ABSTRACT

Title of Document: **CHARACTERIZATION OF LUMINOUS BACTERIA AS A BIOSENSING ELEMENT FOR DETECTION OF ACRYLAMIDE IN FOOD**

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World Health Organization (WHO) has called for further research into acrylamide, a known carcinogen and neurotoxin in animals, following emergency consultations to review data from studies that revealed the presence of acrylamide in starch-based foods fried and baked at high temperatures. The presence of acrylamide in food is recognized as a major concern in humans based on the ability to induce cancer and heritable mutations in laboratory animals. The objective of this study was to characterize the cellular-level damage of acrylamide by bioluminescence stress fingerprinting. Five genetically engineered strains containing selected stress-responsive *E. coli* promoters fused to the *luxCDABE* reporter were employed. One of the strains containing DNA damage responsive promoter, DPD2222, was also employed in conjunction with alkaline and neutral comet assay to assess respective single- and double-stranded DNA damages. Results showed that Luminous *E. coli* DPD2222 containing DNA damage responsive promoter, recA, yielded the highest

response followed by luminous *E. coli* DPD2234 which contained protein damage responsive promoter, grpE. Moreover, acrylamide stress response of the cells up to 14 days old was the same as that of the overnight culture. Furthermore, it was revealed that the *E. coli* DPD2222 and DPD2234 were capable of detecting acrylamide between 1 and 10,000 μg/L upon contact, with the response signals proportional to acrylamide concentration. The most severe cellular damage of these two strains was achieved after 100 min of contact, as indicated by the highest signals. In addition, temperature-wise, 37 °C resulted in the most significant light emission of *E. coli* DPD2222. No single strand break was observed at acrylamide concentrations <1000 μg/L by using alkaline comet assay. However, neutral comet assay performed at acrylamide concentrations >10,000 μg/L (ambient temperature) showed a more severe DNA double strand breaks in the cells, so did 1000 μg/L acrylamide at 37 °C. Therefore, it is evident that exposure of bioluminescence sensing cells to acrylamide causes severe DNA damage at either high concentration at room temperature or reduced concentration at body temperature. Quantitative and fingerprint assessment of acrylamide damage could be achieved by optimizing bioluminescence cells constructed with different stress-responsive reporter plasmids.

CHARACTERIZATION OF LUMINOUS BACTERIA AS A BIOSENSING ELEMENT FOR DETECTION OF ACRYLAMIDE IN FOOD

By

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Chapter 1: Introduction

1. 1 Introduction

On April 24, 2002, the formation of acrylamide in starchy foods, which had been heat-treated like a variety of baked and fried foods cooked at high temperature, was reported by researchers from the Swedish National Food Administration (SNFA) and the University of Stockholm. Since the Swedish report, similar findings have been reported by many other countries (Tareke et al., 2002; Keramat et al., 2010). Acrylamide is naturally formed in food as a byproduct of Maillard reaction at high temperature (120 to 170°C) in presence of glucose and asparagine (Mottram et al., 2002). Acrylamide concentrations often exceeded 1000 μg/kg, which caused a worldwide concern because acrylamide is classified as probably carcinogenic to humans (Group 2A) by the International Agency for Research on Cancer **(**IARC, 1994). Food safety is a matter of public concern and monitored closely by national authorities. Thus, the interest of customers' preferences for safe and healthy food products drives the food industry to seek for more efficient, cost-effective, faster, and powerful analytical methods for detecting toxic compounds such as acrylamide in food. Therefore, the development of biosensors, an amalgamation of signal transducers and biocomponents, has been the center of scientist's attention for recent decades. Biosensor has many advantages compared to GC and HPLC, which are current methods for detecting toxicants. Their advantages are contained ease of mass

fabrication, cost reasonability, field applicability, ability to give response in real time, and ability to give information about the effect of stimulate on living systems (Malhotra et al., 2005). Annual worldwide investment in biosensor R&D is estimated to be $300 US million (Weetall, 1999; Alocilja and Radke, 2003). Today, in consideration of the need of suitable biosensors for real-time toxicity monitoring, bacteria as test organisms have been greatly proposed. The advantages offered by microbial toxicity testing include high sensitivity, low costs, large homogenous test populations and most importantly rapid responses (Bitton and Dutka, 1986). Probably today, the most common microbial toxicity available tests are based on the use of luminous bacteria as an optical microbial biosensor. Light generation is sensitive to a large number of environmentally significant contaminants, and the bioassay, rather than measuring long-term effects on viability, determines short-term effects on light production. In addition, convincing correlations have been reported between the results obtained using microbial tests and those derived from long-term assays using higher organisms (Ribo and Kaiser, 1987). Also, the use of bacteria as test organisms has an additional unique advantage, being amenable to sophisticated genetic manipulations; bacteria can be engineered to generate easily assayed signals in response to specific classes of compounds. One approach to achieve this is to fuse two genetic elements together: the first, the promoter, reacts to the presence of the toxicant and "turns on" the expression of the second, which acts as the reporter. Since light emission is easy to monitor and quantify (Stewart and Williams, 1992; Chatterjee and Meighen, 1995), bioluminescence genes are excellent candidates for the reporting element. This approach has recently been utilized in several cases to

construct microbial tools capable of sensitively reporting on the presence of specific compounds such as naphthalene (King *et al.,* 1990), mercury (Selifonova *et al.,* 1993) or other metals (Guzzo *et al.,* 1992; Corbisier *et al.,* 1993; Guzzo and DuBow, 1994). Therefore, a panel of genetically modified luminous *E. coli* strains, which contains different stress responsive promoters, can be a good candidate to work on in order to develop a novel biosensing element to detect acrylamide in food and characterize damage mechanisms of this toxic chemical.

1.2 Research Objectives

The ultimate goal of this project was to develop a novel biosensing element using stress- responsive luminous bacteria for real-time detection of acrylamide, a toxic chemical formed in starch-based foods fried and baked at high temperatures. The Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) Consultation on Health Implication of Acrylamide in Food has recognized the presence of acrylamide in food as a major concern in humans, based on its ability to have genetic and non- genetic harmful effects on laboratory animals (FAO/WHO, 2005). There is a pressing need for robust, rapid, cost reasonable and easy methods to achieve early detection of acrylamide in food to prevent adverse effects and reduce health risks.

In order to accomplish the ultimate goal, the proposed work can be divided into three specific objectives:

Objective 1: Identification of the stress fingerprints induced by acrylamide using bacterial bioluminescence.

Objective 2: Assessment of the effects of internal and external factors on stress response of the specific bioluminescent *E. coli*.

Objective 3: Elucidation of toxicity by understanding correspondent cell repair mechanisms.

Chapter 2: Literature Review

2.1 Acrylamide

2.1.1 Background

In 1893, acrylamide was produced by Moureu in Germany for the first time. In 1952, Hercules Company started making research quantities of acrylamide, and after that production for commercial use started in 1954. Since the use of acrylamide was primarily for the production of polyacrylamides with broadly different physical and chemical properties, this was the only known acrylamide exposure from industrial products at that time (Smith and Oehme, 1991). To date, acrylamide is used as a binding, thickening, or flocculating agent in grout, cement, sewage, wastewater treatment, pesticide formulations, cosmetics, sugar manufacturing, and soil erosion prevention. The polymers of the compound are used in ore processing, food packaging, plastic products, and molecular biology laboratories gels for separation of proteins and chromatography (WHO, 1985; EU, 2002; IARC, 1994). In April 2002, Swedish National Food Authority reported the presence of acrylamide principally in carbohydrate-rich foods (Tareke et al., 2002), which was led to the intensive investigations into acrylamide, encompassing the analysis, occurrence, chemistry, toxicology, and potential health risk of this contaminant in the human diet.

2.1.2 Chemical characterization of acrylamide

Acrylamide (CH2=CH-CO-NH2; 2-propenamide) is a white crystalline solid with a molecular weight of 71.08. It has a melting point of 84.5±0.3 °C, low vapor pressure (e.g. 0.007 mm Hg at 25 °C, 0.03 mm Hg at 40 °C, 0.07 mm Hg at 50°C and 0.14 at 55 °C), a high boiling point (136 °C at 3.3 kPa/25 mmHg) (Norris, 1967). The solubility in polar and non-polar solvents varies greatly, and it has a very high solubility in water. Acrylamide is a difunctional monomer, containing a reactive electrophilic double bond and an amide group. Acrylamide shows both weakly acidic and basic properties (Haberman, 1991).

2.1.3 Acrylamide formation in food

It has been shown that the Maillard reaction, in the presence of asparagine is the main pathway for acrylamide formation in a wide range of foods processed at high temperatures (Yaylayan et al., 2003; Zyzak et al., 2003). As demonstrated in Figure 2.1, the reaction of a carbonyl compound (a reducing sugar) with the amino acid asparagine starts acrylamide formation, which results in the corresponding N-glycosyl conjugation and the formation of a decarboxylated Schiff base after dehydration at high temperature (Stadler et al., 2002). After its decarboxylation, the Schiff base may lead directly to acrylamide and an imine or followed by hydrolysis to aminopropionamide and carbonyl compounds. In this respect, it should be noted that aminopropionamide may also yield acrylamide after the elimination of an ammonia group (Granvogl and Schieberle, 2006). Acrylamide is chiefly formed during heat processing (>120 ºC) of foods. Mostly those derived from plant origin such as potato cereal products. Its formation increases in range of 120 to 170°C and then decreased

(Stadler et al., 2002; Mottram et al., 2002; Zyzak et al., 2003). Stable isotope-labeled experiments have indicated that the backbone of the acrylamide molecule originates from the amino acid asparagine (Stadler et al., 2002; Zyzak et al., 2003). Asparagine by itself could in principle form acrylamide by direct decarboxylation and deamination, but the reaction is not efficient and with its yield is enormously low (Granvogl and Schieberle, 2006). Conversely, asparagine in the presence of reducing sugars (a hydroxyl carbonylmoiety) or reactive dicarbonyls supplies acrylamide in the range up to 1 mol% in model systems (Stadler et al., 2004). On the other hand, over the past years non-asparagine ways leading to acrylamide have been published (Lignert et al., 2002; Yaylayan and Stadler, 2005) although, these non-asparagine pathways may be considered as of negligible importance because studies in potato- and cereal-based foods have showed the importance of asparagine by effectively decreasing acrylamide through the use of the substrate-selective enzyme asparaginase (Lineback et al., 2012). The presence of Maillard precursors in raw materials is the most important factor for acrylamide formation, e.g. reducing sugar such as glucose and fructose, and amino acids in the form of free asparagine, and the level of the combined temperature and time load (Brathen and Knutsen, 2005). Foods rich in both reducing sugar and asparagine these are mainly derived from plant sources such as potatoes and cereals (barley, rice, wheat), but apparently not animal foods such as poultry, meat, and fish. Generally, high temperature processed foods include French fries, potato chips, tortilla chips, bread crust, crisp (Bermudo et al., 2004) bread, and various baked goods and cereal formulations have high level of acrylamide. However, wide variations in levels of acrylamide in different food categories as well as in

different brands of the same food category (e.g., French fries; potato chips) were observed. Therefore, besides precursors amount, genetic factors and environmental conditions may also affect the level of acrylamide precursors. Moreover, the processing conditions (e.g., temperature; time; nature of frying oil; nature of food matrix), and water activity of foods may also have effect on acrylamide formation (Friedman et al., 1999).

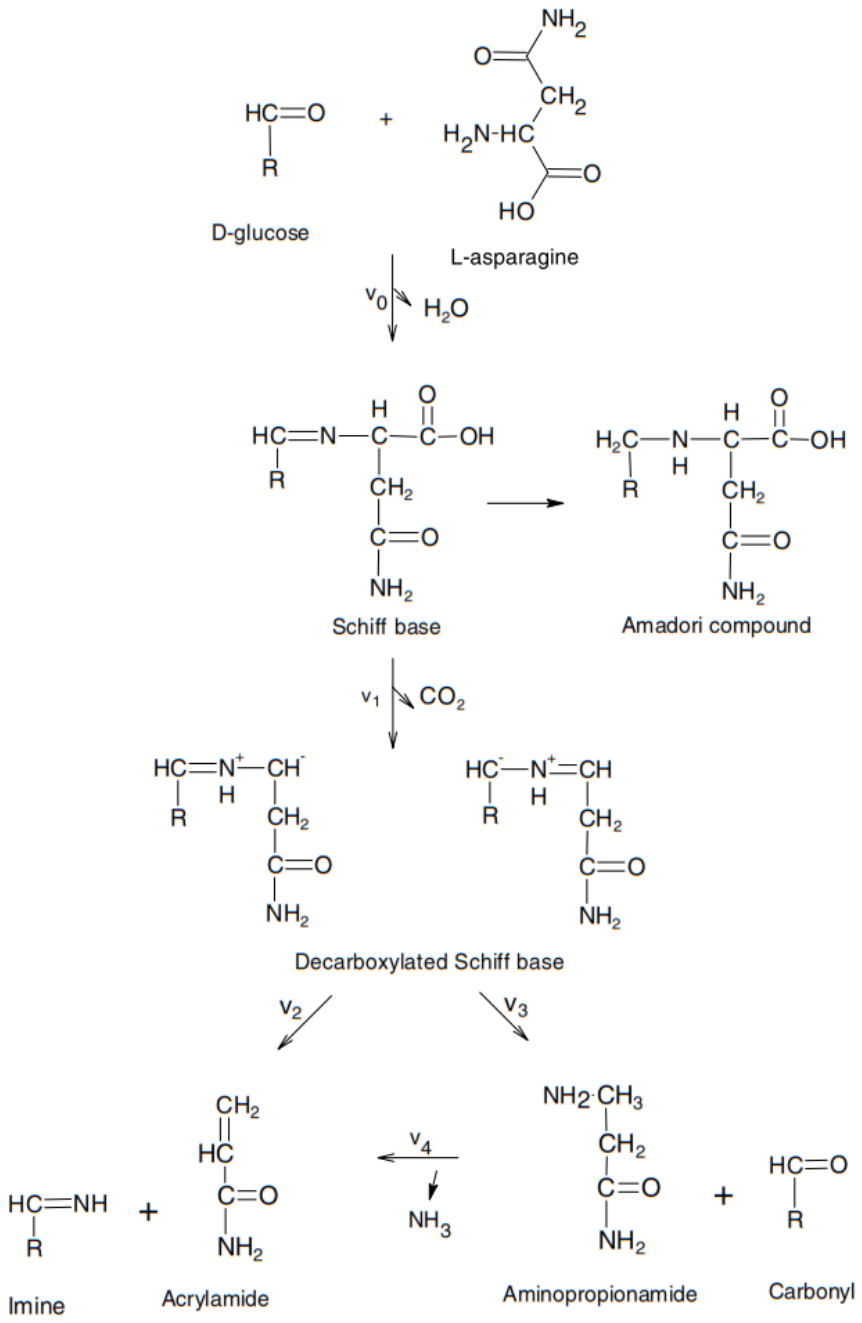

Figure 2.1 Acrylamide formation by Maillard reaction in heated foods (Adopted from Hedegaard et al., 2008).

2.1.4 Toxicology of acrylamide

2.1.4.1 Regularity levels

As mentioned in the previous section, acrylamide is formed in food at high temperature. Food groups containing greater levels of acrylamide can be listed as follows; potato products (French fries, oven-baked chips, potato crisps, and so on), different cereal-based foods such as breakfast cereals, cookies, biscuits, bread (especially toasted bread), pies, cakes, coffee, chicory, and other coffee substitutes, chocolate, teething biscuits, baby rusks, and various other baby foods. A joint FAO/WHO consultation group on the health implications of acrylamide in food has estimated an average daily food intake of acrylamide by the general population in the range of 0.3–0.8 μg/kg bw/day (FAO/WHO, 2005). According to exposure assessments in 2006, the daily dietary intake of acrylamide with diet is approximately 0.4 μg/kg bw with a 90th percentile of 0.95 μg/kg bw (DiNovi, 2006). Permissible levels were established for drinking water by the WHO at 1 μg/L, by the European Union at 1 μg/L, and by the US Environmental Protection Agency (EPA) at 0.5 μg/L. Levels for acrylamide in cosmetics have been set at <0.1 ppm in body care products and <0.5 ppm for other cosmetic products (SCCNFP, 1999). No threshold level exists below which food safety concerns can be marginalized. For that reason the ALARA (‘as low as reasonably achievable’) principle should be followed with regard to levels of acrylamide in food (EC, 2011).

2.1.4.4 Neurotoxicity

The neurotoxicity of acrylamide for animals and humans is clear. However, the neurotoxic effects seem to be only a problem in humans with high-level exposure

(Exon, 2006). Human studies have shown that occupational exposure to acrylamide produces symptoms of peripheral neuropathy (LoPachin and Gavin, 2008). Studies on occupational exposure of Swedish tunnel workers to a grouting agent have indicated that acrylamide can cause mild and reversible peripheral nervous system symptoms (Hagmar et al., 2001). Also another study in China showed that cerebellar dysfunction, followed by neuropathy can suffer workers by longer exposure to acrylamide (He et al. 1989). It was evaluated by Joint Expert Committee on Food Additives (JECFA) of the World Health Organization (WHO) that the most sensitive adverse non-carcinogenic effect could be the morphological changes in nerves of rats with a no-observed-adverse-effect level (NOAEL) of 200 μg kgbw $^{-1}$ d^{-1} (Viswanath, 2012). In order to determine cumulative effects on the nervous system further studies are needed on low-level chronic exposures (Exon, 2006).

2.1.4.3 Reproductive toxicity

Acrylamide produces reproductive toxicity as proved in animal studies. These kinds of effects have not been observed in humans. The neurotoxic effects may or may not be related to the mechanism underlying reproductive effects. Some research shows that reproductive effects may be produced by the neurotoxicity and resultant behavioral changes or by the same mechanisms as neurotoxicity through effects on the kinesin motor proteins in reproductive cells. The mechanism may also be by direct interaction with sulfhydryl groups on proteins essential to the function of germ cells. The amount of acrylamide people expose to by consuming food is not expected to induce any reproductive toxicity, but studies on long-term low doses are still needed (Exon, 2006).

2.1.4.4 Carcinogenicity

Relatively high doses of acrylamide are carcinogen for rodents. There is no certain evidence from dietary or occupational exposures that acrylamide increases cancer risk in humans although some of epidemiologic studies may lack the necessary statistical power to detect small increases in cancer incidence in diet. The mechanism of carcinogenicity is unclear in rodents. Genetic damage is induced in acrylamide exposure, but this may be through indirect effects on proteins involved in cell division or chromosome structure and function. Epigenetic mechanisms involving hormonal imbalance and increased cell division may be the reason of high incidence of hormonal or endocrine tumors. On the other hand, direct effect on DNA can also be a carcinogenic factor, especially from the reactive metabolite, glycidamide. Further research is needed to elucidate the mechanism of carcinogenicity and its relationship to neurotoxicity and reproductive toxicity (Exon, 2006).

