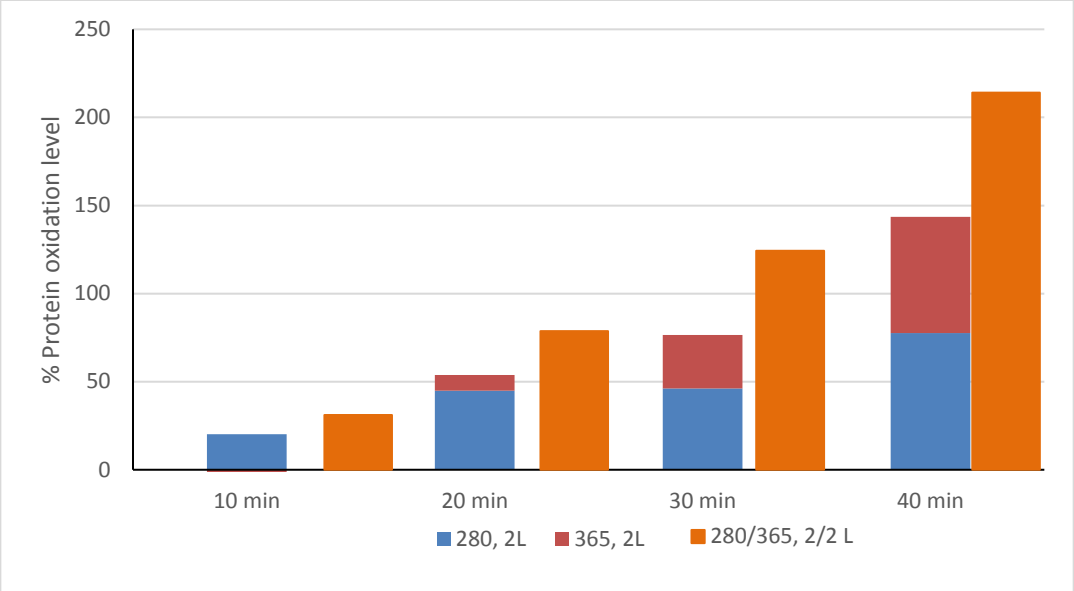


Figure 6.17. The protein oxidation of *E. coli* K12 treated with individually and simultaneously by UV-LED irradiation emitted light at 280 nm and 365 nm.

6.2.4.4. Reactive Oxygen Species (ROS) Generation

The formation of intracellular reactive oxygen species (ROS) in *E. coli* K12 cells subjected to 254 nm (4 lamps), 254 nm (2 lamps), 280 nm (4 lamps), 280 nm (2 lamps), and combining 280 nm and 365 nm wavelengths (2 lamp/2 lamps) were determined by using a fluorescent probe H_2DCFDA (2', 7' dichlorodihydrofluorescein diacetate). The effects of UV-LED irradiation at different wavelength and their combination with different exposure time on the formation of ROS generation in *E. coli* K12 cells depicts in Figure 6.18.

It was clearly observed that the ROS generation in cells was not significantly stimulated following exposure to 2 and 4 lamps emitting light at 254 nm. 4 L or 2 L UV-LED irradiations at 254 nm for 40 min barely enhanced the ROS level to 5%. Similarly, ROS generation after the application of 280 nm UV-LED irradiation for 40 min reached up to 8.96% ($p>0.05$). When the cells subjected to 280 nm with 4 lamps, the ROS generation of the cells stayed at the level of 7% up to 30 min ($p>0.05$), then this percent slightly increased to 13.92% when the exposure time increased to 40 min. Besides, 10 and 20 min 365 nm UV-LED irradiation did not cause any significant enhancement on

ROS generation. But prolongation of the exposure time to 30 and 40 min, the ROS generation level sharply increased and reached to 16.18% and 20.42%, respectively. Santos et al. (2013) examined the individual inactivation mechanisms of low mercury UV lamps emitting light at 254 nm (UV-C), 302 nm (UV-B) and 365 nm (UV-A) wavelengths on 9 different bacterial isolates. The highest reactive oxygen species (ROS) formation was seen in the bacteria exposed to 365 nm wavelength. They reported that intracellular ROS generation which is indicative of indirect UV effects, showed the strongest response to UVA irradiation.

Simultaneous application of 280 nm and 365 nm showed the similar ROS generation trend in the cells when the exposure time increased up to 20 min. The increment similarly reached to 5% within 20 min ($p>0.05$). When the exposure time increased to 30 min, ROS generation suddenly raised to 25.76%. Finally, at the end of 40 min exposure time, ROS generation enhancement reached to the maximum level and recorded as 37.42%.

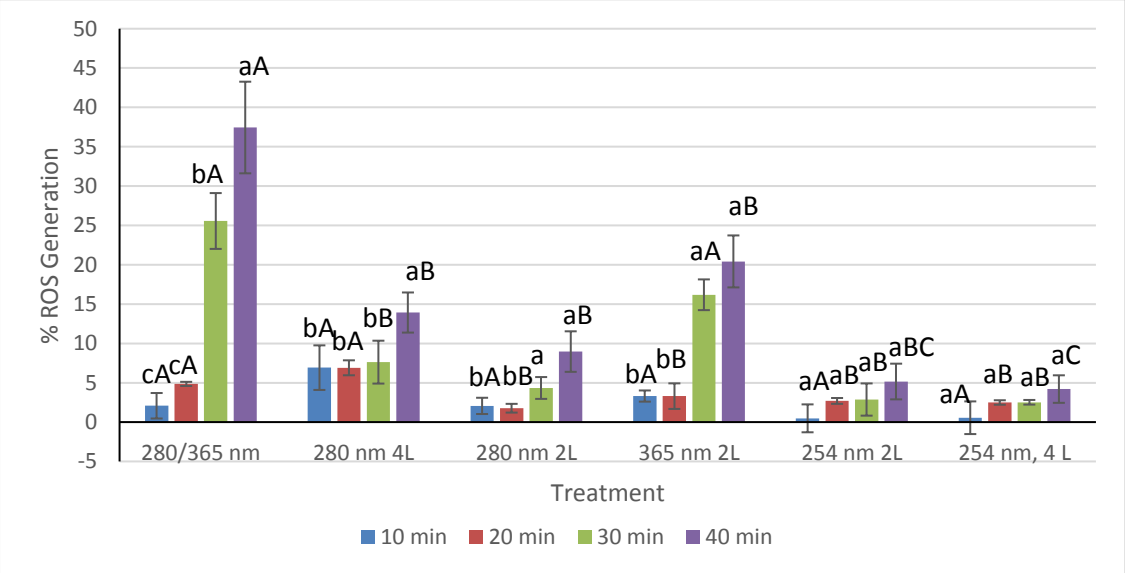


Figure 6.18. Effects of different UV-LED wavelengths on ROS generation in *E. coli* K12 as a function exposure time. Results were presented as "means ± standard error". Least significant difference was determined by Tukey pairwise comparison test. A–C:Values within each exposure time at same wavelength or combination followed by the same letter are not significantly different ($P > 0.05$).

Figure 6.19 demonstrates the % ROS generation in the *E. coli* K12 cells subjected to combination of 280/365 nm (2 lamps/2 lamps). The sum of the % ROS levels of the cells subjected to individual application of 280 nm and 365 nm wavelengths are also

depicted in this figure to determine whether a synergistic effect could be achieved. The percentages of ROS generation obtained by the simultaneous application of 280 and 365 nm were lower than the sum of values obtained by individual application of them for 10 min. It was clearly observed that during the 10 min simultaneous application of the 280/365 nm irradiation, 365 nm did not have any effect on the generation of ROS in *E. coli* K12. For 20 min exposure time, the percentage of ROS generation obtained from simultaneous and the sum of individual applications were approximately at the same level. Therefore, it could be concluded that there is an additive effect on ROS generation when the cells were exposed to simultaneous 280 nm and 365 nm UV-LED irradiation. On the other hand, when the cells were exposed to 280/365 nm simultaneously for 30 and 40 min, the synergistic effect became dominant. The ROS generation levels were 25.76% and 37.44% and these values were higher than the individual sums. Therefore, it could be concluded that a certain exposure time was required for the synergistic effect to take place in ROS generation occurring in *E. coli* K12 cells when the simultaneous 280 nm and 365 nm UV-LED irradiation was applied. UV-A irradiation inactivates the cells indirectly via photooxidation of oxygen causing the reactive intermediates and oxidative damage to DNA and other cellular components (Chatterley and Linden, 2010, Chevremont et al. 2012a, 2012b, Hamamoto et al. 2007, Hwang et al. 2013). ROS are able to oxidize almost every compound in the cell leading to internal damages such as the formation of pyrimidine dimers, the peroxidation of proteins and lipids, losses in membrane permeability or DNA rupture by generating strand breaks. However, this process requires more time than the direct damage produced by UV-C radiation (Chatterley and Linden, 2010). This is because the living organism synthesizes a number of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), which play a role in the defense mechanism that prevents it from entering the autocatalytic phase under oxidative stress (Joshi et al. 2011). During the simultaneous application of 280 nm and 365 nm, 280 nm could stimulate inactivation of these enzymes of defense mechanism by playing an important role in denaturation of the protein type of components (Bowker et al. 2011), whereas, 365 nm cause the increment in ROS levels, mostly •OH, generating oxidative stress. The excess ROS in cells induce oxidative damage to cellular components, such as DNA self-repair enzymes, leading to enzymatic function failure and loss of self-repair ability (Song et al. 2019).