2.1.4.5 Risk and Hazard

In a number of countries, there is not enough information available concerning the amount of acrylamide in different foods. Moreover, the amount that is there varies greatly even within the same brands and batches. In addition, there is not enough information about the health effects of levels of acrylamide in the diet. As a result, no credible food safety group or government agency is recommending any changes in our food choices at this time to avoid foods that contain acrylamide. Because of these reasons, it is premature for groups, such as in California, to insist on labeling of products that contain acrylamide. Amount of acrylamide is various in different ypes of food (Table 2.1). The majority of responsible groups agree that more research is

needed on the health effects of acrylamide. In addition, since the European Chemicals Agency (ECHA) added acrylamide to the list of substances of very high concern, further studies are needed to examine the metabolism, storage, excretion, and metabolic fate of acrylamide and its metabolites (Exon, 2006; ECHA, 2010).

Table 2.1 Maximum amount of acrylamide (µg/kg) in several types of food (adopted from European food safety Authority (EFSA) Journal 2010).

Types of food	**Amount (µg/kg)**
Biscuits	
Crackers	1526
Infant	2300
Not specified	4200
Wafers	1378
Bread	
Bread	2430
crisp Bread	910
soft Non specified	2565
Breakfast cereals	1600
Cereal-based baby food	353
Coffee	
Instant	1047
Not specified	1158
Roasted	958
French fries	2668

Table 2.1(continued)

Types of food	Amount (µg/kg)
Potato crisps	4180
Deep fried	1661
Oven fried	941

2.1.5 Current methods for detecting acrylamide

Since acrylamide has been classified as a probable carcinogenic substance to humans, national and international regulatory agencies have focused their attention on the detection of acrylamide in food items (US EPA, 1994; EC, 2011). It is stated by FDA that "development of rapid or inexpensive screening methods and validating confirmatory methods of analysis" is a major goal for the issue of acrylamide in food (FDA, 2013). The common methods currently used for detecting acrylamide (Table 2.2) include Capillary Electrophoresis, Gas Chromatography, and High-Performance Liquid Chromatography (Wenzel et al., 2003).

Table 2.2 Comparison of common methods for detecting acrylamide

Method	Detection limit (potato chips)	Sample preparation	Cost of installation	Reference
Capillary Electrophoresis	0.1µg/ mL	Not difficult	Expensive	Zhang et al. 2007
Gas Chromatography	5-50 µg/ kg	Very difficult	Very expensive	Fernandes and Soares 2007
High-Performance Liquid Chromatography	0.5 µg/ L	Difficult	Rather expensive	Kaplan et al. 2009

2.1.5.1 Capillary Electrophoresis

For the determination of acrylamide in brownish-colored home-made French fries, microemulsion electrokinetic chromatography was developed (Bermudo et al., 2004). Two in-line pre- concentration capillary zone electrophoresis (CZE) methods field amplified sample injection (FASI) and stacking with sample matrix removal were planned for the analysis of acrylamide in foodstuffs after derivatization with 2-mercaptobenzoic acid (Bermudo et al., 2006). For the analysis of acrylamide after its derivatization with 2-mercaptobenzoic acid, a Capillary Electrophoresis mass spectrometry (CE-MS) method was developed (Bermudo et al. 2007). Micellar electrokinetic capillary chromatography was developed for the separation and determination of acrylamide in potato chips (Zhou et al. 2007).

2.1.5.2 Gas Chromatography

Gas Chromatography (GC) methods have commonly involved the derivatization of acrylamide with potassium bromate and potassium bromide to improve the method's properties. In a recent GC method, a high-resolution time-of-flight mass analyzer was developed for the direct analysis (no derivatization) of acrylamide in different heat-

processed foodstuffs (Dunovska et al., 2006). Extraction with n-propanol followed by solvent exchange acetonitril avoided co-isolation of acrylamide precursors that could yield additional analyte in the hot splitless GC injector. Based on derivatization of the target analyte with bromination and detection by an electron capture detector (ECD) was another GC method (Zhang et al., 2006). Results from the GC-ECD analysis were confirmed with GC-MS. To determine acrylamide in coffee and coffee products another GC-MS method was developed by Soares et al. (2006). After that these researchers focused exclusively on this particular beverage, determining the factors to be considered in the acrylamide extraction, so contributing to a better knowledge of the exposure levels for espresso coffee consumers (Haza et al., 2010). Acrylamide was detected in Espresso coffee by matrix solid-phase dispersion (MSPD) and GC-MS. A GC-MS-MS method was developed to measure acrylamide in aqueous matrices extracted from French fries and potato crisps by using direct immersion solid-phase microextraction (SPME) without derivatization (Lee et al., 2007).

2.1.5.3 High-Performance Liquid Chromatography

While the CE and GC methods for acrylamide determination are in routine use in some laboratories, a great number of laboratories use HPLC methods. LC, coupled with MS-MS detection is mainly preferred. For the analysis of acrylamide and methacrylamide, a method using normal-phase HPLC with UV detection was developed by Paleologos and Kontominas (2005). Also, a HPLC method with UV detection was developed for the determination of acrylamide in deep-fried flour-based leaven dough foods available in Hong Kong (Wang et al., 2008). For the determination of acrylamide in potato-based foods at low levels LC coupled to diode

array detection (DAD) was developed (Gökmen et al., 2005). Moreover, an ion-exclusion liquid chromatography coupled with diode array detection (LC-DAD) method was developed for the determination of acrylamide in starch-based foods (Geng et al., 2008). Further, for the determination of acrylamide in protein-rich foods such as grilled meat and chicken foods as well as carbohydrate-rich foods containing potato chips and biscuit another LC-MS method was reported, (Kaplan et al., 2009). Moreover, for the determination of acrylamide in potato crisps an ultra-performance liquid chromatography coupled to ESP-MS-MS was utilized (Zhang et al., 2007).

Therefore, since these conventional techniques are very complex, tedious, time consuming and require trained personnel, and by considering public concern over acrylamide contamination in food products, the development of simple, rapid, precise, and cost-effective method for detecting acrylamide in food products can have a great benefits.

2.2 Biosensor

2.2.1 Definition

In 1956 Leland Charles Clark Jr., the pioneer of the biosensor research, published his first paper about the electrode to measure oxygen concentration in blood. After that, research communities from various fields such as Physics, Chemistry, and Material Science have come together to develop more stylish, reliable and mature biosensing devices for applications in the fields of medicine, agriculture, biotechnology, as well as the military and bioterrorism detection and prevention.

"A biosensor can be defined as a quantitative or semi quantitative analytical instrumental technique containing a sensing element of biological origin, which is

either integrated within or is in intimate contact with a physicochemical transducer" (Turner et al., 1987). A device that transforms chemical information is chemical sensor, which can be ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. A chemical (molecular) recognition system (receptor) and a physicochemical transducer are two basic components of chemical sensors that are connected in series. In the same way, biosensors are chemical sensors in which the recognition system uses a biochemical mechanism interfacing the optoelectronic system (Cammann, 1977; Turner et al., 1987). A transducer is the key component of a biosensor (Figure 2.2) that makes use of a physical change accompanying the reaction, which may be heat output, changes in electrical or electronic output, redox reaction, light output or light absorbance difference between the reactants and products or based on mass of the reactants or products. In order to get a better reading of the sensors, the data will need to be cleaned, amplified, and processed. This is done because the base signals coming from the transducers are often very weak that includes heavy noise. A "reference" signal from the same transducer is used without any bio catalytic membrane from the sample in order to increase the signal to noise ratio. This way, the noise is known and when compared with the transducer with the bio catalytic signal, it will produce a better result by comparing the two signals. The analog data is then converted to digital and passed through a microprocessor in order to display the concentration units on a display device or to record the data (Chaplin, 2004).

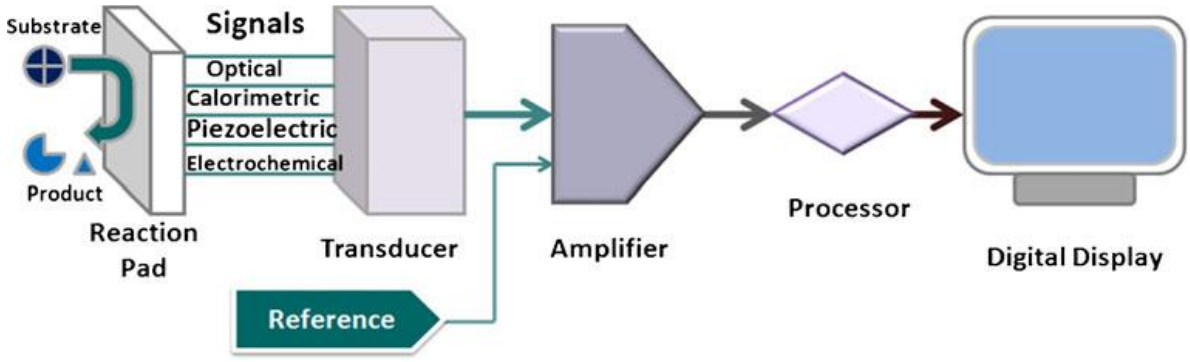

Figure 2.2 Schematic representation of biosensor components (Adopted from Thakur and Ragavan, 2013).

2.2.2 Essential characterization of biosensor

There are six major characters of biosensor containing selectivity, sensitivity, linearity of response, reproducibility of signal response, quick response time and recovery time, and stability and operating life.

1- Selectivity: It is necessary for biosensor device to be highly selective for the target analyte and show minimum or no cross reactivity with substances having similar chemical structure.

2- Sensitivity: The biosensor device should have the ability to measure in the range of interest for a given target analyte with minimum additional steps such as pre cleaning and pre concentration of the samples.

3- Linearity of response: The range in which target analyte is measured should be covered by the linear response of biosensor device.

4- Reproducibility of signal response: We should be able to get the same response from the system when samples having same concentrations are analyzed several times.

5- Quick response time and recovery time: Since the real time monitoring of the target analyte need to be done efficiently, the biosensor device response should be quick enough. Also, for reusability of the biosensor system, recovery time should be small.

6- Stability and operating life: Since, most of the biological compounds are unstable in different biochemical and environmental conditions, the biological element used should be interfaced such that the activity is kept for a long period of time. Therefore, the device can be marketable and practically useful in the field (Mello and Katsu, 2002; Chaplin 2004; Kazemi et al., 2013).

2.2.3 Different types of biosensor

Based on the working principle of biosensors they are classified into different types (Figure 2.3). Some of the significant biosensors are electrochemical, optical, microbial, optical microbial, affinity, surface plasmon resonance (SPR)**,** and acoustic sensors. Three of the most important biosensors are explained below.

2.2.3.1 Optical biosensors

Because of the resemblance of optical biosensors with electrodes, they are also known as “optodes”. This kind of biosensor contains determining changes in light absorption between the reactants and products of a reaction, or measuring the light output by a luminescent process. To identify chemical or biological species, optical biosensors integrate optical technique with a biological element. Numerous

optical biosensors were developed based on surface Plasmon resonance, spectroscopy and evanescent waves etc. (Gouda et al., 1997; Chouhan et al., 2006; Akshath et al., 2012; Selvakumar and Thakur, 2012).

2.2.3.2 Microbial biosensors

An analytical device that combines microorganisms with a transducer to facilitate rapid, accurate and sensitive detection of target is a microbial biosensor. Common microbial biosensors utilized the respiratory and metabolic functions of the microorganisms to detect a substance that is either a substrate or an inhibitor of the processes. Since the construction of enzyme sensors are complex, and costly microbial biosensors have more advantageous compare to them. Amperometric, potentiometric, and conductometric sensors are some main types of microbial biosensors (Babu et al., 2007; Kazemi et al., 2013).

2.2.3.3 Optical microbial biosensor

Optical microbial biosensors are the combination of optical and microbial biosensors. Bioluminescence, which related with the emission of light by living microorganisms, plays a significant role in real-time process monitoring. Therefore, bacterial luminescence *lux* gene has been widely applied as a reporter. Bioluminescence based biosensors are employed for detecting of metal ions, heavy metals, phosphorus, naphthalene, genotoxicants and chlorophenols. They have high potential in monitoring the sanitation and pollution levels in industries (Ranjan et al., 2012).

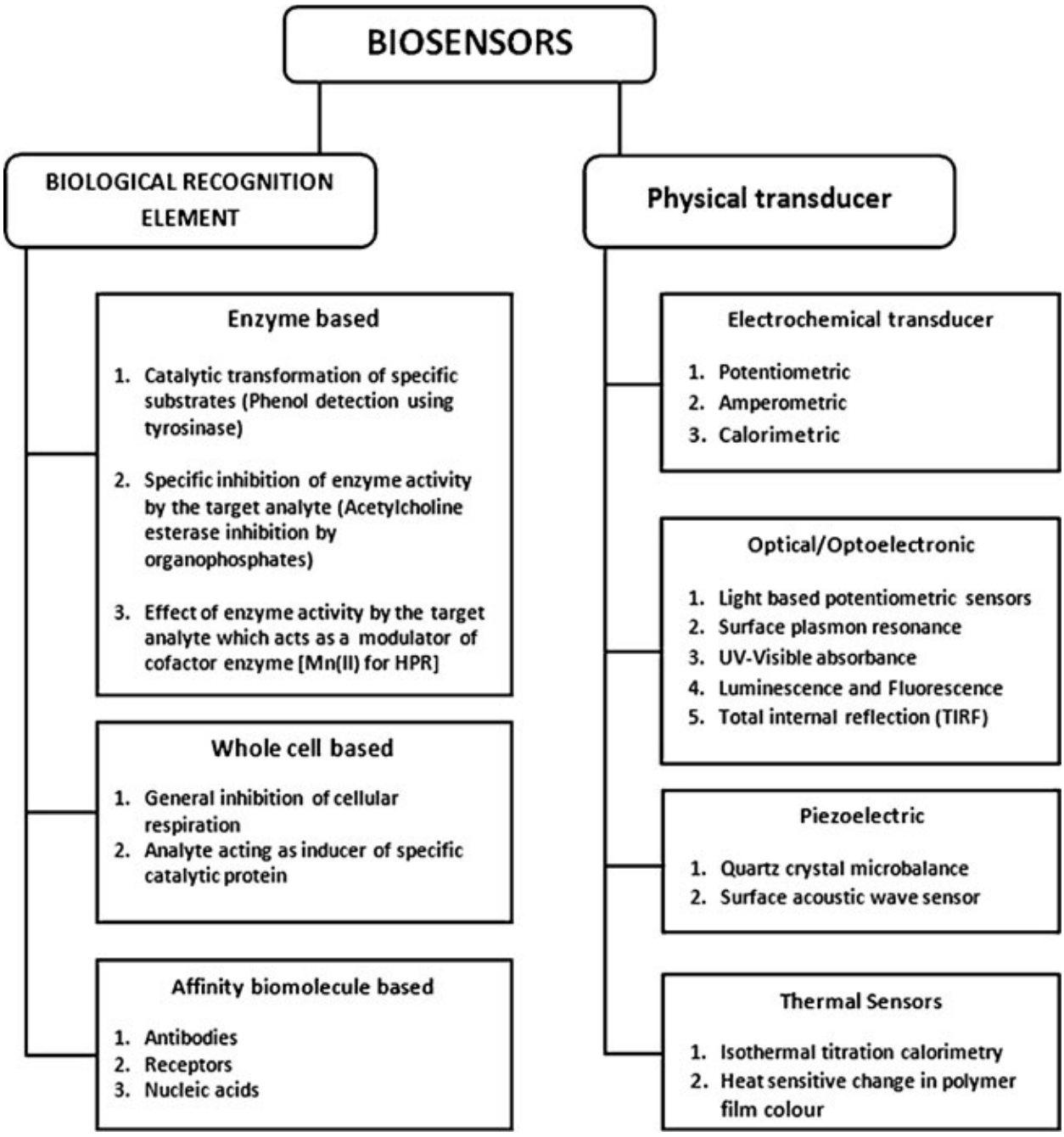

Figure 2.3 Schematic representation of biosensors with various combinations of physical and biological elements (Adopted from Thakur, 2012)

2.3 Bioluminescence

2.3.1 Bioluminescence organisms

Bioluminescent organisms are broadly distributed in nature and include a remarkably various set of species (Hastings et al., 1985; Hastings 1986; Campbell 1989). Bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid are among the light-emitting species. This set of organisms contains terrestrial, freshwater, and marine species from almost fifty percent of the diverse categories in the animal and plant kingdoms. Luciferases are the enzymes that catalyze the bioluminescence reactions in these organisms, and in the majority of cases the substrates are designated as luciferins. Although, the obvious absence of a strong evolutionary relationship between many of the light-emitting systems, important variations exist between the bioluminescence reactions as well as the structures of the Luciferases (enzymes) and luciferins (substrates) from diverse organisms. Moreover, in luminescence systems, the requirement of oxygen for the bioluminescence reactions has been evidently recognized (Meighen, 1991).

2.3.2 Luminous bacteria

symbionts in the teleost fishes and squid are the most common habitats of luminous bacteria (Hastings, 1986). These bacteria are all gram-negative rod shape, and facultative anaerobes (Nealson, and Hastings, 1979; Baumann, 1983). Most of luminous bacteria have been categorized into the three genera *Vibrio*, *Photobacterium*, and *Xenorhabdus*, with most of the species being marine in nature (Baumann, 1983; Campbell, 1989). Just *Xenorhabdus* species infect terrestrial

organisms (Farmer, 1989). The light-emitting bacteria, which have been studied in most detail, are *Vibrio harveyi, V. fischeri, Photobacterium phosphoreum, P. leiognathi*, and *Xenorhabdu*s luminescens. Since there are many various isolates and sources of each species, significant differences may exist between the *lux* systems from luminescent strains of the same species. *V. logei*, *V. splendidus, V. cholerae* (Hada, 1985), a freshwater species, and one species (*Altermonas haneda*) classified in the genus *Alteromonas* are other species of luminescent bacteria (Jensen, 1980). Since the majority of strains classified in the genus *Photobacterium* have been isolated as specific light organ symbionts of fishes, it appears that this may be their natural habitat (Hastings, 1987). Primarily *P. phosphoreum* are luminescent bacteria found as symbionts in deep-water fish while symbiotic luminescent bacteria in fish in shallow and temperate waters are *P. leiognathi* or *V. fischeri* (Thomas and Poinar, 1979).

2.3.2.1 Bioluminescence reaction

The light-emitting reaction in bacteria involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long chain fatty aldehyde with the emission of blue-green light. Because the structure of the substrates are relatively simple ($FMNH_2$ and RCHO) and are part of and/or closely related to the normal metabolites in the cell, the term luciferin has generally not been used to refer to the substrates for the reaction catalyzed by bacterial luciferases. This reaction is as follows:

$$FMNH_2 + RCHO + O_2 - FMN + H_2O + RCOOH + hv\ (490\ nm)$$

The reaction is highly specific for FMNH2. Modification of the flavin ring or removal of the phosphate group decreases the activity significantly (Meighen and MacKenzie, 1973). The natural aldehyde for the bioluminescence reaction is believed

to be tetradecanal on the basis of identification of this compound in lipid extracts, the preference for tetradecanal by luciferases at low (nonsaturating) substrate concentrations, and the specificity of the *lux*-specific fatty acid reductase system, which catalyzes the synthesis of the fatty aldehyde substrate (Shimomura, 1974; Ulitzur and Hastings, 1979; Meighen, 1982; Meighen and Grant, 1985; Rodriguez, 1985). However, differences in aldehyde specificity do exist among different bacterial luciferases.

2.3.2.2 Lux gene organization

From a number of luminescent bacterial strains containing *V. fischeri, V. harveyi, P. phosphoreum, P. leiognathi,* and *X. luminescens lux* genes have been cloned. In all cases, *luxA* and *luxB* genes coding for the luciferase subunits and *luxCDE* genes coding for the fatty acid reductase complex have been identified. Moreover, other *lux* genes including *luxF, luxG, luxH, luxI,* and *luxR* have been identified in specific luminescent strains. The genes coding for the luciferase (l*uxAB*) and fatty acid reductase (*luxCDE*) enzymes have the same order in all operons. *LuxF* is an additional gene, which located between *luxB* and *luxE* in most but not all *Photobacterium* species, and is missing in the *Vibrio* and *Xenorhabdus lux* systems (Illarrionov, et al., 1990).

2.4 Bioluminescence-E. coli

2.4.1 *E. coli*

Escherichia coli (*E. coli*) is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded

organisms (endotherms). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine. *E. coli* and related bacteria constitute about one percent of gut flora, and fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination. The bacterium can also be grown easily and inexpensively in a laboratory setting, and has been intensively investigated for over sixty years. *E. coli* is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA (Han and Lee, 2006; Hartwell et al., 2008).

2.4.2 Genetic engineering of luminous *E. coli*

The ability of bacteria to be engineered to generate an easily assayed signal in response to specific classes of compounds is the unique advantage of them as test organisms, which make them amenable to sophisticated genetic manipulations. According to this feature, bacteria can combine, at least partially, the two traditional approaches to toxicants detection; an observed response will indicate the existence of a toxic compound, as well as provide some information on its nature. One way to attain this is to fuse together two genetic elements. First, the promoter reacts to the

presence of the toxicant and "turns on" the expression of the second, which acts as the reporter. Because light emission is monitored and quantified easily (Stewart and Williams, 1992; Chatterjee and Meighen, 1995), bioluminescence genes are perfect candidates for the reporting element. Recently, in several cases to construct microbial tools capable of sensitively reporting on the presence of specific compounds such as naphthalene (King et al., 1990), mercury (Selifonova et al., 1993) or other metals (Guzzo et al., 1992; Corbisier et al., 1993; Guzzo and DuBow, 1994), this approach has been utilized. A relatively non-specific response was described for a *lux* fusion to a Pseudomonas isopropylbenzene degradation gene (ibp) in 1996 (Selifonova and Eaton, 1996). Different approaches have been attempted. Besides fusing the *lux* system to genes of a relatively narrow response spectrum, they have chosen to utilize wide, global regulatory circuits, which are activated in response to several classes of environmental threats (Van Dyk et al., 1994; 1995; Belkin et al., 1996). Among these circuits is the bacterial heat shock system (Neidhardt and VanBogelen, 1987; Yura et al., 1993; Van Dyk et al., 1994), responsive to protein damage, the SOS machinery involved in protection against DNA damage (Kushner, 1987; Walker, 1996), and others. The engineered organisms enhance their light production in response to the presence of biologically distinguishable categories of toxicants. The strains combined used as an analytical panel for toxicants detection is one of the probable applications of them. These kinds of panel may have several advantages; the most important of these is that a positive response by a panel member will not only show the presence of a toxicant but will also provide some idea as to its character. In this research first, a panel of five bioluminescent strains containing selected stress responsive *E. coli*

promoters fused to the *Photorhabdus luminescens luxCDABE* reporter were used. The panel strains are chosen to represent a range of stress responses such that different stresses are expected to result in different patterns of induced gene expression. Table 2.3 summarizes the stress response, regulatory circuit, promoter fused to *lux*, and the strain name of all the strains employed in the panel for this study. These fusions are: *recA-lux* (in the SOS regulon), *grpE-lux* (in the heat shock regulon), *inaA-lux* (internal acidification responsive and in the Sox and Mar regulon), *yciG-lux* (in the ∂^s-dependent stress response regulon), and *o513-lux* (in the ∂^s-independent, stationary phase inducible stimulon).

Table 2.3 Stress-responsive *E. coli lux* fusion strains (Adopted from Van Dyk, 1998).

Stress Response	**Regulatory Circuit**	**Promoter Fused to *lux***	**Strain Name**
Internal Acidification	Mar/Sox/Rob	*inaA*	DPD2240
DNA damage	SOS	*recA*	DPD2222
Protein damage	Heat shock (δ^{32})	*grpE*	DPD2234
"Super-stationary phase"		*o513*	DPD2232
Sigma S stress Response	Stationary phase (δ^{s})	*yciG*	DPD2233

2.5 DNA damage and repair

Common types of damage are base modifications (methylation, oxidation), mispairs (mistakes in DNA synthesis), cross-linked nucleotides (intrastrand, interstrand covalent links), and double-stranded DNA breaks. Different types of repair fix different types of damage, which in order of complexity of problems contain direct repair of specific modification, base excision repair for missing or altered base, nucleotide excision repair for distortion of B-DNA with damage on one strand, mismatch repair for both bases are, and interstrand cross-link or double-stranded break repair for both strands are damaged (Nickoloff and Hoekstra, 1998).

2.5.1 Recombinational repair capacity of *E. coli* cells

E. coli cells can grow in a nutritionally poor medium (Sinden and Cole, 1978). *E. coli* cells are still viable after repairing 100 to 200 daughter strand gaps per chromosome (Wang and Smith, 1983). Also, *E. coli* cells have the ability to tolerate multiple disintegration of replication forks, because recombinational repair can reattach the resulting double-stranded ends to the circular domains of the chromosome. Among DNA damage, the only two-strand DNA lesion that has proved to be deadly for *E. coli* is a double-strand break. If only two or three double-strand breaks in *E. coil*'s chromosomes occur, it can survive. Therefore, when a double-strand break occurs in an unreplicated portion of the chromosome, it cannot be repaired (Krasin and Hutchinson, 1977; Ulmer, 1979).

2.5.2 SOS Response: Reaction of *E. coli* to DNA Damage

Like many other living organisms *E. coli* is often exposed to a diversity of environmental agents, both by natural and man-made, which perturb the integrity of DNA. These DNA adducts often obstruct continual DNA replication and thus pose immediate threats to continued cell survival. Maybe as a result, *E. coli* (as well as many other prokaryotes) has evolved such that a number of unlinked genes participated in DNA repair, cell division, and damage tolerance are respectively expressed after DNA damage. This global cellular response is called "SOS response". The SOS response, as its name inaccurately implies is by no means a desperate attempt to stay alive (Radman, 1975), but, rather, an arranged and measured reaction of the cell to DNA synthesis inhibition. Two proteins, *RecA* and *LexA* transcriptional

repressor, which are themselves SOS-inducible protein, control the complex SOS regulatory network (Nickoloff and Hoekstra, 1998).

2.5.2.1 RecA protein

Escherichia coli's *recA* protein is a multifunctional protein that is vital to three distinct, but related biological processes containing general genetic recombination, regulation of the coordinated expression of over forty unlinked genes in response to DNA damage, known as the SOS response, and the error-prone replicative bypass of DNA lesions, resulting in a highly mutagenic repair of DNA. Consequently, RecA protein has three major biochemical activities including the homologous pairing and exchange of DNA, ATP and DNA-dependent coproteolytic processing, and interaction with specialized DNA polymerases to facilitate error-prone DNA synthesis past DNA lesions.

The DNA-strand exchange activity is characteristic of groups of proteins that are necessary to genetic recombination, a biological process in which two homologous DNA molecules pair and exchange regions of their DNA strands. The ATP-dependent exchange of single strands of DNA between the participating DNA partners is facilitated by these proteins. The prototypic member of this family of functionally and genetically similar proteins is RecA protein of *E. coli*. Another unique property of RecA protein is coprotease activity. The highly specific catalysis of the self-cleavage of proteins (*LexA* repressor, UmuD mutagenesis factor and phage repressor proteins) is this coprotease activity, which is dependent on both ATP and single-stranded DNA (ssDNA) binding. The last activity of RecA protein is its direct

contribution in the replication-dependent bypass of mutagenic lesions in DNA (Bianco and Kowalczykowski, 1998).

2.5.2.1.1 The structure of RecA protein:

Assembling on ssDNA to form a nucleoprotein filament known as the presynaptic complex is essential for RecA protein to function (Story et al., 1992). This filament is an adaptable structure, which makes *recA* capable of performing its functions.

The active nucleoprotein filament is a helical complex of RecA protein monomers wrapped around ssDNA at a stoichiometry of three nucleotides per monomer and about six monomers per turn. The RecA monomer itself containing of three regions, a large, central region, surrounded by relatively small amino and carboxyl regions. The central region, related to DNA and ATP binding, contains primarily of a twisted beta sheet with 8 β-strands, bounded by 8 α-helices. The amino region includes a large α-helix and short β-strand, this α/β structure being significant in formation of the RecA polymer. Carboxyl region, which contains three α-helices and a three-stranded β-sheet, facilitates interfilament associations.

RecA protein will also form filaments on dsDNA under certain conditions. Filament assembly is greatly cooperative and induces in the 5'→ 3' direction relative to the ssDNA to which it is bound. Consequently, filament has a regular, right-handed, helical structure, and the main feature is a large helical groove. Although one side of the groove is smooth, the other is penetrated by the protrusion of the individual monomers. This groove is the binding site for the LexA repressor and is proposed to be associated with the binding of dsDNA. The binding of the LexA

repressor and dsDNA to the nucleoprotein filament is competitive and it is indicating on the filament that they bind at the same or overlapping site (Ogawa et al., 1993; Yang et al., 2001).

2.5.2.1.2 The regulatory role of RecA protein:

" The SOS regulon is a group of more than forty unlinked genes that are controlled by the LexA repressor, and whose expression is induced to high levels following exposure to DNA-damaging agents" (Courcelle et al., 2001). These genes encoded proteins whose functions are known (Table 2.4) and participating in all aspects of DNA metabolism, acting to excise DNA damage, activate transcription and transport and facilitate the error-prone, recombinational repair of DNA. Since RecA protein functions as a coprotease, stimulating the autocatalytic cleavage of a number of protiens, being a regulator of the SOS regulon and inducible DNA repair is a key role of RecA protein (Ogawa et al., 1979; Roberts et al., 1979). The most important controller of the expression of genes in the SOS regulon is the LexA repressor, which binds to the SOS box of these SOS-inducible genes and limits their transcription. After a DNA-damaging occurrence, such as ultraviolet (UV) irradiation, the coprotease activity of RecA protein becomes ‘activated’. The reason of activation is related to the generation of ssDNA resulting either from the action of nucleases or from stalled replication forks. The ssDNA is bound by RecA protein in the presence of an NTP cofactor, promoting nucleoprotein filament formation that leads to cleavage of the LexA repressor and production of SOS genes, containing *recA*. The first to be expressed completely are genes with operators that bind LexA protein weakly (e.g. *recA*). Whether the damage is continued or high enough quantities of the

damaging agent are applied, the concentration of activated RecA protein increases, leading to further cleavage of LexA protein, and derepression of even those genes whose operators bind LexA tightly. The *recA* expression is repressed and the basal level of RecA protein is maintained at 1000 molecules per cell under normal conditions. Resulting LexA repressor cleavage, the level of RecA protein in the cell increases by as much as twenty times. The raise in the level of RecA protein is fast, and reaches a maximum level within 1 h of a DNA-damaging occurrence. Within 4–6 h following the initial damage event, RecA protein levels return to the basal level. This decrease is presumably due to removal of the inducing signal by repairing of the DNA damage passing over the agent that activated RecA protein. Consequently, since RecA protein is no longer able to induce its cleavage, the intracellular concentration of LexA repressor increases. Therefore, repression of the SOS system is reestablished and the cell is returned to its uninduced state (Tang et al., 1999).

Table 2.4 Genes of the SOS regulon whose function is known (Adopted from Bianco and Kowalczykowski, 2005)

Gene	Gene product/function
dinB	Error-prone DNA polymerase IV
dinG	DNA helicase
dinI	Inhibitor of RecA coprotease activity
dinS	Transposase (IS150)
dnaN	DNAsynthesis as part of DNA polymerase III
dnaQ	DNAsynthesis as part of DNA polymerase III

Table 2.4 (continued)

Gene	Gene product/function
Fis	DNA inversion factor, transcription factor
glvB	PTS system; arbutin-like IIB component
grxA	Glutaredoxin coenzyme for ribonucleotide reductase
ibpB	Heat-shock protein
intE	Prophage e14 integrase
lexA	Represses transcription of _20 genes by binding to operators
ogrK	Prophage P2 protein
oraA	Regulator, OraA protein
polB	DNA polymerase II
potB	Spermidine/putrescine transport system permease
recA	DNA strand exchange; induces SOS; activates UmuD; SOS mutagenesis
recN	Recombination
recQ	DNA-dependent ATPase and helicase; can both promote homologous recombination and disrupt illegitimate recombination
ruvA	Forms a complex with RuvB; recognizes Holliday Junctions
ruvB	Forms a complex with RuvA; branch migration helicase
smpA	Small membrane protein A
smpB	Small membrane protein B

Table 2.4 (continued)

Gene	Gene product/function
Ssb	Binds to ssDNA
sulA (sfiA)	Inhibits cell division
sunB	Enhances synthesis of sigma-32
umuC	Forms a complex with Umu (D')2; subunit of error-prone DNA polymerase V
umuD	Forms complex with UmuC; subunit of errorprone DNA polymerase V
uvrA	Excision-repair nuclease subunit A; part of the UvrABC endonuclease that initiates excision repair
uvrB	Helicase and ATPase; excision-repair nuclease subunit B; part of the UvrABC endonuclease that initiates excision repair
uvrC	Excision-repair nuclease subunit C; part of the UvrABC endonuclease that initiates excision repair
uvrD	Helicase II; required for excision repair

2.6 The heat-shock proteins

By inducing the synthesis of a group of proteins called the heat-shock proteins or hsps all organisms respond to heat. The response is the most highly conserved genetic system known, which exist in all organisms from archaebacteria to eubacteria, and from plants to animals. Almost all responses are universal although certain features of the response vary among organisms. The fact that the hsps protect cells from the toxic effects of heat and other stresses is an early and long-standing assumption about the heat-shock response (Lindquist and Craig, 1988).

Organisms commonly expose to environments that are not optimal for growth. Cells expend energy to maintain homeostasis via specific and general mechanisms, including alterations in gene expression in response to such stressful conditions. For instance, the heat shock response, a process found in all organisms, is thought to make cells capable to respond to protein unfolding (Craig, and Gross, 1991; LaRossa and Van Dyk, 1991)., the synthesis of a number of evolutionarily conserved heat shock proteins is induced when cells experience an abrupt raise in temperature . These stress proteins contain the molecular chaperones, Hsp_{60} and Hsp_{7O}, which have significant cellular functions in protein folding and denaturation during both steady-state growth and heat shock (Mortimoto et al., 1990). Transcriptional regulation by the positively acting RNA polymerase subunit, sigma-32, and accounts for the heat shock induction of about 20 genes occurs in *Escherichia coli* (Neidhardt and VanBogelen, 1987). These contain *dnaK*, encoding Hsp70; *groEL*, encoding Hsp60; and *grpE*, encoding an essential protein known to interact with the *dnaK* and *groEL* gene products (Ang et al., 1986; Ang and Georgopoulos, 1989; Liberek et al., 1991; Langer et al., 1992). Although a hallmark of the sigma-32-mediated heat shock response is the fast, transient raise in the relative rates of heat shock protein synthesis (Lemaux et al., 1978; Yano et al., 1987). The important point is that heat shock can also be induced in presence of chemical pollutant. Therefore, a suitable biosensor for detecting toxic chemical such as acrylamide might be produced by fusing a proper heat shock promoter to *lux* genes (Nickoloff and Hoekstra, 1998).

Chapter 3: Identification of the stress fingerprints induced by acrylamide using bacterial bioluminescence

3.1 Introduction

In view of the need for real-time toxicity monitoring, significant advances have been made in the application of bacteria as test organisms. High sensitivity, low costs, large homogenous test populations and most importantly rapid responses are advantages of microbial toxicity testing (Bitton and Dutka, 1986). Moreover, by sophisticated genetic manipulations, bacteria can be engineered to generate an easily assayed signal in response to specific classes of compounds, which is a unique benefit of bacteria application as test organisms. One approach to use bacteria as a sensing element is to fuse together two genetic elements: first element is the promoter that reacts to the presence of the toxicant and "turns on" the expression of the second element that acts as the reporter. Bioluminescence genes are excellent candidates for the reporting element since light emission is easy to monitor and quantify (Stewart and Williams, 1992; Chatterjee and Meighen, 1995). Thus, in recent years, biosensors based on whole-cell bioluminescence have been shown to be a sensitive substitute to conventional analytical methods in determining concentrations of various compounds with high sensitivity and specificity (Van Dyk et al., 1994; Ramanathan et al., 1997; Buchinger et al., 2010).

In this study, five genetically engineered strains containing selected stress-responsive *E. coli* promoters fused to the *luxCDABE* reporter were employed. Use of

the five-gene *lux* reporter system allows facile monitoring of gene expression because all components necessary for light production are present in the cell. The responses were found to be biologically appropriate when stressed by internal acidification, protein damage, DNA damage, super-stationary phase, and δ^{s} dependent stress.

3.2 Materials & Methods

3.2.1 Regent and Chemicals

Acrylamide (for electrophoresis, ≥99% (HPLC), powder, SIGMA-ALDRICH), Ampicillin (Ready Made Solution, 100 mg/mL, 0.2 μm filtered, SIGMA- ALDRICH), Luria- Bertani Broth (LB broth, Lennox (Granulated), FISHER SCINTIIFIC).

3.2.2 Bioluminescent *E. coli* strains

Preparation of culture for measuring relative light unit of strains was adopted from Fukushima (2004) and modified in this study.