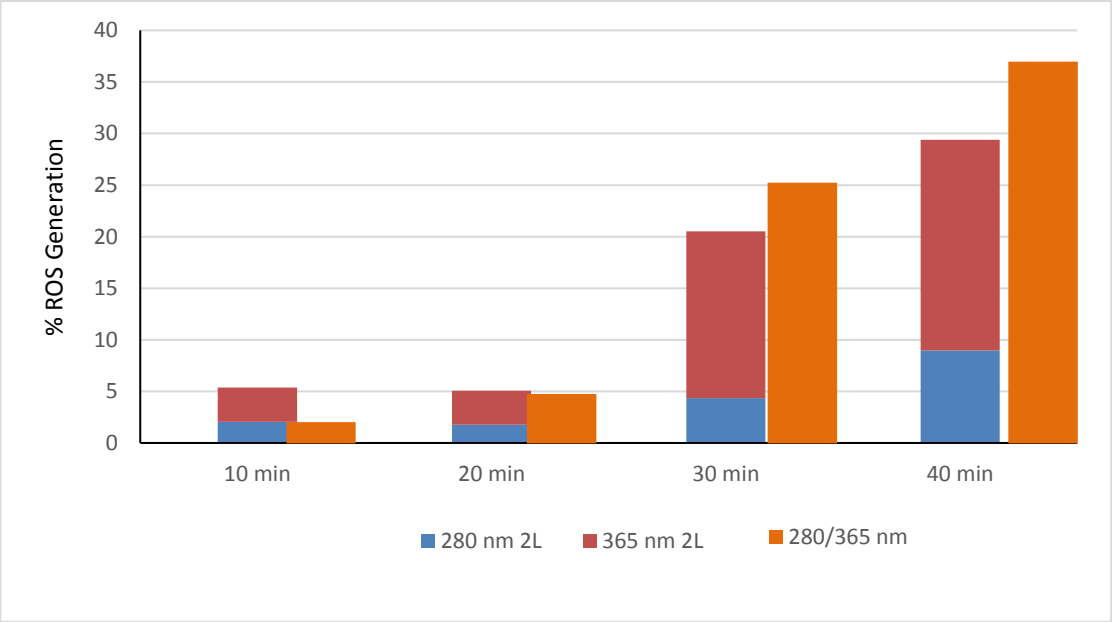


Figure 6.19. The ROS generation of *E. coli* K12 treated with individually and simultaneously by UV-LED irradiation at 280 nm and 365 nm.

6.2.4.5. Scanning Electrone Microscopy

The morphological changes on *E. coli* K12 subjected to 254 nm (4 lamps), 254 nm (2 lamps), 280 nm (4 lamps), 280 nm (2 lamps), 365 nm (2 lamps) and combining 280 nm and 365 nm wavelengths (2 lamp/2 lamps) for 40 min exposure time were determined by using SEM analysis. The images directly illustrate the destructive effects of the UV-LED irradiation on bacteria. Untreated cells (control) were intact and showed a smooth surface (Fig. 6.20), Fig. 6.21, 6.22 and 6.23 depict treated cells having different surface morphologies treated at different wavelengths and number of lamps.

Figure 6.21 (A) shows the morphological changes occurred in *E. coli* K12 cells after exposure to 254 nm (4L) for 40 minutes. It is obvious that cell membrane integrity destroyed in most of the bacteria and the holes was clearly seen in the SEM images. Most of the cells were observed to get clustered and stick to each other. Previously colony formation method was used and 4.23 log CFU / mL reduction for *E. coli* K12 cells in PBS after exposure to 254 nm was reported. These images supporting this result. The cell death may have been the result of the extensive loss of cell integrity and cell contents, the exit of critical molecules and ions, or the initiation of autolytic processes (Denyer, 1990). Figure 6.21 (B) represents damaged *E. coli* K12 cells exposed to 254 nm (2L). Bacteria

maintained their membrane integrity better than the ones treated with 4-lamp at 254 nm. Although, the bacteria cells tended to aggregate, this was not severe as much as 4-lamp application. Small deformities such as punctuated holes and wrinkled cell surface were observed on the membrane of the cells.

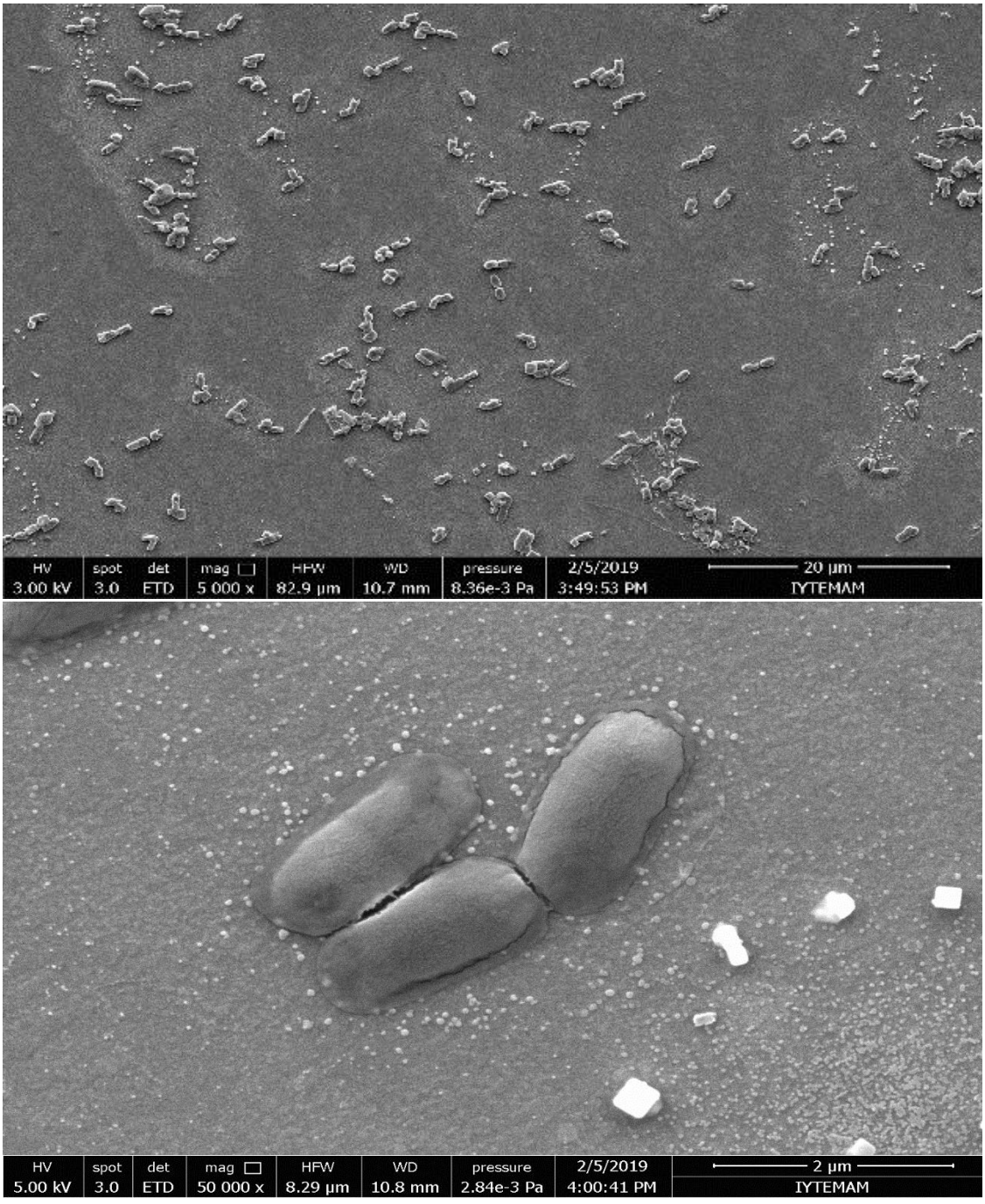
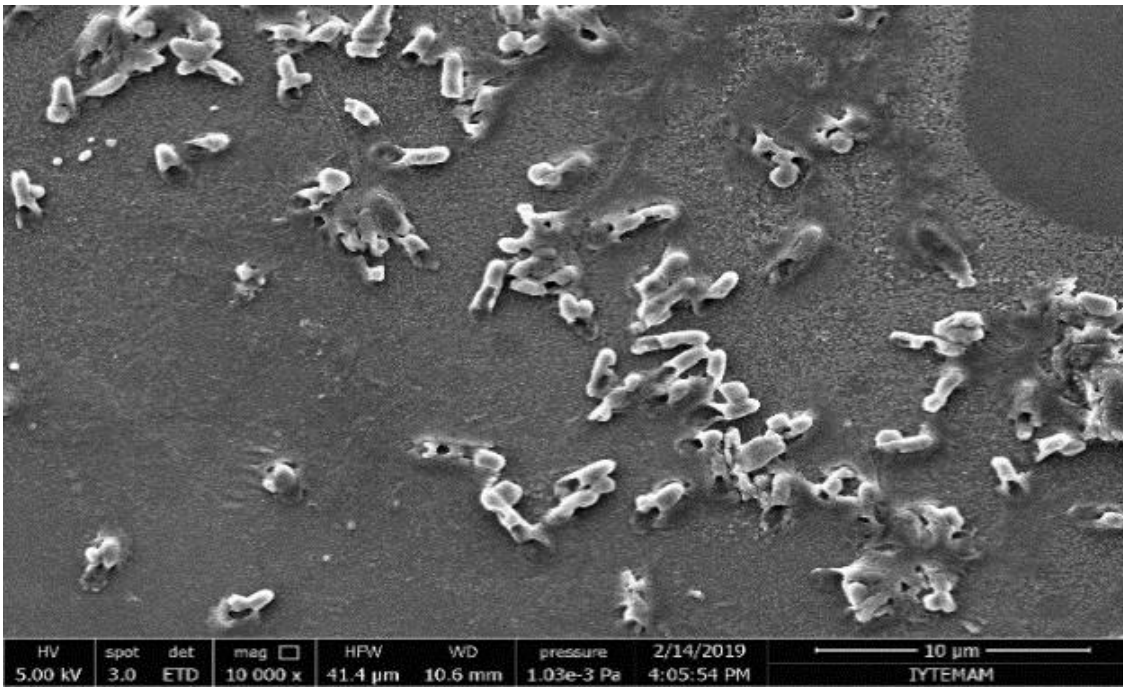