Five bioluminescent *E. coli* strains obtained from DuPont Genetics Lab (DuPont Company, Wilmington, DE) are used in this study. Each strain contained different selected stress-responsive promoter fused to the Photorhabdus luminescence *luxCDABE* reporter. The panel strains were chosen to represent a range of stress responses to result in different patterns of internal induced gene expression. Each strain responds respectively to internal acidification, DNA damage, protein damage, "super- stationary phase" and sigma S Stress (Table 2.3). The *E. coli* strains were maintained in a 50% glycerol suspension at -80°C. Prior to the assay, the stock cultures are transferred to Petri dish containing Luria Bertani (LB) medium (pH7) and

incubated for 17 hours at 37 °C on a model 1575 orbital shaking incubator (VWR Scientific, Cornelius, OR). Then Transfer one colony from each Petri dish to 250 ml flask with 100 ml sterilized LB broth and incubated for 17 hours at 300 rpm (OD= (600nm)=0.15 approximately 10^7 cell/ ml). To ensure stability of the plasmids containing *lux* genes, ampicillin (10 µl/ ml) was added in the growth media.

3.2.3 *E. coli* stress fingerprinting

Culture of each *E. coli* strain was diluted with sterile distilled water at ratio of 1: 10. Then, 10 µl of culture solution was transferred to 1.5 ml Eppendorf microtube. Luminescence from *E. coli* of each tube was measured by GloMax® 20/20 Single Tube Luminometer (Promega; Madison, WI, USA) and the luminescence values were presented as instrument's arbitrary relative light unit (RLU). RLU values were recorded for two set of samples, one group without adding acrylamide (control) and another group samples containing 5, 10, 20, 40, 80, 160, 320, 640, and 2560µg/ L acrylamide. The different and ratio of the two RLU values (" RLU without acrylamide" and "RLU with acrylamide") were both calculated to indicate the stress responses from the bioluminescent *E. coli* strains.

ΔRLU = "RLU with acrylamide" - "RLU without acrylamide"

RLU ratio = "RLU with acrylamide" / "RLU without acrylamide"

If RLU ratios are greater than one and ΔRLUs are bigger than zero (" light- on"), it indicates that the *lux* gene fusion is expressed because of the acrylamide. If RLU ratios are less than one and ΔRLUs are less than zero (" light- off"), it suggests a dampening of bioluminescent *E. coli* strains, which means that cells could not repair damages, in the presence of acrylamide.

3.2.4 Statistical Analysis

Data are presented as mean± the standard errors of the mean (S.E.M.). Statistical analysis was performed using Paired T-Test and ANOVA Tukey's Multiple Comparison test utilizing analyzing software SPSS version 20.

3.3 Results and Discussion

Results showed (Figure 3.1 and Figure 3.2) that for all strains, the highest significant difference with probability of 95% were observed at concentrations 40, 320, 1280 µg/L of acrylamide. The light emitting of both *grpE* and *recA* -*lux* fusion strains significantly increased at 5 µg/L concentration of acrylamide since ΔRLU values and RLU Ratios of them showed significant difference between 0 and 5 µg/L amount of acrylamide (P<0.05). For strains containing YciG and o513 promoters significant increase in ΔRLU values and RLU Ratios were observed at 40 µg/L concentration of acrylamide, but for *inaA*-*lux* fusion strain was observed at 20 µg/L concentration of acrylamide (P<0.05). ΔRLU and RLU Ratio of none of the strains showed significant differences between 1280 and 2560 µg/L concentrations of acrylamide (P>0.5). Among these five strains, ΔRLU values and RLU Ratios of *recA*-*lux* fusion strain at all concentrations of acrylamide showed significant differences with their adjacent concentrations (P<0.05). In addition, for *grpE*-*lux* fusion the significant differences were observed at all concentrations except 20 and 40 µg/L concentrations of acrylamide.

The strains containing recA, grpE, and yciG promoters yielded the high response ratios. The strain containing *recA*-*lux* fusion is in the SOS regulon and showed DNA damage in presence of acrylamide. Although, at a less extent compared

to *recA-lux*, the strains containing *yciG-lux, grpE-lux, inaA-lux, o512*3-*lux* fusion also, responded to acrylamide stress. Expression of the yciG gene is under control of σ^s. Thus, the *yciG-lux* fusion reported on the activation of the σ^s-dependent stress response. While, grpE is in the heat shock regulon controlled by σ^{32}, this response indicates that presence of acrylamide also induced the protein-damage responsive regulon. On the other hand, activation of inaA and o5113 promoters showed internal acidification and Super-stationary phase damage. Moreover, it was observed that by increasing amount of acrylamide light emitting of bioluminescent *E. coli* strains increased significantly and in whole range of examined acrylamide concentrations ΔRLU values are positive and RLU ratios are greater than one, which means that no cell dampening occurs up to adding 2560 μg/ L concentration of acrylamide.

In a previous study conducted by Van Dyk et al(2000), these five strains were used for detecting limited specific amount of other toxicants including H_20_2, Nalidixic acid, Ethanol, Sodium Salicylate, Methyl violgogen, dichlorophenoxyacetic acid, aluminum chloride, cadmium chloride, and aflatoxin B1. Comparing results of this study with those obtained by Van Dyk et al(2000), it was observed that in presence of Nalidixic acid and aflatoxin B1like acrylamide the highest RLU ratio was related to *recA-lux* fusion strain, but no significant RLU ratio was observed in presence of Nalidixic acid for other strains. In addition, in presence of H_20_2 *recA* and *grpE-lux* fusion strains showed high level of RLU ratios. Moreover, Ethanol, Methyl violgogen, and cadmium chloride could increase RLU ratio of *grpE-lux* fusion strain more than other ones, but other strains showed no response in presence of ethanol. On the other hand, i*naA-lux* fusion strain showed the highest response to Sodium

Salicylate, and dichlorophenoxyacetic acid and the second place belonged to *grpE-lux* fusion. For aluminum chloride, the highest response belonged to *yciG* and second level of RLU ratio was related to *grpE* (Van Dyk et al., 2000).

Moreover, another study, Fukushima research, showed that the highest RLU ratios in presence of 0.3 mg/ml ephedrine and pseudoephedrine (Two toxic compounds in traditional Chinese medicinal herb) were related to *recA-lux* fusion strain followed by *grpE-lux* fusion strain (Fukushima, 2004).

Therefore, recA and *grpE-lux* fusion strains showed the high response to most toxicants, which contains acrylamide. As a result, it could be concluded that the DNA and protein damage are the major types of damages which induced by many toxicants.

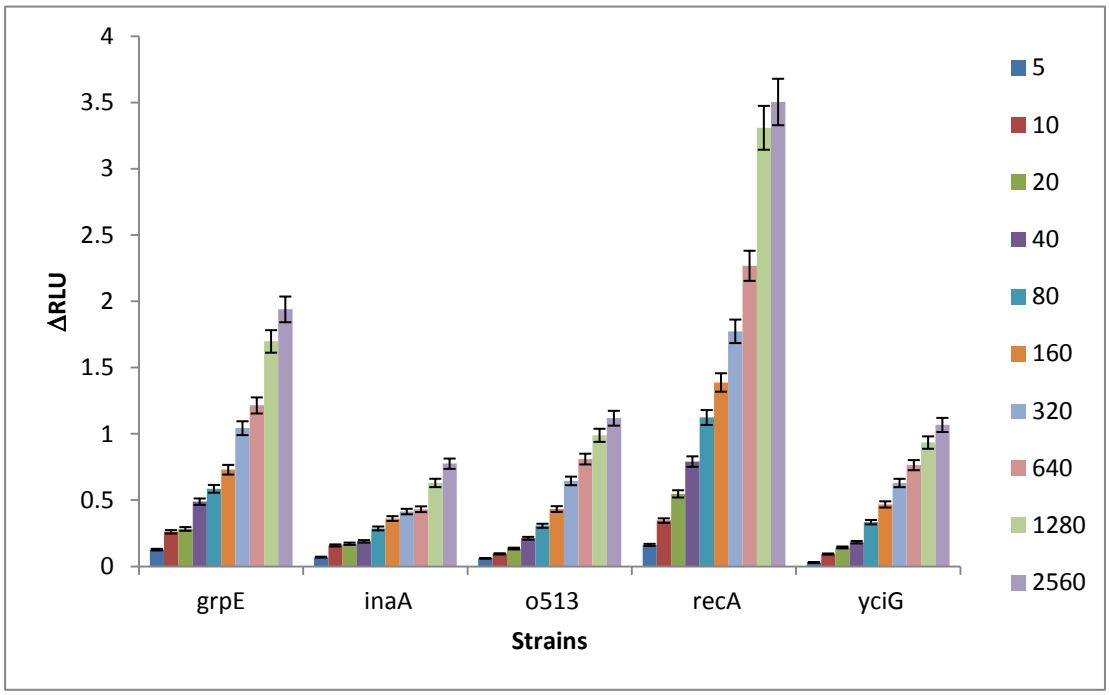

Figure 3.1 ΔRLU of five strains when different amount of acrylamide (µg/L) was added. Shown are mean ± S.E.M. (n=3).

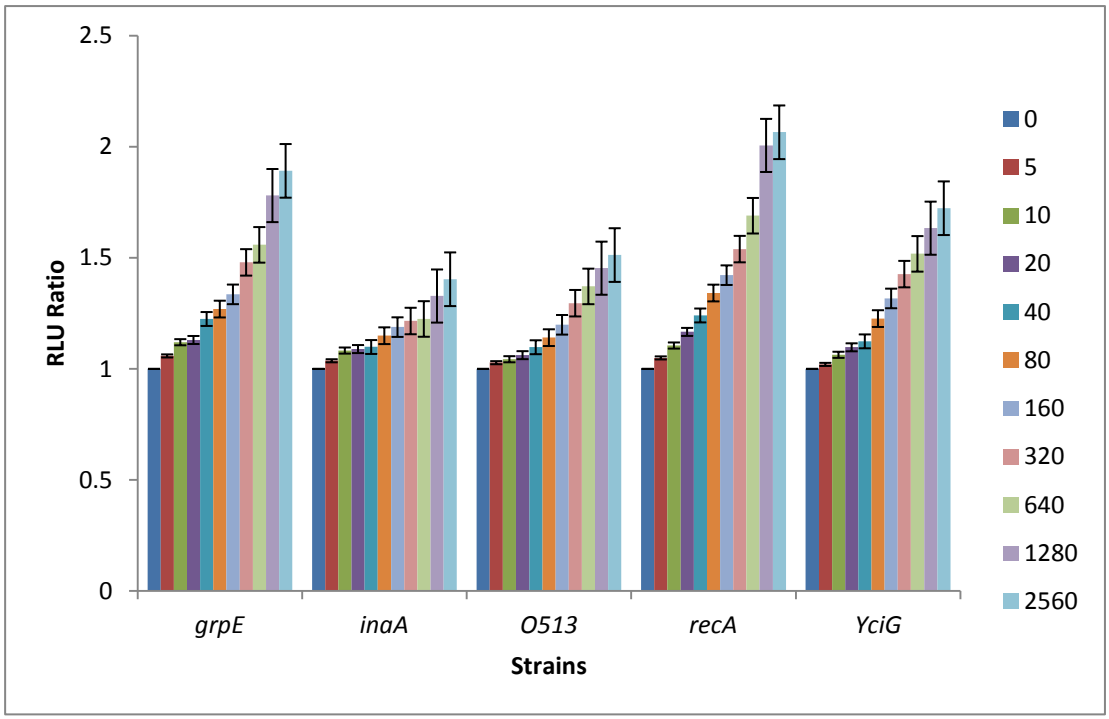

Figure 3.2 RLU ratio of five strains when different amount of acrylamide (µg/L) was added. Shown are mean ± S.E.M. (n=3).

3.4 Conclusion

No previous study had used these five genetically engineered strains of luminous *E. coli* to detect acrylamide. The approach presented for the first time in this research, has four unique attributes. First, luminous *E. coli* panel was capable to detect low amount of acrylamide, since acrylamide concentrations below 50µg/L were detected by *yciG-lux*, *inaA-lux*, and *o513-lux* fusion strains and even as low level as 5 µg/L acrylamide concentration was detected by *recA-lux* and *grpE-lux* fusion strains. Second, wide range of toxicant could be detected by this panel since light emitting of these five strains were measured in presence of acrylamide concentrations from 5 to 2560 µg/L. Third, this study revealed that light emitting of these five luminous *E. coli* increased accordingly by increasing amounts of

acrylamide. Fourth, the highest light emitting was related to the strains containing recA and grpE promoters and hence the most serious damages of bacterial cells caused by acrylamide might be DNA and protein damages. Therefore, based on highest and most distinguished light emitting of *recA-lux* and *grpE-lux* fusion strains in presence of acrylamide, these two strains will be the subjects of research in next chapters.

Chapter 4: Assessment of internal and external factors' effects on stress response of recA -lux fusion and grpE- lux fusion strains of bioluminescent *E. coli*

4.1 Introduction

4.1.1 The *recA*- *lux* fusion strain

For detection of unknown DNA damaging agents and for evaluation of the related DNA damage potency, a biological response SOS response, which may occur in various bacteria, is exploited to develop a novel type of biosensing element to achieve this purpose. SOS response occurs in prokaryotes, when their cells suffering from substantial DNA damages. During normal growth, cells undergo little DNA damage and SOS genes are negatively regulated by LexA repressor protein. The LexA protein binds to a consensus sequence (the SOS box) in the promoter region for those SOS genes. The DNA replication will be blocked at DNA damage sites when DNA damage occurs. Consequently, large amounts of single strand DNA will appear which needs more RecA protein to bind to them. The resulted RecA-ssDNA filaments present the activated form RecA protein that interacts with the LexA repressor to assist the LexA repressor's self-cleavage from the SOS promoters (Nelson and Cox, 2005; Friedbergm et al., 2006). At the preliminary stage of SOS response, RecA protein can increase its amount significantly for several times. Since the amount of

RecA protein is closely related with the activity of *recA* promoter, *recA* promoter can be a wise choice for the development of an effective biosensor for detection of DNA damage. The reporter Lux proteins under the control of *recA* promoter can manifest the expression of RecA protein in the *lux*-fusion strain. Since the luminescence of *lux*, system can easily be tested by a luminometer, the luminescent intensity can representative the activity of *recA* promoter, further displaying the level of SOS response of cells treated by chemicals. Therefore, if we identified the range of luminescence produces by a specific toxic chemical such as acrylamide, we can use the *recA*- *lux* fusion strain as a biosensing element to detect that toxic chemical. Furthermore, the results of objective1of this study showed that among all five *luminous E. coli* strains the highest ΔRLU values and RLU ratios were related to *recA*- *lux* fusion strain.

Therefore, this strain can be a good candidate to work with in order to develop a novel biosensing element for detection acrylamide in food. Moreover, to get optimal response, it is necessary to optimize the condition for this strain. Thus, in this part of study effects of internal and external parameters (Dose of acrylamide, Time, Temperature, Light, number of cells per ml, Cell age) on ΔRLU value and RLU ratio of *recA*- *lux* fusion strain were assessed in presence of acrylamide.

4.2.2 The *grpE-lux* fusion strain

A subset of proteins in *E. coli*, induced by heat shock also is induced by various starvation conditions and by the presence of chemical pollutants (Blom et al., 1992; Jenkins et al., 1991; VanBogelen et al., 1987). The induction of the heat shock response, typically observed at subinhibitory concentrations, is thought to be a critical

mechanism for survival in the presence of environmental insults. This common induction profile by various stresses suggests that monitoring the heat shock response may be a sensitive method for detecting environmental stress. Simplistic detection of transcriptional activation is achieved by fusion of promoter elements to reporter genes. A particularly useful reporter system is made up of the structural genes, *luxCDABE,* of the bioluminescence operon derived from the marine bacterium (Meighen, 1991; Stewart and Williams, 1992). As mentioned before, rapid response, excellent sensitivity, large dynamic range, and noninvasive continuous measurements amenable to automated data collection with minimal manipulations are advantageous of the bioluminescence reporter. Requirements for light production from the proteins encoded by these reporter genes include expression in actively metabolizing cells at temperatures below 30°C (Boylan et al., 1985; Hill et al., 1993). Two main heat shock promoters of *Escherichia* are *dnaK* and *grpE*. By considering the fact that light production from the *dnaK* fusion was much lower than that from the *grpE* fusion (Van Dyk et al., 1994), in this study, *grpE- lux* fusion strain of *E. coli*, was used, which also can be a good candidate to use as a novel biosensing element for detection of acrylamide in food. Therefore, the same as what was done for *recA- lux* fusion strain, we need to optimize the condition for this strain in order to get the optimum response. Thus, in this section, effects of internal and external parameters (Dose of acrylamide, Time, Temperature, Light, Number of cells per ml, Cell age) on ΔRLU value and RLU ratio of *grpE- lux* fusion strain were evaluated in presence of acrylamide .

4.2 Materials & Methods

4.2.1 Regent and Chemicals

Acrylamide (for electrophoresis, ≥99% (HPLC), powder, SIGMA- ALDRICH), Ampicillin (Ready Made Solution, 100 mg/ mL, 0.2 μm filtered, SIGMA-ALDRICH), Luria- Bertani Broth (LB broth, Lennox (Granulated), FISHER SCINTIIFIC).

4.2.2 Bioluminescent *E. coli* strains

Preparation of culture for measuring relative light unit of strains was adopted from Fukushima (2004) and modified in this study.

Two bioluminescent *E. coli*, *recA lux-fusion* and *grpE lux-fusion*, strains obtained from DuPont Genetics Lab (DuPont Company, Wilmington, DE) are used in this part of study. *RecA lux-fusion* and *grpE lux-fusion*, which respectively contained DNA damage and protein damage stress-responsive promoter fusion to the Photorhabdus luminescence *luxCDABE* reporter (Table 2.3). The *E. coli* strains were maintained in a 50% glycerol suspension at -80 °C. Prior to the assay, the stock cultures are transferred to Petri dish containing Luria Bertani (LB) medium and incubated for 17 hours at 37 °C on a model 1575 orbital shaking incubator (VWR Scientific, Cornelius, OR). Then Transfer one colony from each Petri dish to 250 ml flask with 100 ml sterilized LB broth and incubated for 17 hours at 300 rpm (OD= (600nm)=0.15 approximately 10^7 cell/ ml). To ensure stability of the plasmids containing *lux* genes, ampicillin (10 µl/ ml) was added in the growth media.

4.2.3 Luminous *E. coli* stress response

4.2.3.1 Acrylamide concentrations effects

To assess the effect of acrylamide concentrations, culture of each *E. coli* culture was diluted with sterile distilled water at ratio of 1: 10. Then, 10 µl of culture solution was transferred to 1.5 ml Eppendorf microtube. Luminescence from *E. coli* of each tube was measured by GloMax® 20/20 Single Tube Luminometer (Promega; Madison, WI, USA) and the luminescence values were presented as instrument's arbitrary relative light unit (RLU). RLU values were recorded for two sets of samples, one group without adding acrylamide (control) and another group samples containing different concentrations of acrylamide. These concentrations were selected based on the dose-response results, which were obtained in proceeding study as reported in chapter 3 of this document. Finally, ΔRLU values and RLU ratios of samples were measured.

4.2.3.2 *E. coli* cell number effects

To detect wider range of acrylamide concentrations, different dilutions of the strain culture were used. Then, ΔRLU values and RLU ratios were measured after adding different amount of acrylamide to the 0-fold, 10-fold, and 100-fold dilutions of *E. coli* culture with deionized water (approximate number of cells are respectively 10^7, 10^6, 10^5 CFU/ml).

4.2.3.3 Time effects

To assess the effect of time, 1000 µg/L of acrylamide was added to the strain culture. After 5, 20, 40, 60, 80, 100, 120, 240, and 1440 min (24 hours), ΔRLU values and RLU ratios of the strain were measured.

4.2.3.4 Cell age effects

To assess the effect of cell age and figure out how long the strain culture can effectively response to acrylamide. Acrylamide (1000 µg/L) was added to the *E. coli* culture, which had been kept in refrigerator for 1, 2, 3, 7, 14, 21, and 28 days.

4.2.3.5 Light effects

Acrylamide is sensitive chemical to light and its solution is kept in dark bottles. Therefore, to make sure that during preparing samples light had no effect on results of the experiments, RLU values and RLU ratios of culture containing1000 µg/L were measured after keeping samples in three different conditions: dark room, light room (under bio cabinet), and directly put under lamp (with 10 cm distance to lamp).

4.2.3.6 Temperature effects

To assess the effect of temperature ΔRLU values and RLU ratios of *recA-lux* fusion strain with 1000 µg/L acrylamide, which was kept 24 hours in refrigerator, room temperature, and 37 ºC were measured. Based on the importance of acrylamide effects on cells at 37 ºC, which is body temperature, the effects of time and acrylamide concentrations at this temperature were assessed. This assessment is also unique and there is no reference to compare with.

4.2.3.6.1 Acrylamide concentrations effects at body temperature

ΔRLU values and RLU ratios of *recA-lux* fusion strain were measured when 1, 10, 100, 1000, 10,000, 100,000, 1000,000, 10,000,000 and 100,000,000 µg/L concentrations' of acrylamide were added to the culture and kept for 24 hours.

4.2.3.6.2 Time effects at body temperature

To assess the effect of time at body temperature (37 ºC), 100 and 1000 µg/ L of acrylamide was added to *recA lux-fusion* strain. After 5, 20, 40, 60, 80, 100, 120, 240, and 1440 min (24hours) leaving at 37 ºC, ΔRLU values and RLU ratios of the strain were measured.

4.2.4 Statistical Analysis

Data are presented as mean± the standard errors of the mean (S.E.M.). Statistical analysis was performed using Paired T-Test and ANOVA Tukey's Multiple Comparison test utilizing analyzing software SPSS version 20.

4.3 Results & Discussion

4.3.1 The *recA-lux* fusion strain

4.3.1.1 Acrylamide concentrations effects

Results of adding different amount of acrylamide to ten-fold dilution (with deionized water) of the *E. coli* culture (approximate cell number=10^6 CFU/ml) showed in presence of 1 µg/L acrylamide, light emitting of the strain was too low. Moreover, significant increase in ΔRLU values and RLU ratios were observed between 10 to 1000 µg/L of acrylamide 10,000 µg/L concentration of acrylamide light emitting decreased (ΔRLU was negative and RLU ratio < 1), which showed cell dampening (Figure 4.1 and Figure 4.2). Therefore, in order to measure light emitting lower than 10 and higher than 1000 µg/L of acrylamide amount, it could be useful to try different dilution of the strain broth.

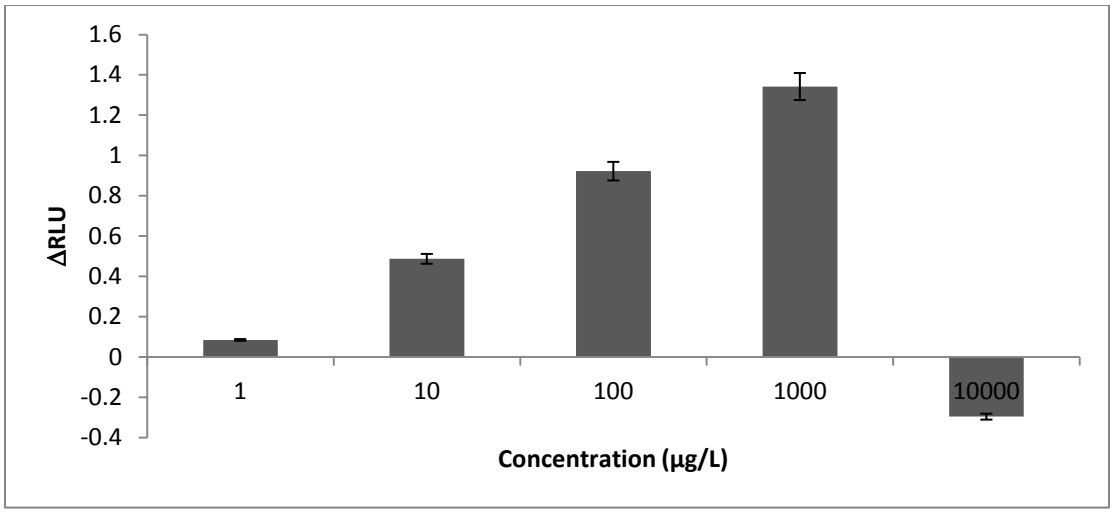

Figure 4.1 ΔRLU of *recA-lux* fusion strain after adding different amount of acrylamide (µg/L) to 10-fold dilution of the culture. Shown are mean± S.E.M. (n=3).

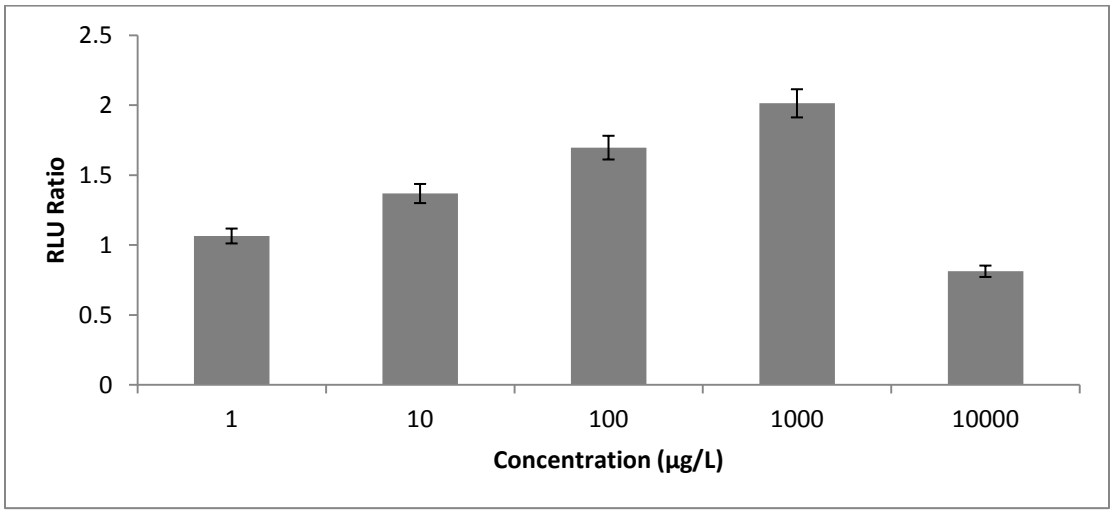

Figure 4.2 RLU ratio of *recA-lux* fusion strain after adding different amount of acrylamide (µg/L) to 10-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

4.3.1.2 *E. coli* cell number effects

To detect lower amount of acrylamide different concentrations of acrylamide (1, 10, 100 µg/L) were added to 100-fold dilution of the strain culture and ΔRLU values and RLU ratios of the stain were measured. By increasing the dilution, 10 times the number of bacteria decreased 10 times, so each cell got a chance to be affected by greater amount of acrylamide. Therefore, even by adding as low level as 1 µg/L acrylamide significant light emitting was observed (Figure 4.3 and Figure 4.4). On the other hand, because of greater effects of acrylamide on each cell at this dilution, cell dampening occurred at the presence of lower level of acrylamide (1000 µg/L) compare to previous part of the experiment in which bacterial broth culture with less dilution and higher number of cells was used .

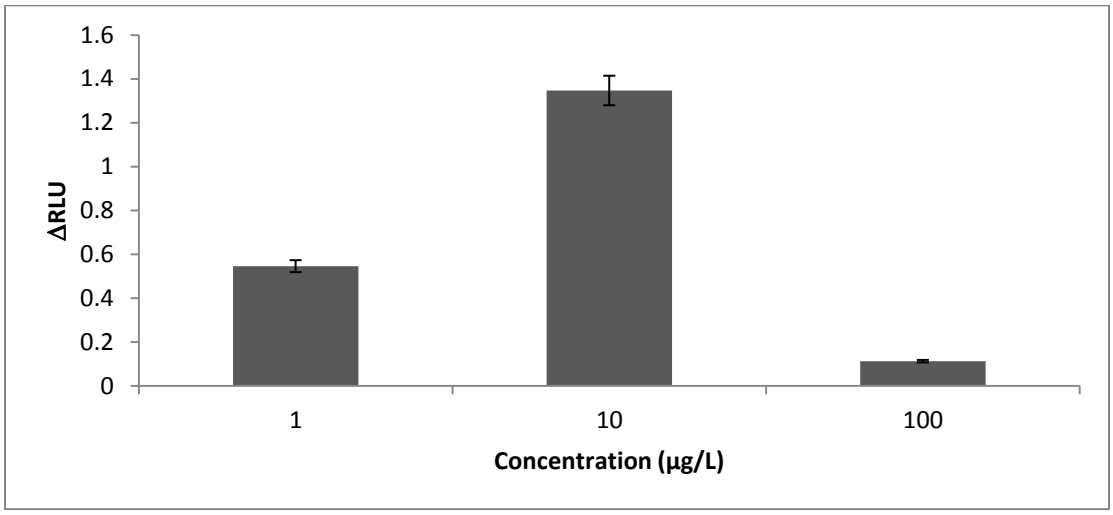

Figure 4.3 ΔRLU of *recA-lux* fusion *strain* after adding different amount of acrylamide (µg/L) to 100-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

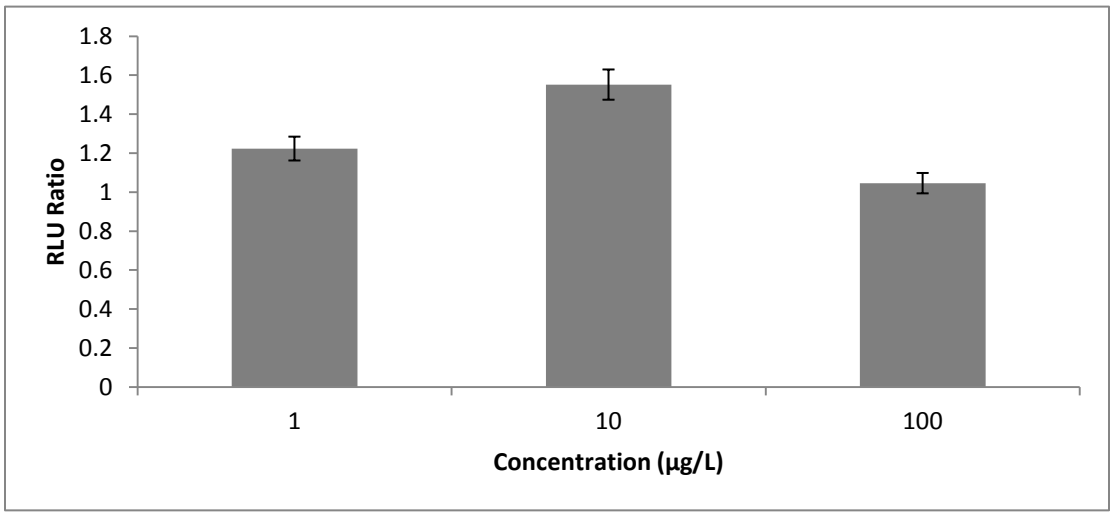

Figure 4.4 RLU ratio of *recA-lux* fusion strain after adding different amount of acrylamide (µg/L) to 100-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

In order to detect higher level of acrylamide, different amount of acrylamide were added to Origin bacterial broth culture without any dilution. Then, ΔRLU and RLU ratio of the strain were measured. Therefore, the number of cells affected by acrylamide increase so less sensitivity to acrylamide concentration, which led to not observing cell dampening up to 10,0000 µg/L amount of acrylamide (Figure 4.5 and Figure 4.6).

Previous studies about the application of radiation in food industries, also showed that by increasing the number of cells, the given radiation dose would be less effective (Jay et al., 2005).

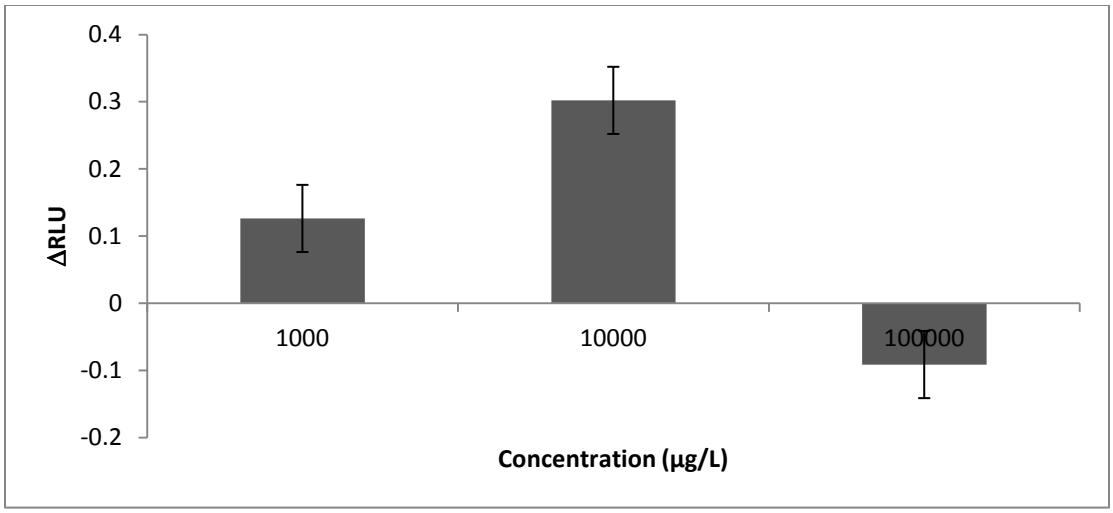

Figure 4.5 ΔRLU of *recA-lux* fusion strain after adding different amount of acrylamide (µg/L) to 0-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

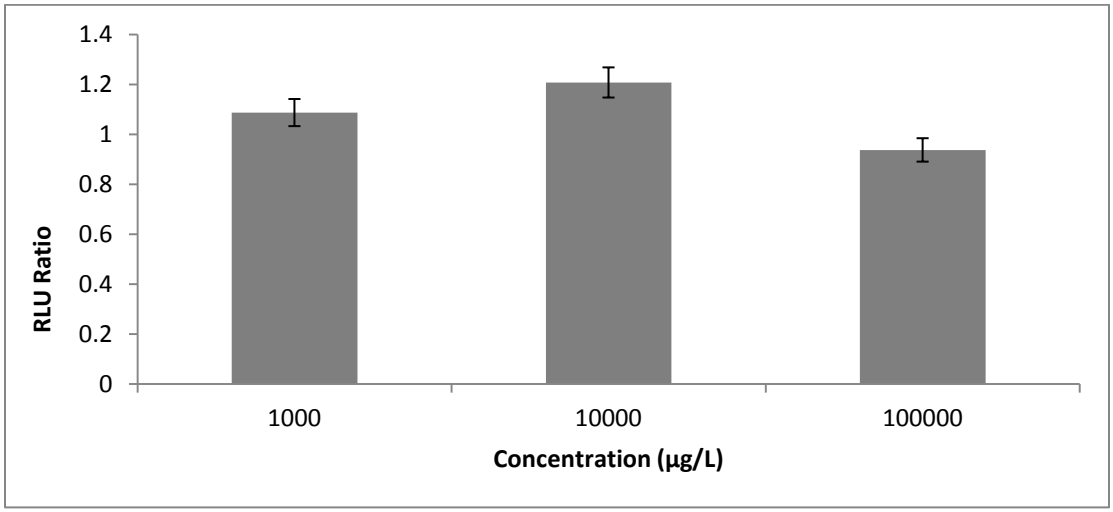

Figure 4.6 RLU ratio of *recA-lux* fusion strain after adding different amount of acrylamide (µg/L) to 0-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

4.3.1.3 Time effects

To assess the effect of time, ΔRlU values and RlU ratios of the strain were measured 5, 20,40, 60, 100, 120, 240, 1440 min after adding 1000 µg/L of acrylamide to the strain culture. As seen in (Figure 4.7 and Figure 4.8), up

to 40 min no significant change was observed between light emitting of the strain before and after adding acrylamide and also no significant difference was observed between ΔRLU values and RlU ratios. Therefore, it took about 40 min for acrylamide to cause damage on this strain. Then, by passing time from 40^{th} to 100^{th} min after adding acrylamide to bacterial broth culture, ΔRLU values and RLU ratios of the strain increased. After 100 min, ΔRLU values and RLU ratios decreased. Thus, from the moment of observing damage (passing 40 min), took 60 min to see the highest level of light emitting. No significant differences were observed between light emitting of the strain before and after adding acrylamide at 240^{th} and 14400^{th} min. In addition, there was not any significant difference between ΔRLU values and RlU ratios after passing 120 min (P>0.05). These changes could be related to the fact that when cells faced to a toxic and DNA damage occurred, it took time for cells to start repair mechanism (activation of *recA*), so at the beginning light emitting was low. It took about 40 min for acrylamide to cause damage on this strain since no significant changes were observed between ΔRLU values and RLU ratios until 40^{th} min. After that, by proceeding repair mechanism and elevating the activation of recA fusion to *lux* gene, light emitting increased. It took 60 min for the strain to reach the highest level of ΔRLU values and RLU ratios, which means the highest level of recA activation.

These results can confirm the previous studies, which mentioned that it took about 60 min for recA promoter to reach its maximum activation (Nickoloff and Hoekstra, 1998; Tang et al., 1999; Volshin et al., 2001).