Figure 6.20. SEM images of untreated the *E. coli* K12



A-254 nm-4L

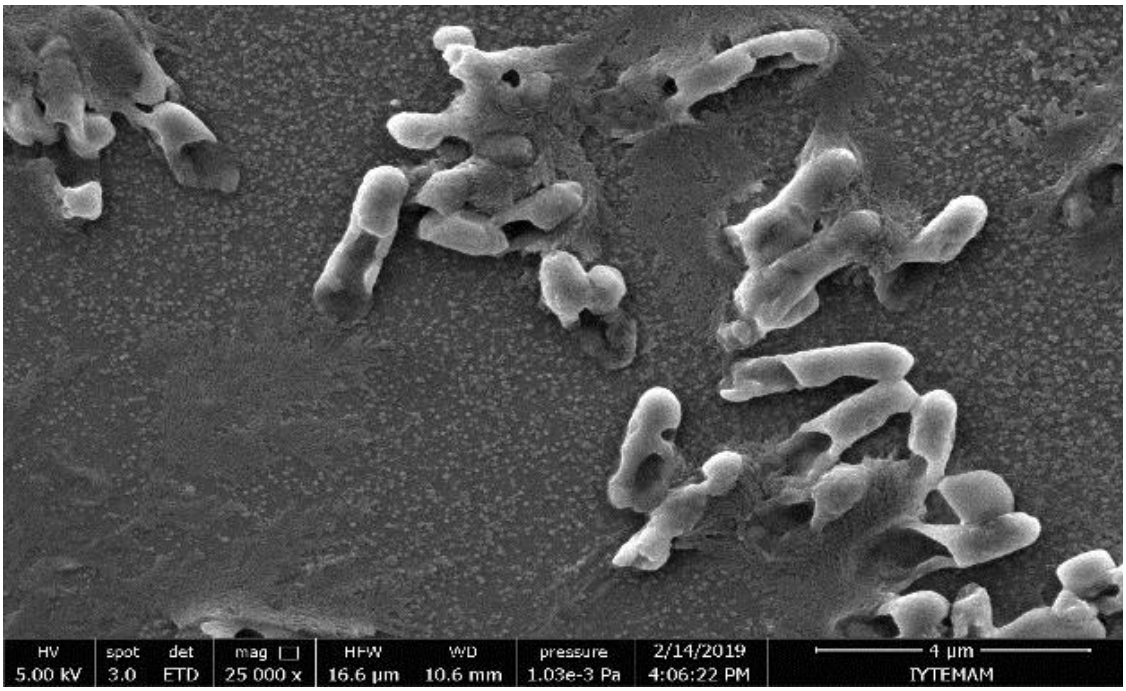
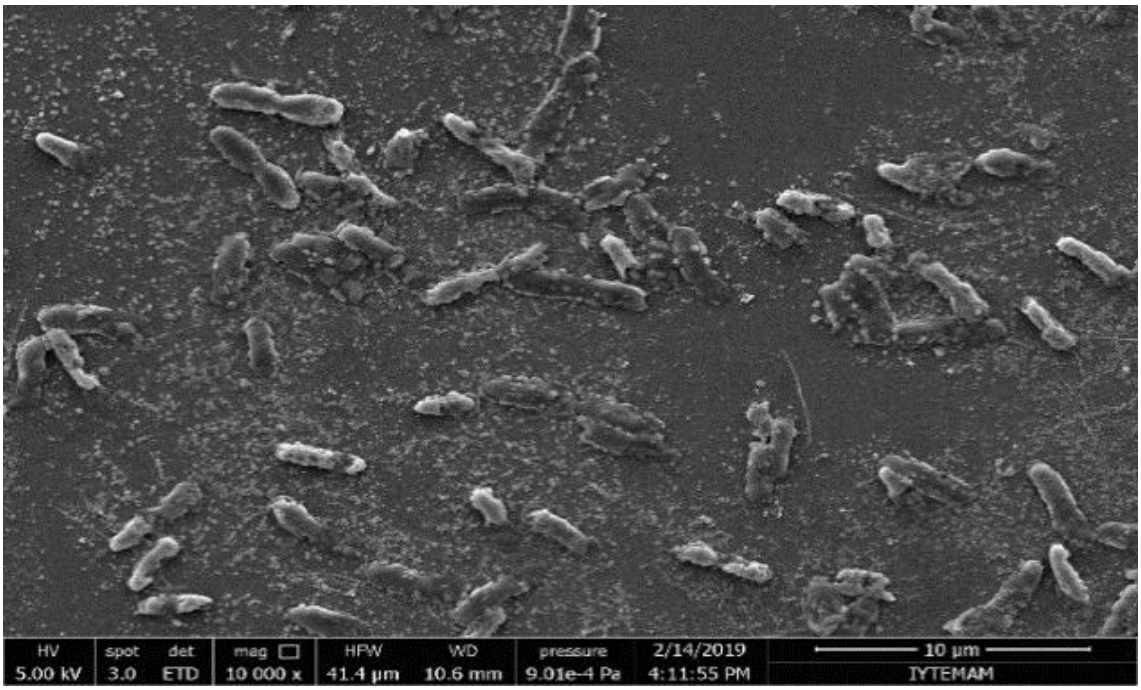


Figure 6.21. SEM images of *E. coli* K12 subjected to 254 nm UV-LED irradiation for 40 min using 4 lamps (A) and 2 lamps (B)

(cont. on the next page)



B-254 nm-2L

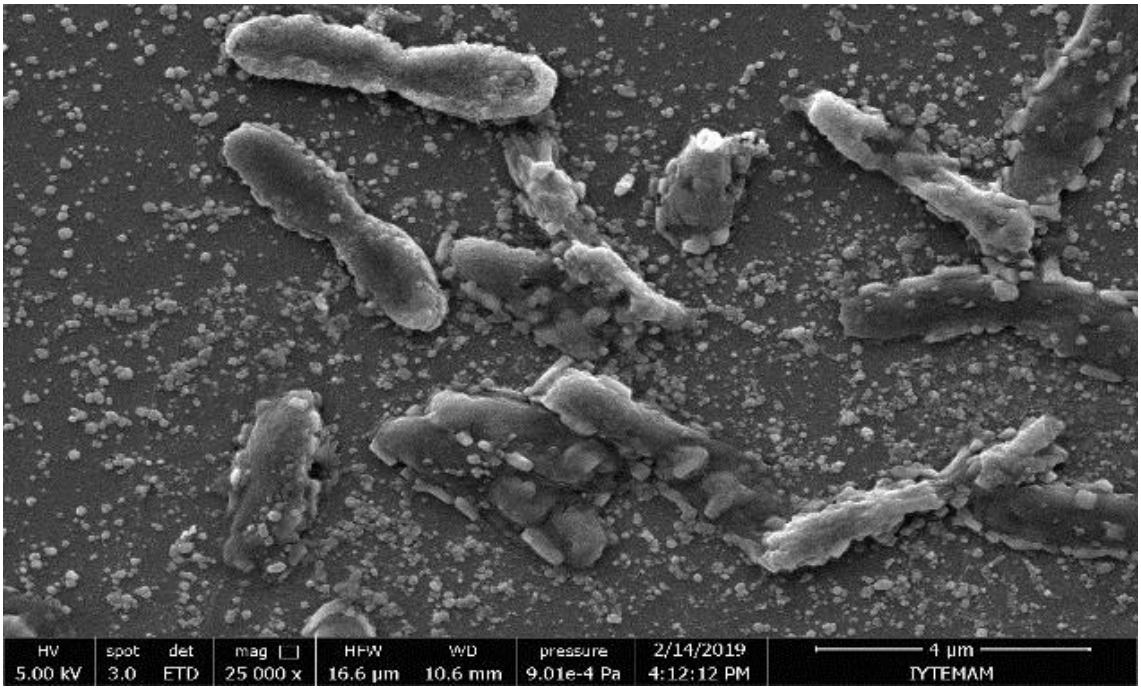


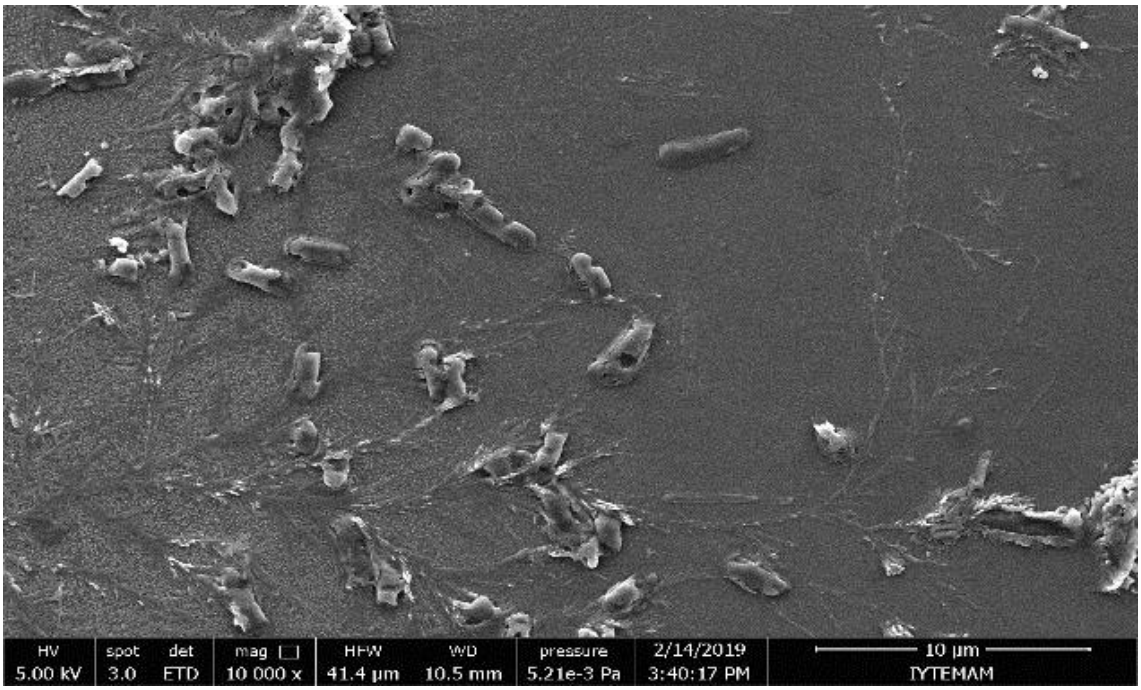
Figure 6.21. (cont.)

Figure 6.22 (A) represents the changes in the surface morphology of *E. coli* K12 cells subjected to 280 nm (4L) for 40 minutes. The image in the figure clearly shows the loss of the membrane integrity. This was found to be more severe compared with the cells treated with 254 nm (4L). Inactivated cells were also aggregated. Additionally, big cavities appeared on their cell membrane. Figure 6.22 (B) shows damaged *E. coli* K12

cells exposed to 280 nm (2L). This treatment did not cause significant damages on cell membrane. The number of lamps, i.e., applied UV intensities, affected the cell deformity especially roughness. Indeed, 4-lamp 280 nm illumination changed the cell membrane structure more severely, causing more leakage of cell components such as proteins, nucleic acids and inorganic ions than 2 lamps. When the results of spread plating method were compared, the illumination PBS containing *E coli* K12 cells at 280 nm using 4-lamps and 2 lamps resulted in 4.92 and 4.21 log CFU/mL reductions, respectively. These data were supported by SEM images.

E. coli K12 severely lost their cell integrity after exposure to 280/365 nm for 40 minutes (Fig. 6.23 (A)). It was resulted in higher numbers of hole formation on cell surfaces. Large surface collapse was observed in the cells and this abnormal cell breakage caused the complete lysis or dead of cell. Previously, spread plating method was used and 280/365 nm application resulted in lower inactivation (4.5 log CFU/mL) compared to reduction achieved at 280 nm (4.92 log CFU/mL) for *E. coli* K12 cells in PBS. These images were supporting and in good agreement with these results. Fig 6.23 (B) shows damaged *E. coli* K12 cells after exposure to 365 nm for 40 min using only 2 lamps. As shown in the figure, although the bacteria have maintained their integrity, the leakage of the cell constituents was clearly observed. This result showed similarity with the results obtained by the spread plating method. According to this method, 0.21 log CFU/mL reduction was achieved. It was concluded that the application of 365 nm with 2 lamps had almost no effect on bacterial inactivation.

It is known that outer membrane of Gram-negative bacteria such as *E. coli* K12 contain a lipopolysaccharide layer. It allows passage of hydrophobic molecules from the external environmental medium. Additionally, the cytoplasmic membrane is responsible for the regulation of osmotic pressure (Kim et al. 2016). The noticeable damages were detected in *E. coli* K12 cells irradiated with 254 (4 L), 280 (4 L) and 280/365 nm (2/2 L) LEDs', although the degree of damages varied with the types of UV-LED and number of the lamps.



A-280 nm- 4 L

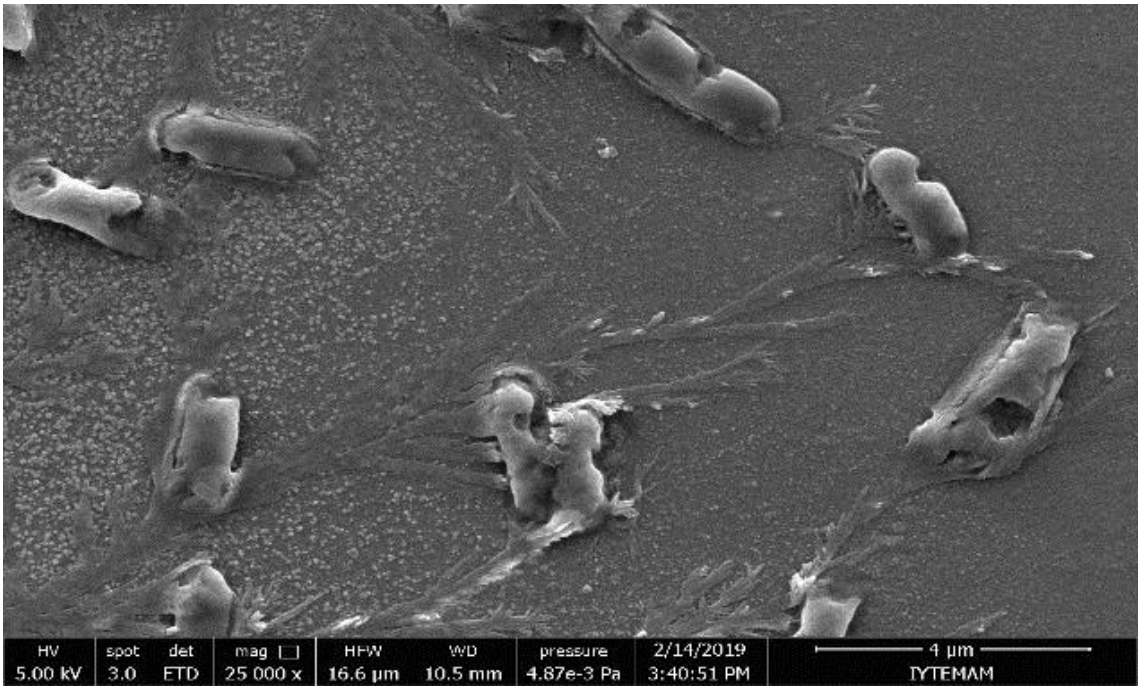
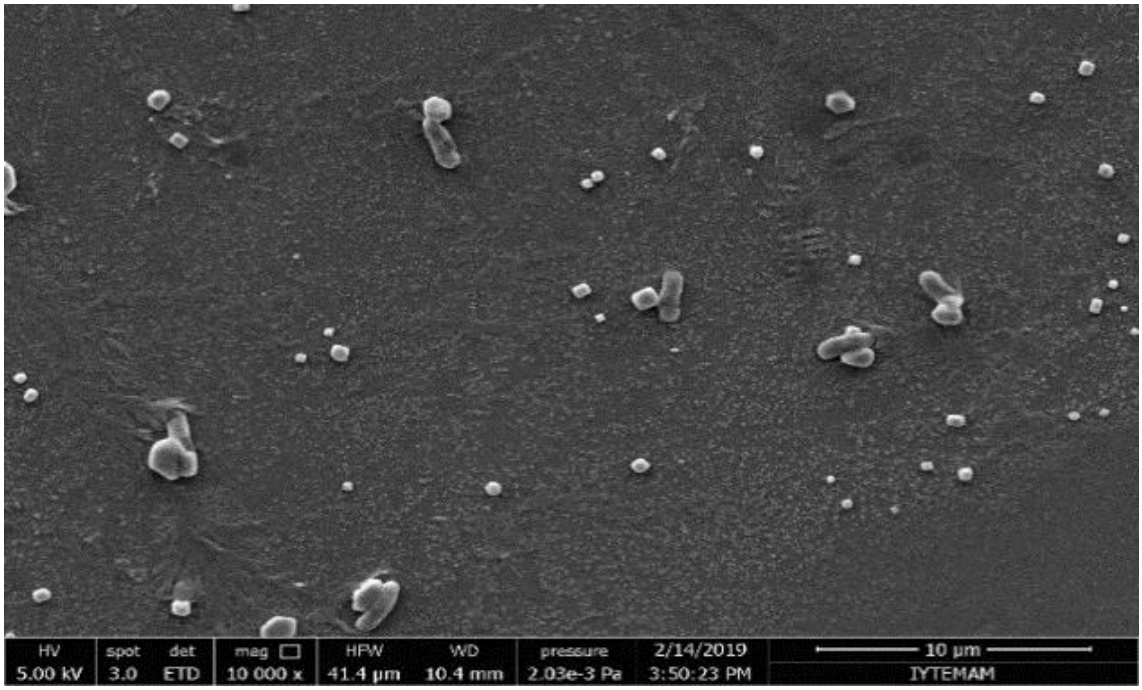