After 60 min, most parts of damage were repaired, so activation of recA as a DNA damage stress responsive promoter decreased and because of the fact that recA in this strain fused to lux gene, light emitting began to reduce. Finally, after 120^{th} min that no significant differences were observed between light emitting before and after adding acrylamide and almost all damages were repaired. Therefore, it took about 140 min for this strain to repair DNA damage.

This research is unique about the effect of time on *recA*- *lux*-fusion strain in presence of acrylamide. A previous research in which *recA*- *lux* fusion strain was exposed to X-rays showed that the bulk of the DNA repair occurred within the first 15 min, while by 120 min after exposure to damaging agent, all of repair was essentially completed. However, some cells showed no repair during this period of time (Singh et al. 1988).

In another research *recA*-*lux* fusion strain responded within 2 hours to the presence of genotoxicants such as phenol (Belkin et al., 1997).

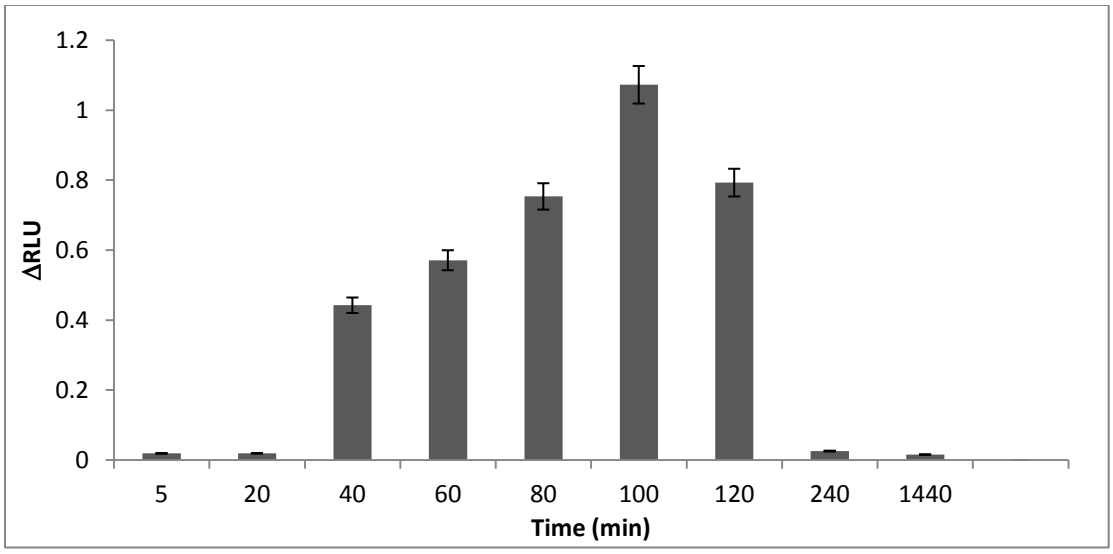

Figure 4.7 ΔRLU of *recA-lux* fusion strain 5, 20, 40, 60, 100, 120, 240, 1440 min after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

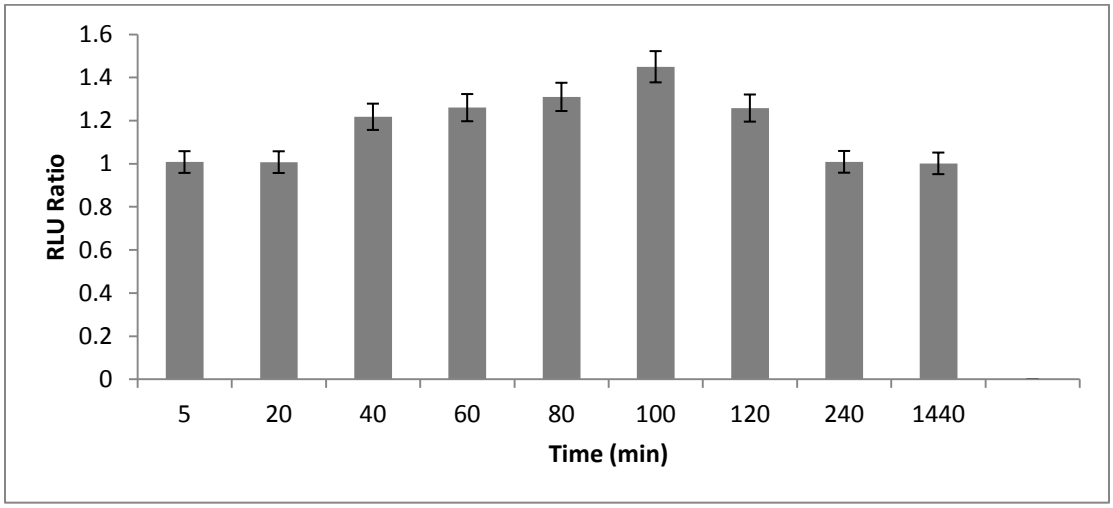

Figure 4.8 RLU ratio of *recA-lux* fusion strain 5, 20, 40, 60, 100, 120, 240, 1440 min after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

4.3.1.4 Cell age effects

To detect the effect of cell age, 1000 µg/L of acrylamide was added to the bacterial strain cultures, which were stored in refrigerator for 1, 2, 3, 7, 14, 21, 28 days. As seen in figures 4.9 and 4.10 no significant difference in ΔRLU values and

RLU ratios of *recA*- *lux* fusion strain was observed up to 14th (P>0.05). After day 14th, light emitting of the strain decreased. Therefore, this bacterial broth culture could be used efficiently up to two weeks, which is a novel approach about the cell age of genetically engineered strain culture to detect toxicants.

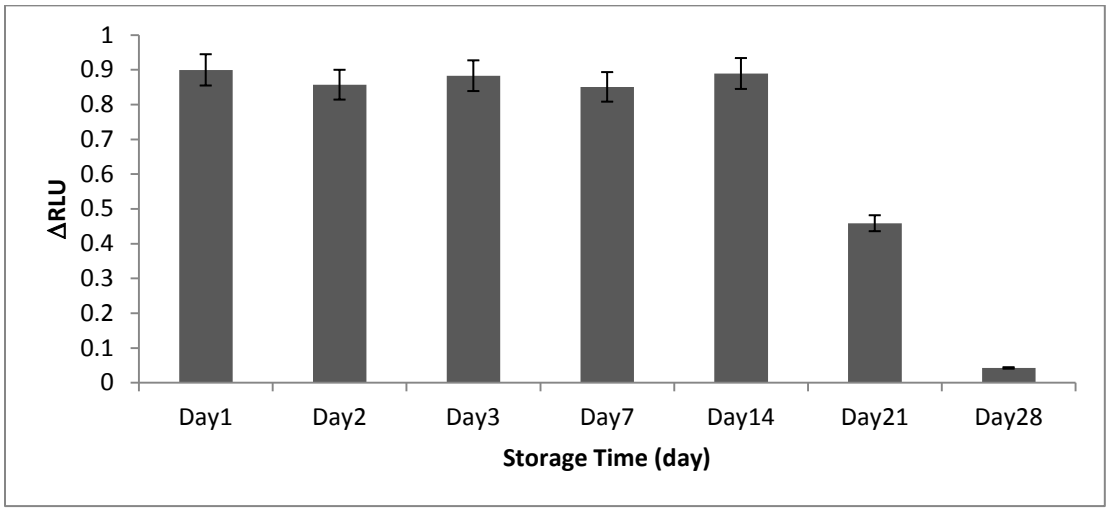

Figure 4.9 ΔRLU of *recA-lux* fusion strain after adding 1000 µg/L acrylamide to the culture, which was stored for different periods. Shown are mean ± S.E.M. (n=3).

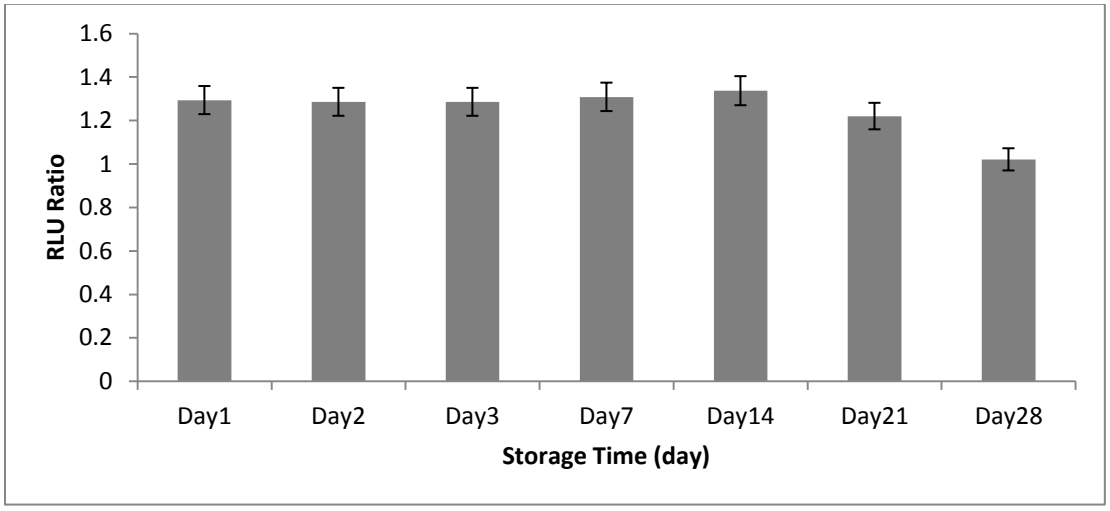

Figure 4.10 RLU ratio of *recA-lux* fusion strain after adding 1000 µg/L acrylamide to the culture, which was stored for different periods. Shown are mean ± S.E.M. (n=3).

4.3.1.5 Light effects

To figure out the effect of light on light emitting of *recA-lux* fusion strain in presence of acrylamide, ΔRLU values and RLU ratios of culture containing1000 µg/L were measured after keeping in 3 different conditions: dark room, light room (under bio cabinet), and directly put under lamp (with 10cm distance to lamp). As seen in Figures 4.11 and 4.12 and statistical calculation showed in presence of acrylamide, light did not have significant effect on ΔRLU value and RLU ratio of *recA- lux* fusion strain of *E. coli* when comparing to ΔRLU value and RLU ratio of this strain at dark condition (P>0.05). Only under, direct light with close distance to sample, ΔRLU value was too low, which might be related to deactivation of acrylamide. Therefore, in natural microbial lab condition (under bio cabinet) light had no effect on results.

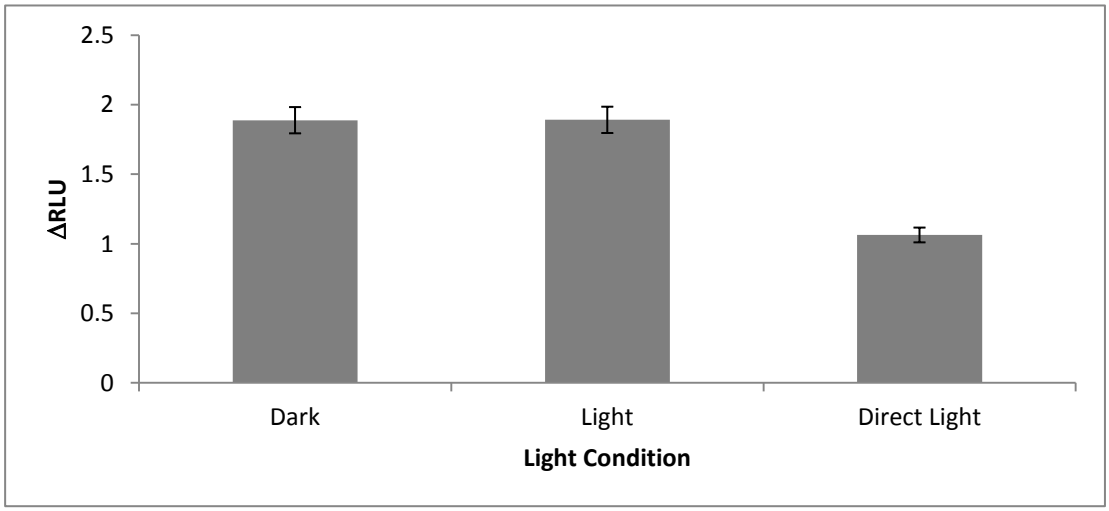

Figure 4.11 ΔRLU of *recA-lux* fusion strain in different light condition after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

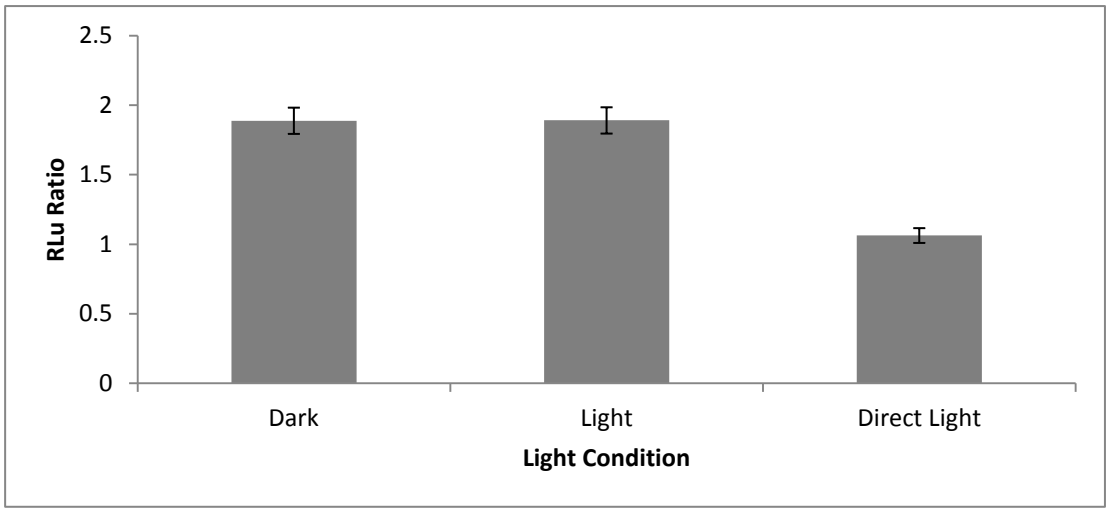

Figure 4.12 RLU ratio of *recA-lux* fusion strain in different light condition after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

4.2.3.6 Temperature effects

To assess the effect of temperature on light emitting of *recA-lux* fusion strain in presence of acrylamide, ΔRLU values and RLU ratios of culture containing1000 µg/L were measured after keeping 24 hours in 3 different temperature conditions: Refrigerator (about 4 °C), Room (about 21 °C), and Body Temperature (37 °C). As seen in figures 4.13 and 4.14 no significant increase was observed in ΔRLU value and RLU ratio after keeping the culture at room temperature in presence of acrylamide (P>0.05). Therefore, after 24 hours *recA-lux* fusion strain could repair DNA damage caused by 1000 µg/L, which confirmed the previous results of this research (4.3.1.3). In contrast, by keeping the culture in refrigerator, as results showed ΔRLU value and RLU ratio decreased significantly (P<0.05). This might be related to the fact that non-spore-forming bacteria are more sensitive to toxicant at temperature below or above their growth temperature range since they have to spend most of their energy to survive (*E. coli* growth temperature range is between 5 to 45 °C). Moreover, results

showed more decrease in ΔRLU value and RLU ratio at 37 °C compared to room temperature. This might be related to the fact that a number of organisms can do recovery better when incubated at temperature below the optimum growth temperature (Jay et al., 2005). There is no research available about the effect of body temperature on DNA damage caused by acrylamide. However, the reason of the more severe effect of acrylamide on cells at 37 °C compared to ambient temperature might be related to longer lag phase at room temperature, compared to 37 °C (the optimum temperature for *E. coli*) since Lag phase is the most resistant phase to toxicants in bacterial growth curve. In addition, results showed that ΔRLU values and RLU ratios of both refrigerator and body temperatures are negative so they could have dampening effect on cells. Since 37 ºC is the temperature of human body, it is important to figure out its effects on cells in presence of acrylamide. Therefore, in following parts it was focused more on the effects of acrylamide on cells at 37 ºC. The effect of toxicants on light emitting luminous *E. coli* at 37 ºC was performed for the first time in this research and no similar research is available in this field.

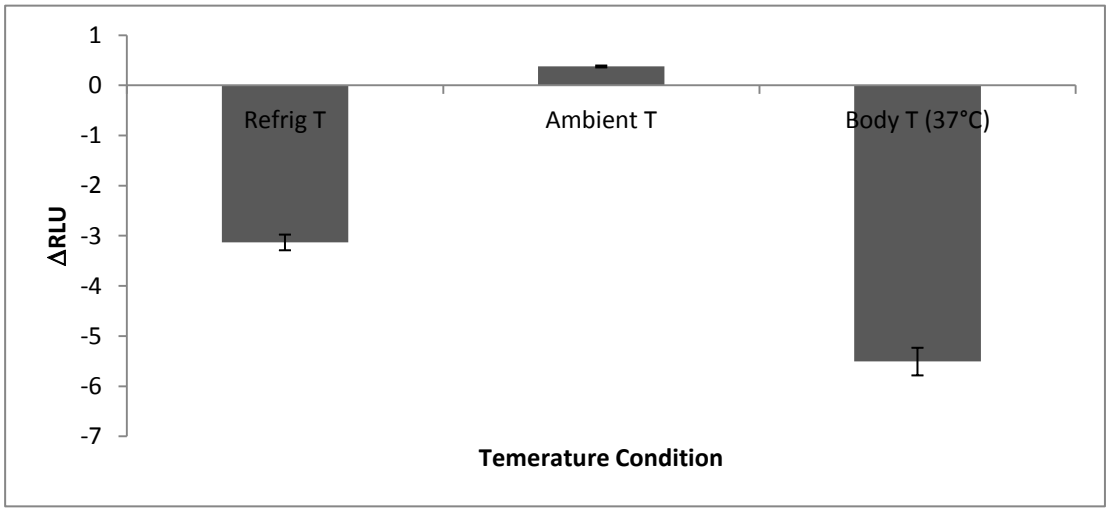

Figure 4.13 ΔRLU of *recA-lux* fusion strain after adding 1000 µg/L acrylamide and 24 hours keeping in different temperature. Shown are mean ± S.E.M. (n=3).