Figure 6.22. SEM images of *E. coli* K12 subjected to 280 nm UV-LED irradiation for 40 min using 4 lamps (A) and 2 lamps (B)

(cont. on the next page)



B-280 nm- 2 L

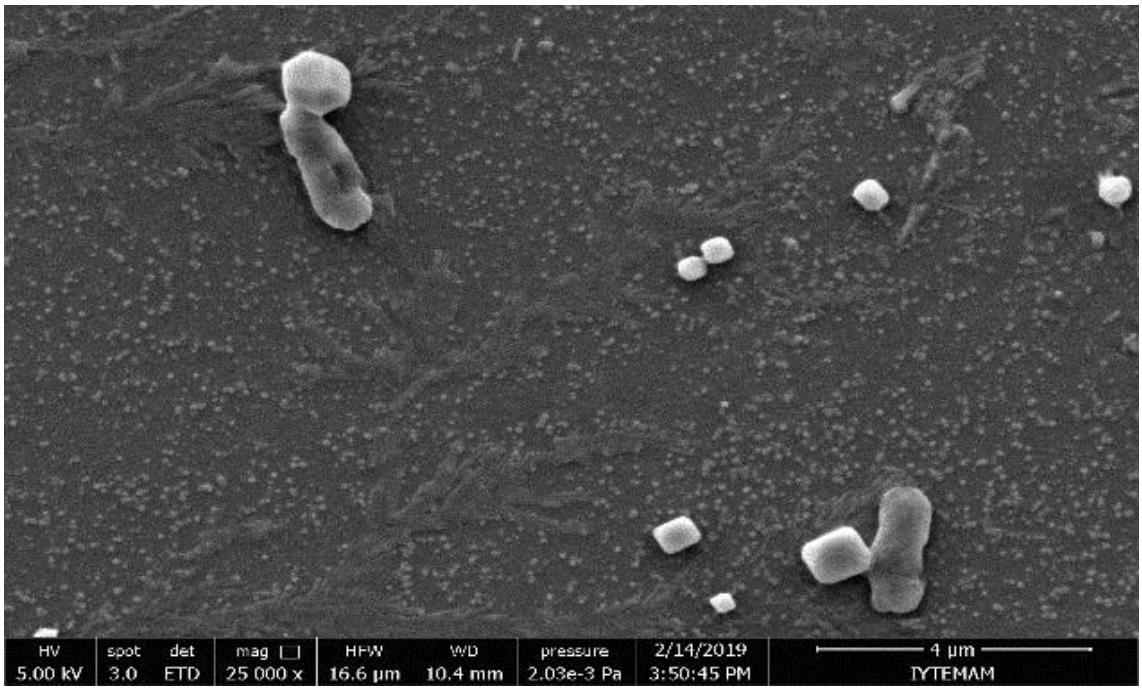
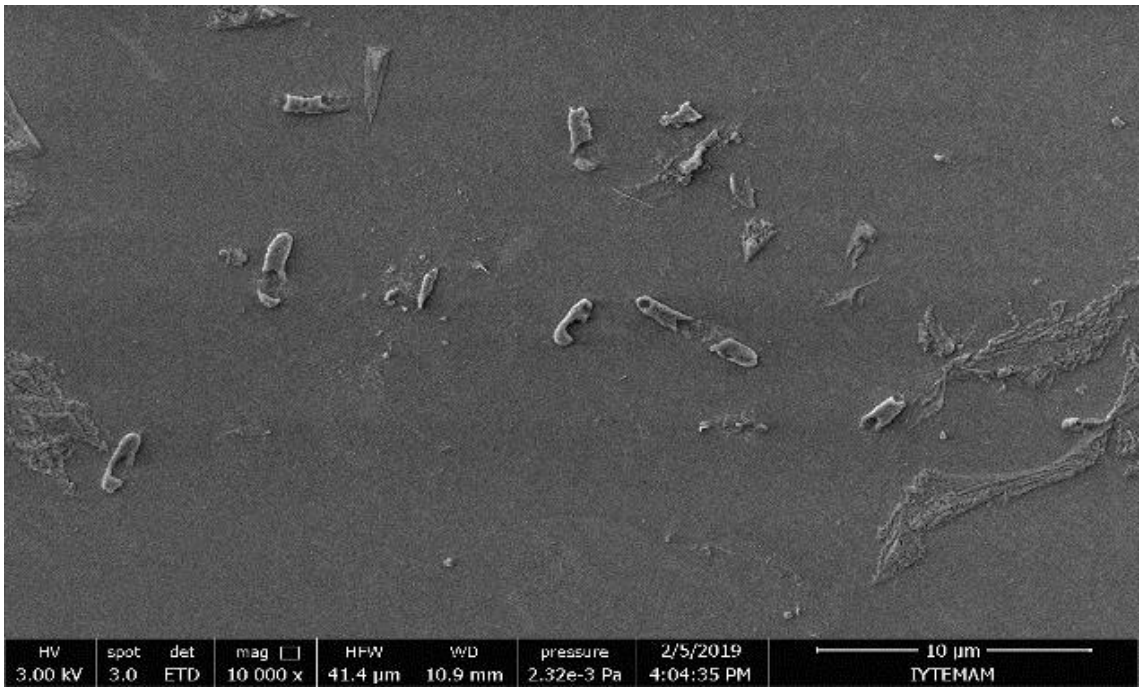


Figure 6.22. (cont.)



A-280/365 nm- 2/2 L

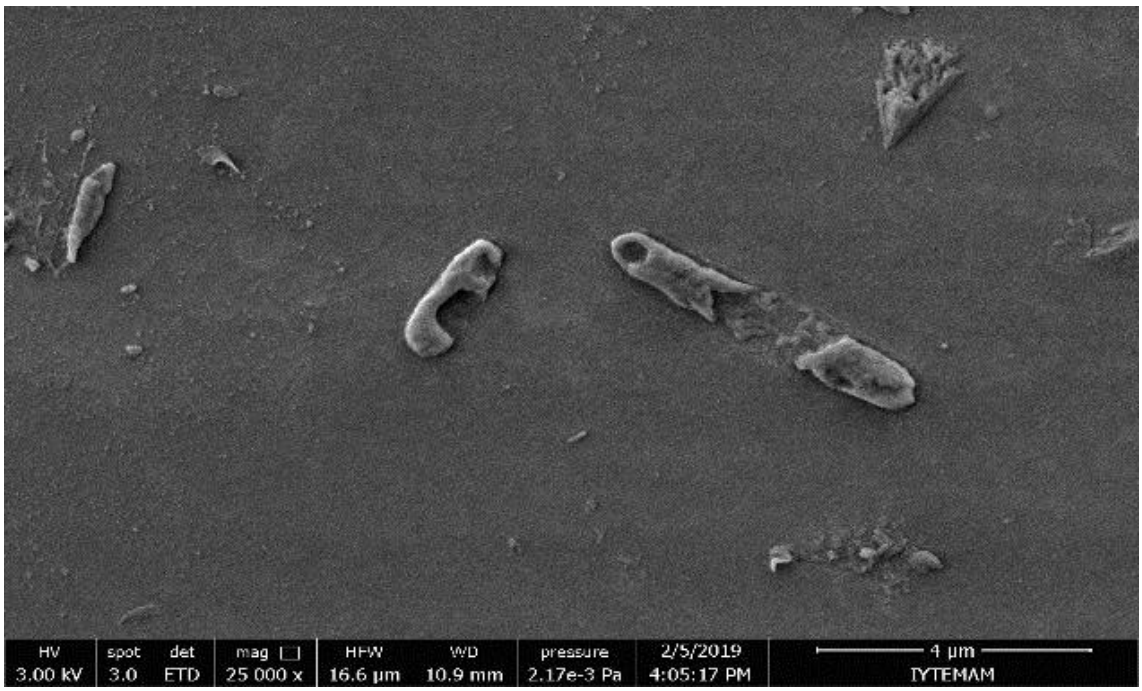


Figure 6.23. SEM images of *E. coli* K12 subjected to combination of 280/365 nm (A) and 2 lamp 365 nm (B) UV-LED irradiation