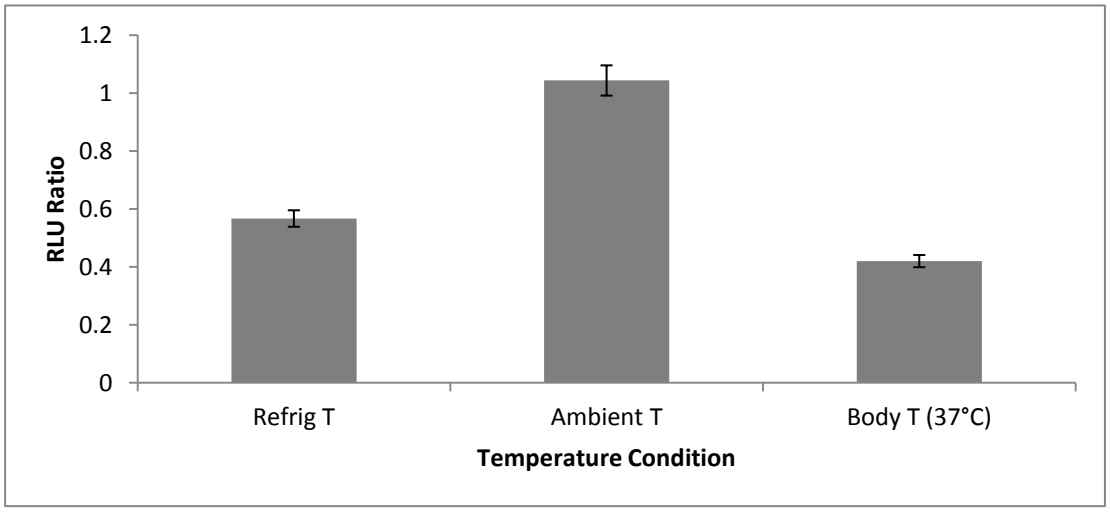

Figure 4.14 RLU ratio of *recA-lux* fusion strain after adding 1000 µg/L acrylamide and 24 hours keeping in different temperature. Shown are mean ± S.E.M. (n=3).

4.3.1.6.1 Acrylamide concentrations effects at body temperature

To assess the effect of acrylamide concentration at 37 ºC on *recA-lux* fusion strain, ΔRLU values and RlU ratios of the strain were measured at concentration of

acrylamide between 1 and 1000 µg/L after keeping 100 min at 37 ºC. As seen in figures 4.15 and 4.16, by increasing amount of acrylamide (1-100 µg/L), both ΔRLU values and RlU ratios increased the same as what was observed at ambient temperature. By increasing the concentration of acrylamide, significant increase in light emitting of the strain (P<0.05) was detected because of the elevation in recA promoter activation in order to repair DNA damage. As results showed, in contrast of room temperature at 37 ºC, in presence of 1000 µg/L concentration of acrylamide, ΔRLU of the strain is negative and RLU ratio is less than one, which showed cell dampening. Therefore, at 37 ºC cell dampening occurred at lower concentration compared to ambient temperature (1000 µg/L compared to 10,000µg/L). This might be related to longer lag phase at temperature below the optimum temperature of bacteria and the highest resistant of bacteria to toxicant in their lag phase (Jay et al., 2005).

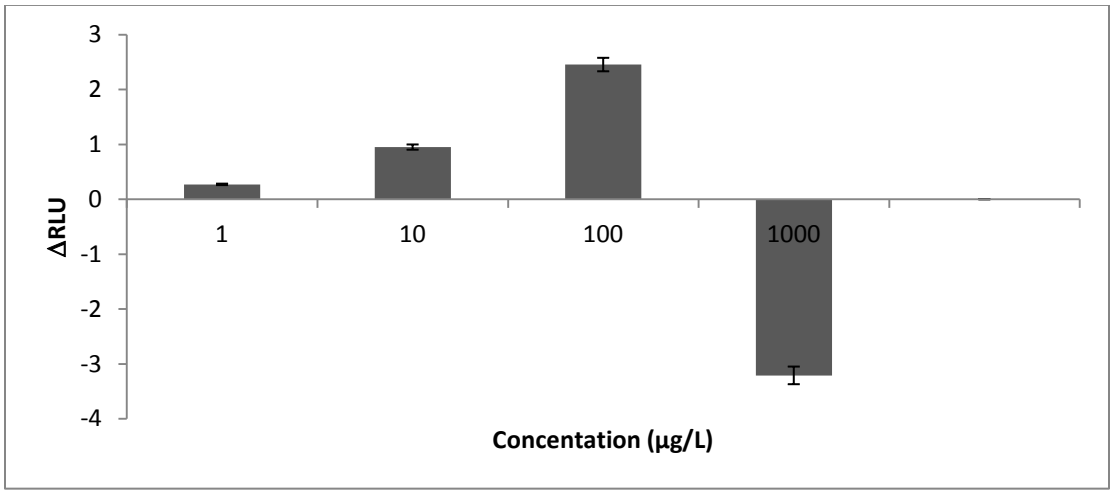

Figure 4.15 ΔRLU of *recA-lux* fusion *strain* after adding 1, 10, 100, and 1000 µg/L acrylamide and keeping 100 min at 37 ºC. Shown are mean ± S.E.M. (n=3).

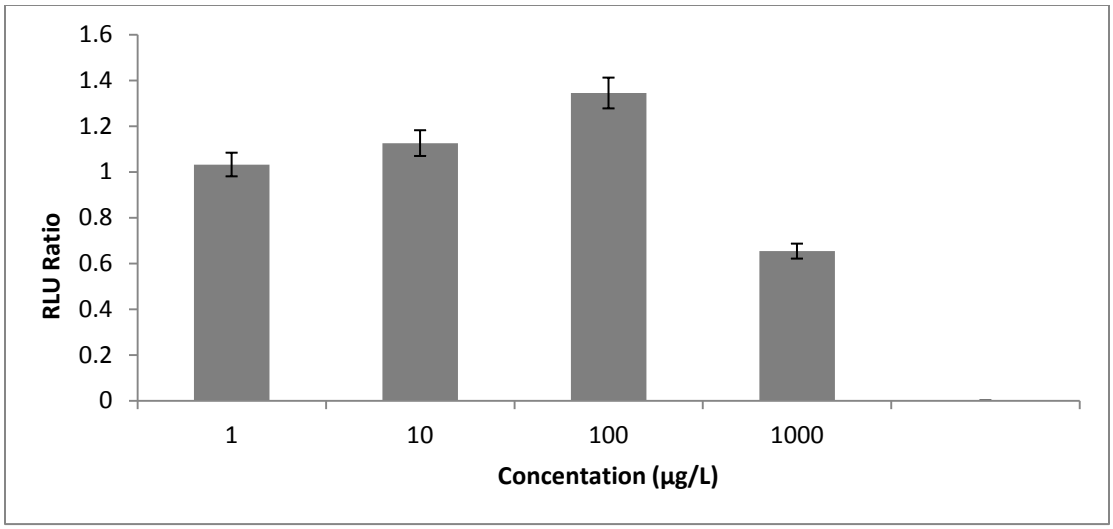

Figure 4.16 RLU ratios of *recA-lux* fusion *strain* after adding 1, 10, 100, and 1000 µg/L acrylamide and keeping 100 min at 37 ºC. Shown are mean ± S.E.M. (n=3).

In order to see the effect of higher amount of acrylamide on *recA-lux* fusion strain at 37 ºC, ΔRLU values and RLU ratios of the strain were measured between 10,000 and 100,000,000 µg/L concentrations of acrylamide after keeping 100 min at 37 ºC. As seen in figures 4.17 and 4.18 at all concentrations ΔRLU values were negative and RLU ratios were below one. In addition, no significant difference was observed between these concentrations (P>0.05). Therefore, at all of these high concentrations of acrylamide cell dampening occurred, which means that recA promoters did not activate because cells could not repair DNA damage.

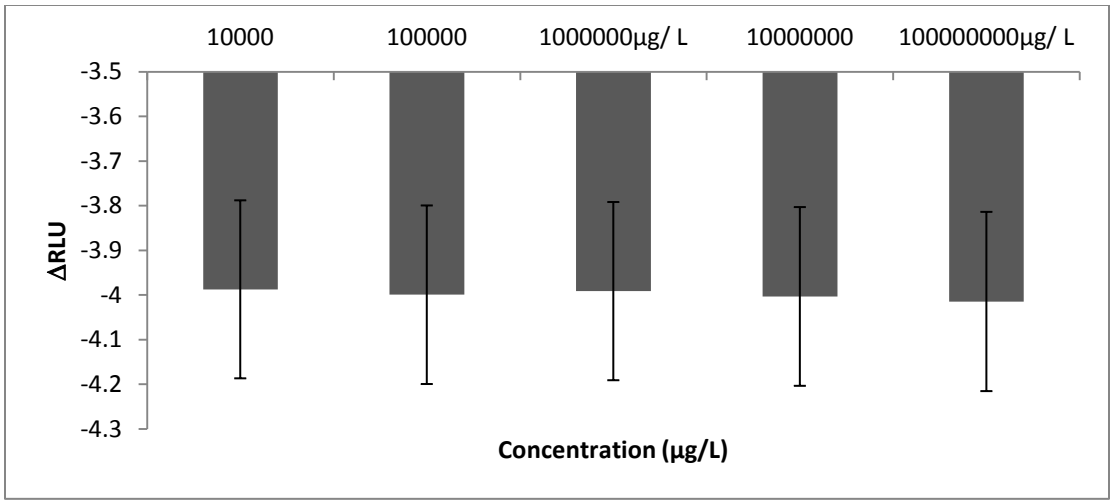

Figure 4.17 ΔRLU of *recA-lux* fusion strain after adding 10,000, 100,000, 1000,000, 10,000,000, and 100,000,000 µg/L acrylamide and keeping 100 min at 37 ºC. Shown are mean ± S.E.M. (n=3).

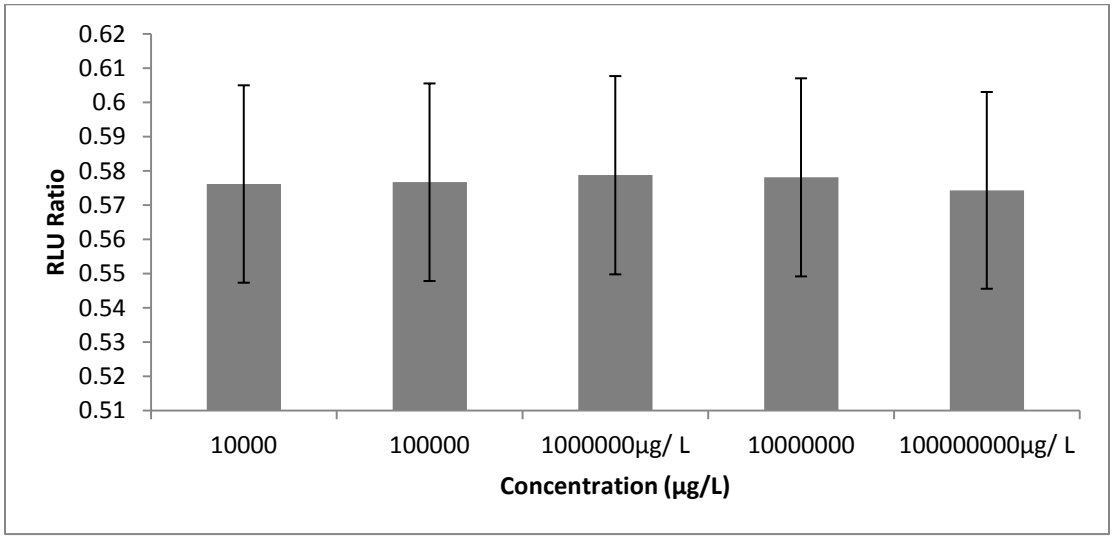

Figure 4.18 RLU ratios of *recA-lux* fusion strain after adding 10,000, 100,000, 1000,000, 10,000,000, and 100,000,000 µg/L acrylamide and keeping 100 min at 37 ºC. Shown are mean ± S.E.M. (n=3).

4.3.1.6.2 Time effects at body temperature

To assess the effect of time on *recA-lux* fusion strain at 37 ºC in presence of 100 µg/L of acrylamide concentration, ΔRLU values and RLU ratios of the strain were measured after keeping culture for different period of time (5, 20, 40, 60, 80, 100, 120, 240, and 1440 min). As seen in figures 4.19 and 4.20, no significant difference were observed between light emitting of the strain before and after adding acrylamide values until 20^{th} min (P>0.05). From 40^{th} min to 100^{th} min, both ΔRLU values and RLU ratios were increased. The maximum values could be observed at 100^{th} min and after that, they were decreased up to 300^{th} min. Therefore, it took about 40 min for 100 µg/L acrylamide to cause damage on *recA-lux* fusion strain at 37 ºC. The maximum activation of *recA* was observed at 100^{th}, which was the same as the results of previous studies, which mentioned that it took about 60 min for recA promoter to reach the highest level of activation (Nickoloff and Hoekstra,1998; Tang et al., 1999;Volshin et al., 2001). No significant difference was observed between 300^{th} min and 1440^{th} min. In addition, no significant difference was observed between light emitting of the strain before and after adding acrylamide at 300^{th} and 1440^{th} (P>0.05). Thus, it took about 200 min for this strain to complete the DNA damage. These results were similar to the results of time effect at ambient temperature in presence of 1000 µg/L. Therefore, the effects of time on *recA-lux* fusion strain at 37 ºC below 100 µg/L of acrylamide concentration was almost the same as its effects on this strain below 1000 µg/L at ambient temperature. The only difference between the effect of time at these two conditions was related to the longer time of completing repair at 37 ºC compared to ambient temperature (200 min compared to 140 min),

since some bacteria could recover injured cells better if incubated at temperature below their optimum temperature (Jay et al., 2005).

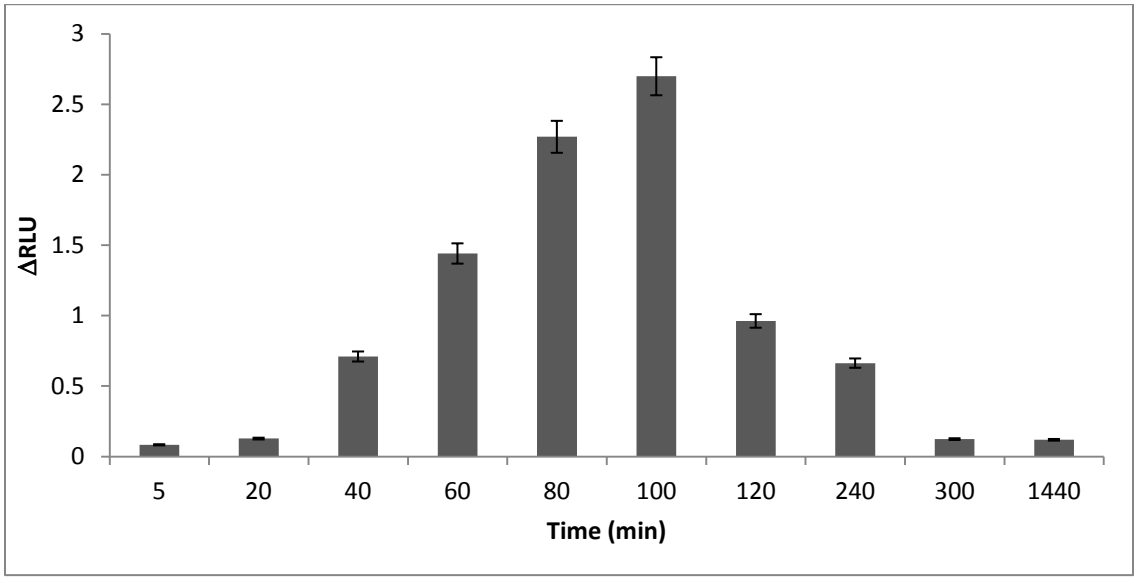

Figure 4.19 ΔRLU of *recA-lux* fusion strain after adding100µg/L acrylamide and keeping 5, 20, 40, 60, 80, 100, 120, 240, and 1440 min at 37 ºC. . Shown are mean ± S.E.M. (n=3).

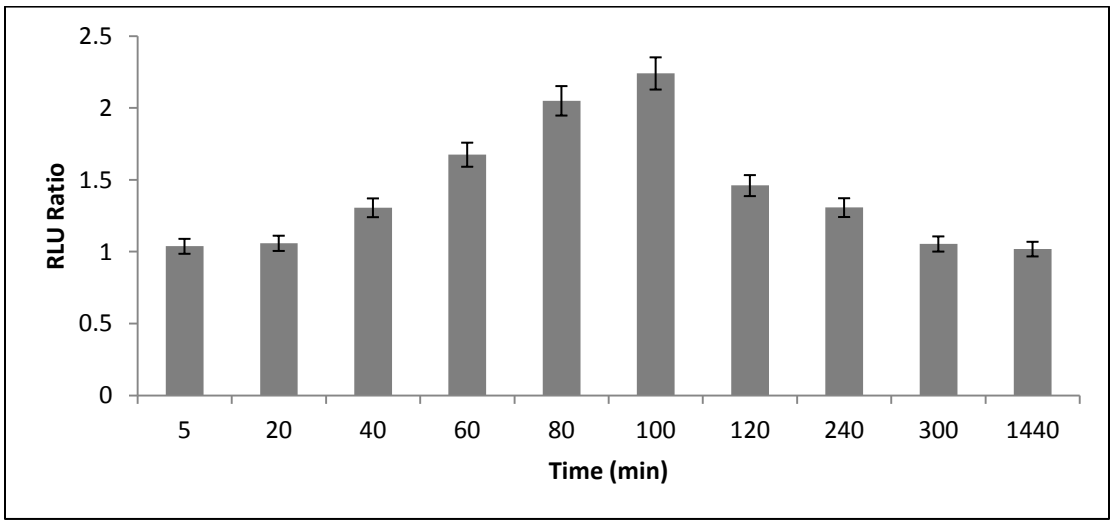

Figure 4.20 RLU ratio of *recA-lux* fusion strain after adding100 µg/L acrylamide and keeping 5, 20, 40, 60, 80, 100, 120, 240, and 1440 min at 37 ºC. . Shown are mean ± S.E.M. (n=3).

To assess the effect of time at 37 ºC in presence of the concentration of acrylamide at which cell dampening was observed (1000µg/L), ΔRLU values and RLU ratios of the strain were measured after keeping the culture for different period time in presence of 1000µg/L of acrylamide concentration. As seen in figures 4.21 and 4.22, no significant difference were observed between light emitting of *recA-lux* fusion before and after adding acrylamide up to 40th min. After t40th min, the values increased up to 80th min. Then, from 100th min to 1440th ΔRLU values were negative and RLU ratios were below one. No significant difference was observed between values from 100th min to 1440th. Therefore, it takes about 40 min for acrylamide to complete the damage. In addition, the maximum activation of recA promoter was observed at 80th min and the cell dampening occurred at 100th. Thus, before 100th cell tried to repair the damage, but after that damage might be more severe than the ability of cell to repair it.