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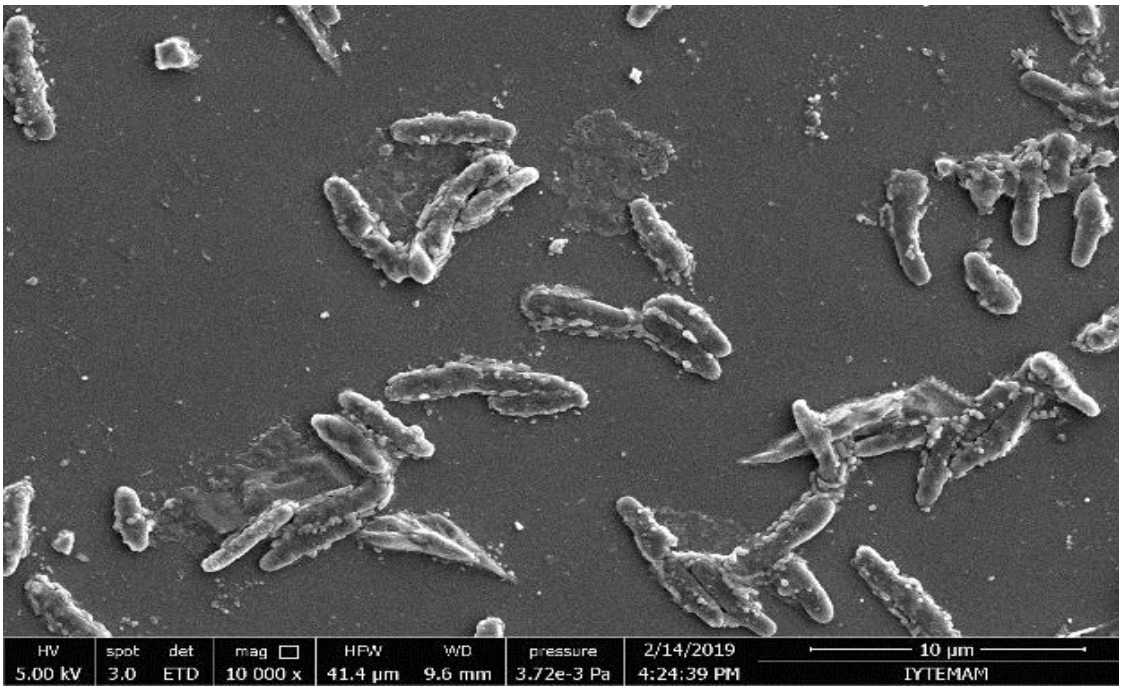

B-365 nm- 2 L

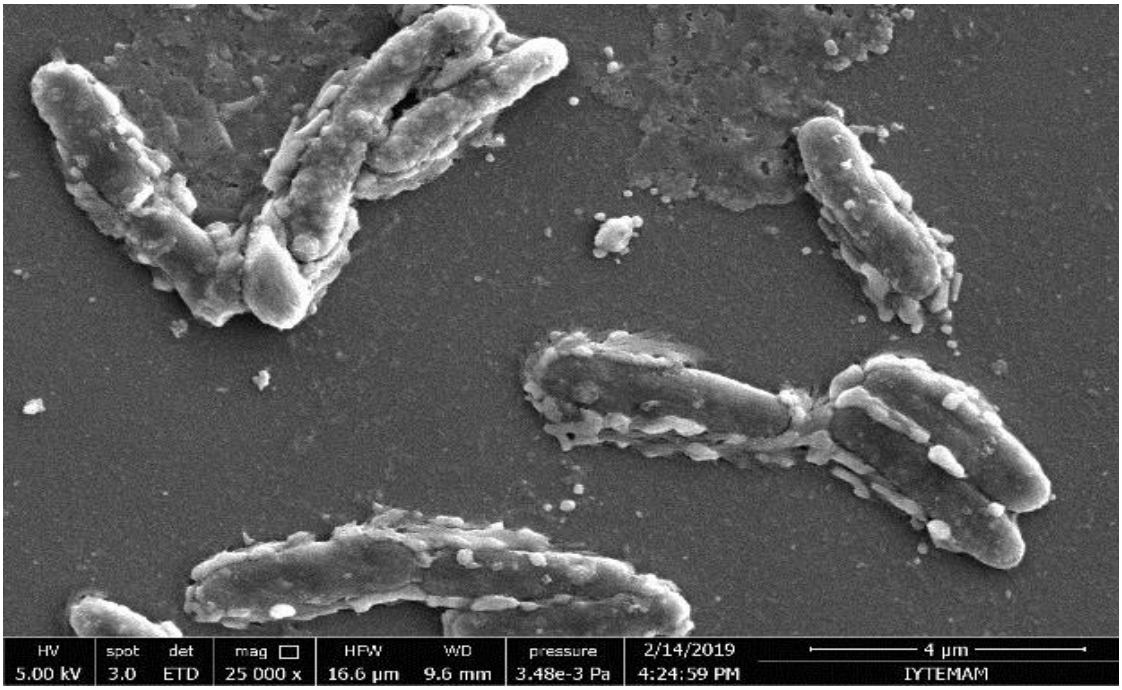

Figure 6.23. (cont.)

There are many possible explanations for these observations. Some authors have indicated that the damage to the cell wall and cytoplasmic membrane was due to the loss of structural integrity (de Billerbeck et al. 2001, Filipowicz et al. 2003, Packiyasothy et al. 2002). Others attributed these cell damages to the effect of UV light on inhibiting proton motivation, respiration chain, electron transfer and substrate oxidation.

Uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides might follow these effects (Farag et al. 1989, Kim et al. 1995).

These images confirm the loss of shape and integrity of cells. Cell death may have been the result of the extensive loss of cell contents, the exit of critical molecules and ions, or the initiation of autolytic processes (Denyer, 1990).

6.2.4.6. Overall Evaluation of Inactivation Mechanisms

In this part of the thesis, the mechanism of UV-LED irradiation at different wavelengths on inactivation *E. coli* K12 cell was reveal by examining the double strand breakage in the cell DNA, the oxidation of lipid and protein, and the formation of reactive oxygen species formed in the intracellular structure. In view of the above results, Table 6.2 was created for better understanding of the mechanism of 280/365 nm (2-2 L), 254 nm (2L), 280 nm (2L) and 365 nm (2L) UV-LED irradiation on *E. coli* K12 inactivation.

Table 6.2. Major inactivation mechanism of UV-LEDs' at different wavelengths

Treatments (40 min exposure time)	**Inactivation Mechanism**	**Inactivation (Log CFU/mL)**
254 nm (2L)	DSB, Protein oxidation	4.13
280 nm (2L)	Protein oxidation	4.21
365 nm (2L)	ROS generation, Lipid oxidation	0.21
280/365 nm	DSB, Protein oxidation, ROS generation	4.36

The effective mechanisms that play important role in the inactivation of *E. coli* K12 are summarized in Table 6.2 by considering the levels of DNA Strand Breakages, lipid and protein oxidation, ROS generation occurred in each UV-LED application. For example, when two-lamp applications are considered, the percentage of formation of double strand breakages on DNA was greater at 254 nm (2 L) than other wavelengths. This means that the formation of DSB played a major role in 2 L-254 nm UV-LED irradiation. Besides, the application of 254 nm (2L) wavelength caused to induction of protein oxidation. The data also showed that formation ROS generation and lipid oxidation did not have a significant lethal effect on inactivation of *E. coli* K12 subjected to UV illumination at 254 nm (2L). On the other hand, the formation of ROS and lipid

oxidation in 365 nm (2L) irradiation was important and resulted in 0.21 log CFU/mL reduction of *E. coli* K12 cells in PBS.

It was found that the 4.21 log CFU/mL of inactivation was mainly as a result of protein oxidation of the cells following the application of 280 nm (2L). In the light of the microbial results, the combination of 280/365 nm irradiation had an additive lethal effect on the inactivation of *E. coli* K12. 4.36 log CFU/mL reduction was directly obtained by means of formation of DSB, protein oxidation, ROS generation. Additionally, the lipid oxidation took part in inactivation of the cells indirectly by stimulating the protein oxidation.

The highest damage in the DNA strand of *E. coli* K12 cells obtained when the cells were treated at 254 nm. Therefore, the wavelength of 254 nm (UV-C) is directly absorbed by DNA, which causes damage by altering the nucleotide base, thereby forming cyclobutane-pyrimidine dimers (CPD) between adjacent nucleotides on the same DNA strand (Abdallah et al. 2012). The formation of CPDs prevent the proper replication of DNA during reproduction and ultimately lead to the death of the microorganisms (Quek and Hu, 2008). In addition, UV-B and UV-C wavelengths have been reported to be absorbed by proteins (Zimmer and Slawson, 2002). Therefore, protein oxidation is an effective inactivation mechanism of UV-C wavelength. The wavelengths in the UV-A range affect the cell membranes and membrane functions. UV-A wavelengths lead to the formation of single oxygen or free radicals known to destroy high energy short wavelength photons absorbed by chromophoric molecules, membranes and other cellular components. UV-A light damages the cell repair mechanism, induce irreversible physiological changes in the cells including metabolic inhibition (Zimmer and Slawson, 2002). To our knowledge, the inactivation mechanism of LEDs emitting at UV-A wavelength. has not been determined so far. However, there are some studies on commonly used mercury lamps and the results are consistent with our findings. For example, Santos et al. (2013) examined the individual inactivation mechanisms of mercury UV lamps emitting light at 254 nm (UV-C), 302 nm (UV-B) and 365 nm (UV-A) wavelengths on 9 different bacterial isolates. They reported the highest inactivation at 254 nm, where DNA damage was the responsible mechanism of cell death. The highest protein oxidation was observed at wavelength of 302 nm (UV-B). On the other hand, the highest lipid oxidation and reactive oxygen species (ROS) formation was seen in the bacteria exposed to 365 nm wavelength. In addition, it was found that the application of

365 nm wavelength did not have any lethal effect on the inactivation of bacteria as other wavelengths. All these results were in good agreement with our findings.

The results of this study showed that combination of 280/365 nm caused damage on DNA and played a major role in inactivation of *E. coli* K12. This mechanism of action is attributed with the formation of oxidative products (ROS) as a result of protein and lipid oxidation triggered at 280 nm and 365 nm wavelengths. It was speculated that the resulting products may have influenced cell DNA. Because the wavelengths of 280 nm and 365 nm increase the production of reactive oxygen species (ROS) that cause oxidative damage in lipids, proteins and DNA. These reactive metabolites can react with sugars, purines and pyrimidines and indirectly damage to DNA. He and Häder, (2002) examined the inactivation mechanism of the *Anabaena* subspecies of UV lamps that emit light in UV-B and UV-A wavelengths. They found that UV-A wavelength stimulated ROS formation. The metabolites formed after ROS generation further increased the cell DNA damage.

6.3. Conclusion

The aim this chapter of the thesis is to elucidate the inactivation mechanism of UV-LED irradiation at different wavelength and their combination on the inactivation of *E. coli* K12. For this purpose, the inactivation efficiency of UV-LEDs' emitting light at 254 nm (4 lamps), 280 nm (4 lamps), 254 nm (2 lamps), 280 nm (2 lamps) and 365 nm (2 lamps) and the combination of 280 nm and 365 nm on *E. coli* K12 in PBS were initially determined. It was revealed that 280 nm (4 L) LEDs for 40 min exposure time was more effective than UV-LED illumination combined at 280 and 365 nm and 254 nm (4 L) wavelengths for inactivation of *E. coli* K12 cells in PBS. Moreover, UV-LED irradiation at 365 nm could not provide any lethal effect on *E. coli* K12. Also, it was observed that 365 nm illumination for up to 20 min had helped to inactivate microorganisms and showed synergistic effect when used together with 280 nm wavelength. But over the 20 min exposure time, it was speculated that 365 nm wavelength play a major role in the repair mechanism of the cell and helped the cells to repair themselves. The sublethal injury and dark and photoreactivation behavior of *E. coli* K12 were also determined following the UV-LED irradiations to support this finding. Although 280 nm UV-LED irradiation with 4 L caused higher sublethal injury on cells, the cells could not repair

themselves under light and dark condition compared to 254 nm and 280/365 nm. A moderate repair under light and dark condition was observed in cells irradiated with combination of 280/365 nm.

In order to get a better understanding of inactivation mechanisms, the results were supported by the investigation of the formation of double strand breakages on DNA, protein and lipid oxidation, ROS generation in cell. The formation of double strand breakages is widely known as the important inactivation mechanism of 254 nm irradiation. It was revealed that the combination of 280/365 nm also resulted in DNA damages in the cells as in the case of 254 nm illumination. Also, protein oxidation and formation of the ROS generation played roles in inactivation of *E. coli* K12 subjected to 280/365 nm irradiation. Formation of the lipid oxidation got indirectly involved in the inactivation of the cells by taking a part in the stimulation of the protein oxidation and ROS generation. The structural membrane damages of the cells were also monitored by Scanning Electron Microscopy technique, especially the cells subjected to 280/365 nm severely lost their cell integrity; a number of holes had formed on their surface, large surface collapse and abnormal cell breaking caused the complete lysis or death of cells.

CHAPTER 7

CONCLUSION

In this Ph. D. thesis, the assessment of applicability of UV-LED irradiation with using different wavelength and combination for pasteurization of fresh fruit juices was aimed in respect to microbial safety, enzymatic activity and product quality. Second aim of the study was to investigate the effect of different wavelengths and their combinations (UV-A and UV-C) on the mechanism of microbial inactivation by elucidation of the formation of DNA breakages, protein and lipid oxidation, ROS generation in cell.

Firstly, the antimicrobial efficacy of UV-LED irradiation was investigated on *E. coli* K12 in cloudy and clear apple juices. For this purpose, the fresh apple juice extracted from Starking Delicious variety of apples, firstly pasteurized at 70°C for 120 s and inoculated with *E. coli* K12 at a level of 10^7 CFU/mL was subjected to different UV-LED treatments. In the first treatment, the apple juice was exposed to UV-LED emitting light at 254 nm wavelength for different time periods (5 to 50 min) to optimize the exposure time of treatments. The exposure time that was applied longer than 40 minutes did not change the inactivation efficiency of the lamps significantly. Thus, the maximum exposure time for UV-LED treatments was determined as 40 min. In the other treatments, the microbial efficiency of different wavelength combinations such as 254 nm, 280 nm, 280/365 nm, 280/405 nm, 254/365 nm, 254/405 nm, 254/280/365/405 nm were investigated under the same process conditions, i.e. 40 min exposure time. The results showed that using the 4 UV-LEDs were not enough to provide a 5-log reduction of *E. coli* K12 in CAJ and AJ. The highest inactivation on *E. coli* K12 in both juice samples was obtained at 280 nm wavelength using 4 lamps for 40 min exposure time. However, coupled wavelength 280/365 nm was more influential on inactivation of *E. coli* K12 than that of 254 nm UV- LEDs. Also, inactivation efficacy of UV-LEDs' on PPO in freshly squeezed CAJ was investigated. The maximum PPO inactivation was achieved when the juice samples were subjected to combination of UV-LEDs emitted light at 280 and 365 nm with 40 min exposure time. It was clearly observed that UV-LED irradiation by coupling UV-A and UV-C rays showed a better inactivation efficiency on PPO enzyme than the one used only UV-C rays. Whereas, the heat treatment on the inactivation of PPO was more effective than UV-LEDs irradiation. The potential of photoreactivation and

dark repair ability of *E. coli* K12 in cloudy (CAJ) and clear apple juice (AJ) were also revealed by the way of incubated at ambient (22ºC) and refrigerated (4ºC) temperatures under the visible light or in the dark. The photoreactivation ability of *E. coli* K12 was only observed in both CAJ and AJ following exposure to 280/365 nm UV-LEDs irradiation after 6 h incubation at 22ºC. Contrarily, dark repair was repressed at studied incubation temperatures, furthermore, a decrease in the survival ratio was recorded in both mediums.

Secondly, it was aimed to increase the microbial efficiency of UV-LEDs irradiation at 280/365 nm by the way of a combination with mild heat. Individual UV-LED irradiation at 280/365 nm and mild heat treatment at different temperatures (40ºC, 50ºC, and 55ºC) were insufficient for pasteurization of CAJ. Simultaneous application of UV-LED irradiation and mild heat treatment significantly increased the inactivation of *E. coli* K12 in CAJ. Application of UV-LED+MH at different temperatures for 5 min met the pasteurization requirement recommended by the FDA (5 log reduction). Moreover, the efficacy of simultaneous application of UV-LED and mild heat treatment on the inactivation of natural microflora of freshly squeezed CAJ was also investigated. The fresh juice was subjected to UV-LED irradiation, mild heating at 50 ºC and 55 ºC and a combined UV-LED+MH treatment for 5, 10 and 15 min. UV-LED (280/365 nm) irradiation, and mild heating at 50ºC did not have a pronounced lethal effect on total mesophilic aerobic bacteria of freshly squeezed CAJ. Moreover, a combination of UV-LED irradiation with mild heat at 50ºC for 15 min was inadequate to inactivate the natural microflora in CAJ. Whereas, when the mild heat temperature was increased to 55ºC and combined with the UV-LED irradiation, the maximum reductions of TAC and YMC in freshly squeezed CAJ were achieved. Thus, 280/365 nm UV-LED irradiation assisted by mild heat at 55ºC for 15 min was selected as the best conditions for inactivation of natural microflora in CAJ. Under this condition, the residual activity of PPO was also reduced to 24.57%.

Thirdly, the effect of 280/365 nm UV-LED irradiation assisted by mild heat at 55ºC on the microbiological, some physicochemical, optical and phytochemical properties such as pH, color, ascorbic acid content, polyphenol oxidase activity and total phenolic content of CAJ were monitored during 40 days under refrigerated storage conditions. Thermally pasteurized (70ºC, 120 sec.) and untreated juice samples were used as the positive and negative controls. UV-LED+MH and thermal pasteurization processes were able to inactivate the natural microbial of CAJ prior to storage. While,

untreated sample was spoiled completely within the 3 days, no significant growth (under the detection limits <30 CFU/mL) of TA and YM was detected in either UV-LED+MH or thermal pasteurized juice samples up to 30 days of refrigeration storage. Although the pH value of UV-LED+MH treated CAJ samples did not change markedly during storage period, the optical properties were slightly affected. Ascorbic acid content of the samples was fully degraded within the first days of the storage. The residual activity of PPO in CAJ treated with UV-LED+MH process remained constant around the 21-22% during 40 days of storage. Thermal pasteurization and UV-LED+MH treatments increased the total phenolic content of CAJ samples. TPC values of treated samples were still higher than that of untreated ones during the storage period. The simultaneous application of UV-LED and MH extended the shelf life of CAJ from 3 to 30 days by maintaining the quality losses to a minimum level. Additionally, polyphenol oxidase enzyme activity in CAJ was substantially degraded by the applied process. Thus, simultaneous application of UV-LED and mild heat can be an effective method for fruit juice pasteurization, if an appropriate equipment can be designed by considering optimal process parameters and a suitable juice type.

Finally, within the scope of investigation of inactivation mechanism, firstly the inactivation efficiency of UV-LEDs' emitted light at 254 nm (4 lamps), 280 nm (4 lamps), 254 nm (2 lamps), 280 nm (2 lamps) and 365 nm (2 lamps) and the combination of 280 nm and 365 nm on *E. coli* K12 in PBS were determined. It was revealed that 280 nm (4 L) LEDs for 40 min exposure time was more effective than UV-LED illumination combined at 280 and 365 nm and 254 nm (4 L) wavelengths for inactivation of *E. coli* K12 cells in PBS. Moreover, UV-LED irradiation at 365 nm could not provide any lethal effect on *E. coli* K12. Also, it was observed that 365 nm illumination for up to 20 min had helped to inactivate microorganisms and showed synergistic effect when used together with 280 nm wavelength. But over the 20 min exposure time, it was speculated that 365 nm wavelength play a major role in the repair mechanism of the cell and helped the cells to repair themselves. The sublethal injury and dark and photoreactivation behavior of *E. coli* K12 were also determined following the UV-LED irradiations. Although 280 nm UV-LED irradiation with 4 L caused higher sublethal injury compared to 254 nm and 280/365 nm, the levels of photoreactivation and dark repair after 280 nm LEDs irradiation were lower than the other wavelengths. For a better understanding of inactivation mechanism of UV-LED irradiation emitted light at different wavelengths and their combination, the formation of DNA breakages, protein and lipid oxidation, ROS

generation in cell was also investigated. The formation of DSB played a major role on the inactivation of cells subjected to 254 nm UV-LED irradiation, whereas the stimulation of the protein oxidation in the cells exposed 280 nm was the main mechanism on the inactivation of *E. coli* K12. Besides, the formation of ROS and lipid oxidation in cells following 365 nm (2L) irradiation did not have a significant lethal effect on inactivation of *E. coli* K12. In the 280/365 nm combined irradiation, the inactivation occurred by means of formation of DSB, protein oxidation, ROS generation. Additionally, the lipid oxidation took part in inactivation of the cells indirectly by stimulating the protein oxidation. The structural membrane damages of the cells were also monitored by Scanning Electron Microscopy technique. The images directly illustrated the destructive effects of UV-LED irradiation on *E. coli* K12. Untreated cells (control) were intact and showed a smooth surface while the cells exposed to UV-LED irradiation underwent severe deformities. The holes and cavities had formed on their surface, large surface collapse and abnormal cell breaking caused the complete lysis or death of cells.

In conclusion, this thesis is the first study investigating the applicability of different wavelengths of UV-LEDs for pasteurization of fresh fruit juices in terms of microbial safety, enzymatic activity and product quality. When the microbial and enzyme inactivation efficacy and reactivation potential were considered, it was concluded that the applications of UV-LEDs emitting light at 280 nm and 280/365 nm combined wavelengths are promising methods for fruit juice processing. The results of this study enlighten some engineering aspects of UV-LED treatment for juice pasteurization. It is suggested that future UV-LED systems should take into account the uniform light distribution and the inactivation mechanism at different wavelengths. The inactivation efficacy of these systems should be tested by considering different type of microorganisms inoculated into liquids with different optical properties. Also, the toxic effect of these wavelengths is required to be investigated.

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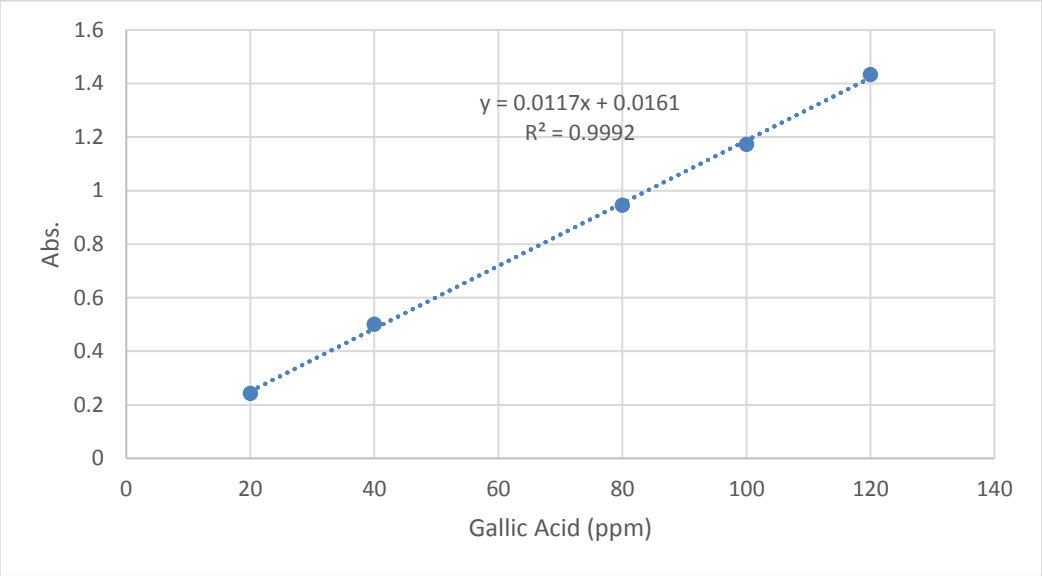
APPENDIX A

CALIBRATION CURVE FOR GALLIC ACID



Appendix A. Calibration curve for gallic acid.

VITA

Merve AKGÜN was born on October 6, 1986 in İstanbul, Turkey. She graduated with a bachelor's degree in the Department of Food Engineering in Celal Bayar University, Manisa in 2008. She started the Master programme of the Department of Food Engineering in İzmir Institute of Technology in September of 2009 and received the Master of Science degree as a food engineer in February of 2012. She studied the new technique for the antibiotic residue analysis defined as biocrystallization in her Master of Science period. The Food Engineering Department in İzmir Institute of Technology accepted her to study the Philosophy of Doctorate programme in 2012. Currently, she has been working on the area of UV-LED irradiation method on fruit juice pasteurization on inactivation mechanisms of them. During her Ph.D. period, she participated a lot of international congress, conferences, workshops and training, as well as presented many oral presentations and posters about her work area. Besides, she has one published SCI articles about the nonthermal UV-LED irradiation.