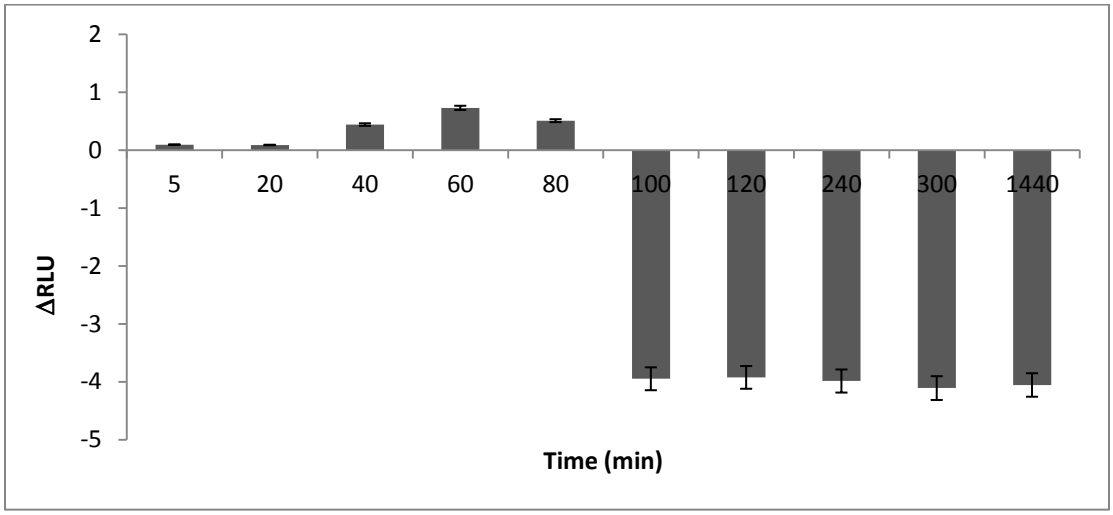

Figure 4.21 ΔRLU of *recA-lux* fusion strain after adding1000 µg/L acrylamide and keeping 5, 20, 40, 60, 80, 100, 120, 240, 300 and 1440 min at 37 ºC. Shown are mean ± S.E.M. (n=3).

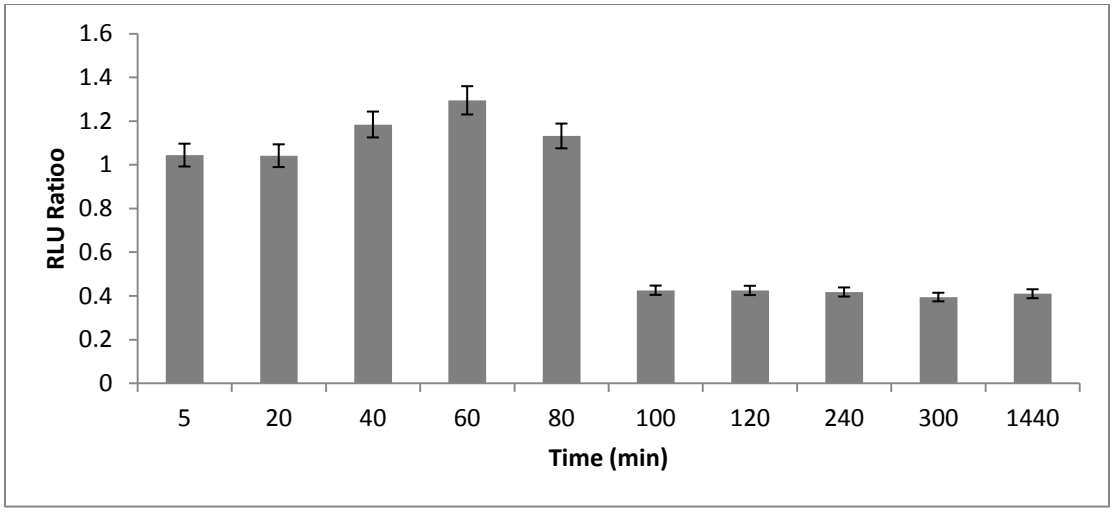

Figure 4.22 RLU ratio of *recA-lux* fusion strain after adding1000µg/L acrylamide and keeping 5, 20, 40, 60, 80, 100, 120, 240, 300 and 1440 min at 37 ºC. Shown are mean ± S.E.M. (n=3).

4.3.2 The *grpE- lux* fusion strain

4.3.2.1 Acrylamide concentrations effects

To detect the effect of acrylamide concentration on *grpE-lux* fusion strain different amount of acrylamide were added to ten-fold dilution (with deionized water) of the *E. coli* culture (approximate cell number= 10^6 CFU/ml). Results show that in presence of 1 µg/L acrylamide, light emitting of the strain was too low and between 10 to 1000 µg/L of acrylamide significant increase in ΔRLU values and RLU ratios was observed. In contrast, at 10,000 µg/L concentration of acrylamide light emitting decreased (ΔRLU was negative and RLU ratio <1), which showed cell dampening (Figure. 4.23 and Figure. 4.24). Therefore, in order to measure light emitting lower than 10 and higher than 1000 µg/ L concentration of acrylamide, it can be useful to try different dilution of the strain broth culture. The results of acrylamide concentration effects on *grpE-lux* fusion strain were the same as those for *recA-lux* fusion strain.

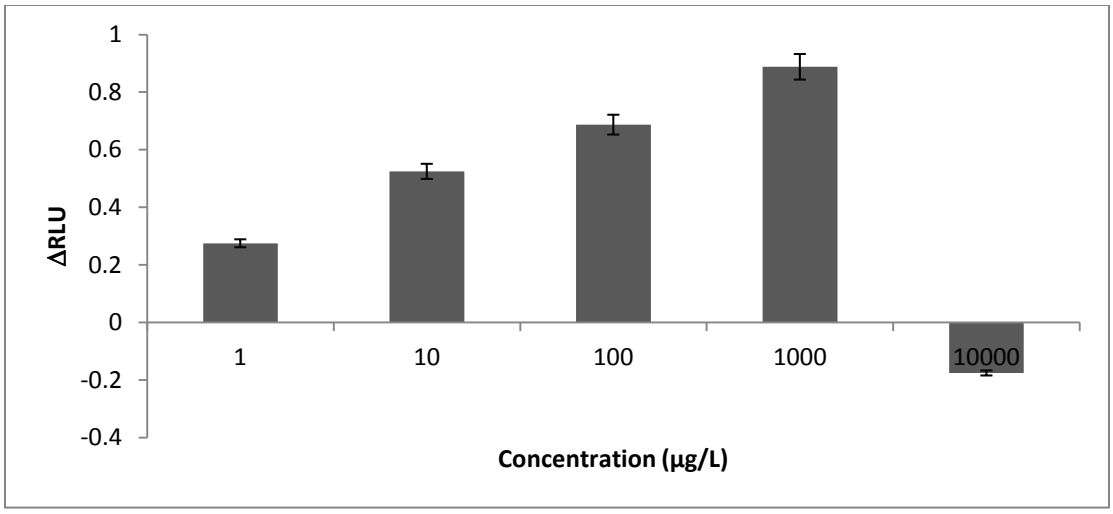

Figure 4.23 ΔRLU of *grpE-lux* fusion strain after adding different amount of acrylamide (µg/L) to 10-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

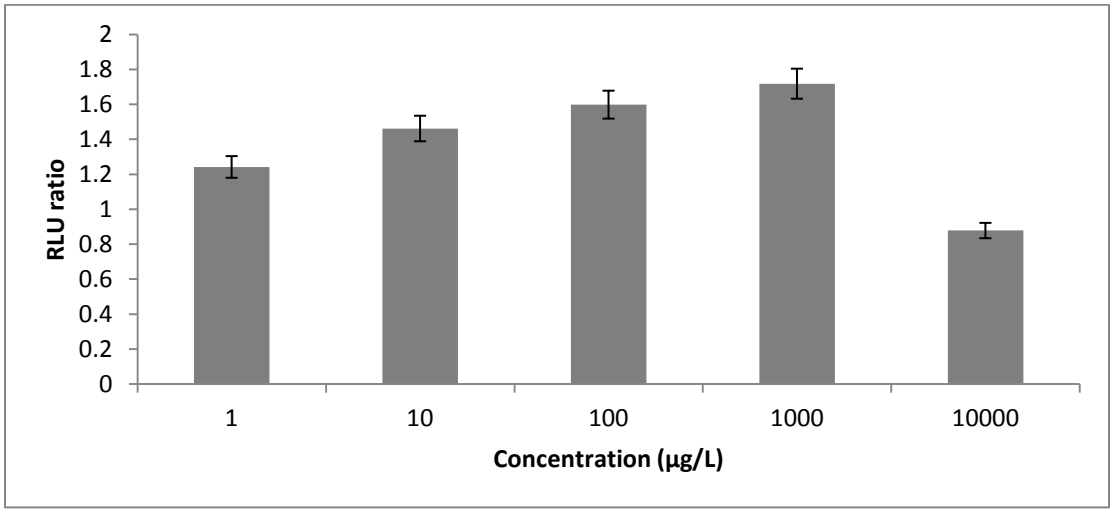

Figure 4.24 RLU ratio of *grpE-lux* fusion strain after adding different amount of acrylamide (µg/L) to 10-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

4.3.2.2 *E. coli* cell number effects

In order to detect lower amount of acrylamide different concentrations of acrylamide (1, 10, 100 µg/L) were added to 100-fold dilution of the strain culture and ΔRLU and RLU of the stain were measured. By increasing the dilution, 10 times the number of bacteria decreased 10 times, so each cell got a chance to be affected by greater amount of acrylamide. Therefore, even by adding as low level as 1 µg/L acrylamide significant light emitting was observed (Figure. 4.25 and Figure 4.26). On the other hand, because of the greater effect of acrylamide on each cell at this dilution, cell dampening occurred at the presence of lower level of acrylamide (1000 µg/L) compare to previous part of the experiment in which less diluted bacterial broth was used with higher number of cells. These results are similar to the results of cell number effects on light emitting of *recA-lux* fusion strain in presence of 1, 10, 100 µg/L of acrylamide concentration.

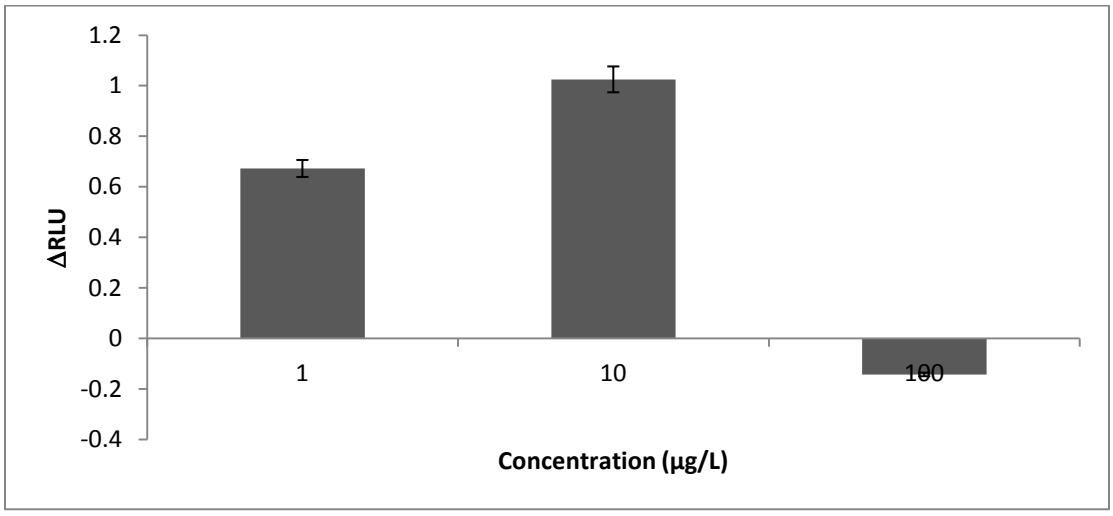

Figure 4.25 ΔRLU of *grpE-lux* fusion strain after adding different amount of acrylamide (µg/L) to 100-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

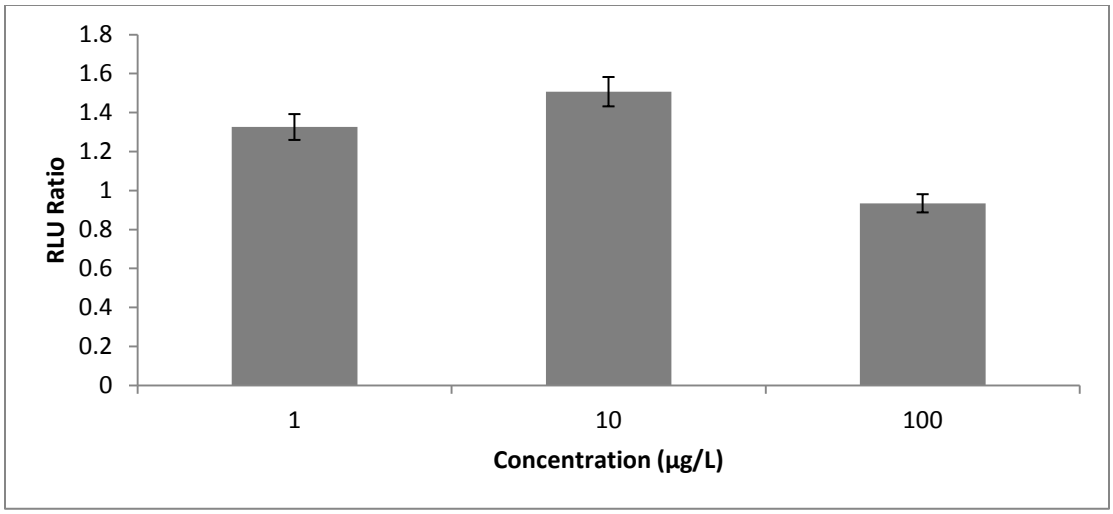

Figure 4.26 RLU ratio of *grpE-lux* fusion strain after adding different amount of acrylamide (µg/L) to 100-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

To detect higher level of acrylamide, different amount of acrylamide were added to origin bacterial broth culture without any dilution. Then, ΔRLU values and RLU ratio of the strain were measured. Therefore, the number of cells affected by acrylamide increased so less sensitivity to acrylamide concentration, which led to not observing cell dampening up to 100,000 µg/L amount of acrylamide (Figure 4.27 and Figure 4.28). Previous research on radiation application in food industries, also showed that the given radiation dose was less effective for the larger number of cells (Jay et al., 2005). These results are similar to the results of cell number effects on light emitting of *recA-lux* fusion strain in presence of 1000, 10,000, and 100,000 µg/L amount of acrylamide.

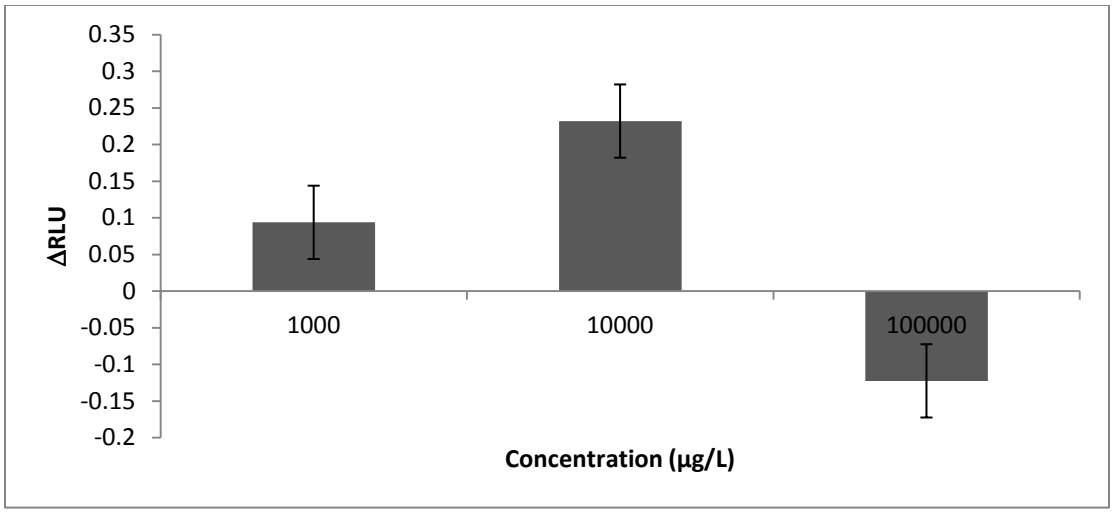

Figure 4.27 ΔRLU of *grpE-lux* fusion strain after adding different amount of acrylamide (µg/L) to 0-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

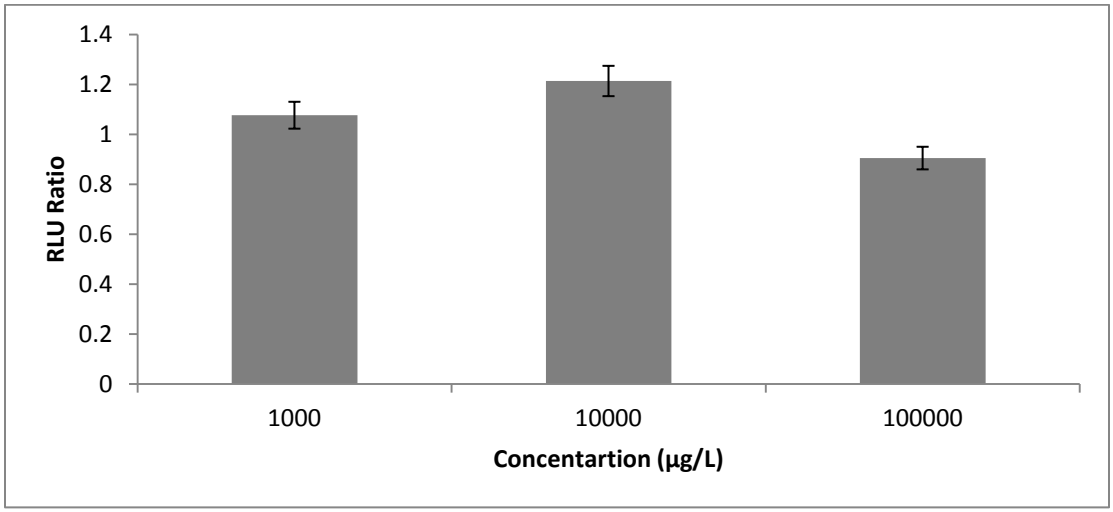

Figure 4.28 RLU ratio of grpE-*lux* fusion strain after adding different amount of acrylamide (µg/L) to 0-fold dilution of the culture. Shown are mean ± S.E.M. (n=3).

4.3.2.3 Time effects

To detect the effect of time ΔRlU values and RlU ratios of the strain were measured after passing different min from the moment of adding 1000 µg/L of acrylamide to the strain culture. As seen in Figures 4.29 and 4.30, until passing 20 min no significant change was observed between light emitting of the strain before and after adding acrylamide and no significant difference was observed between ΔRlU values and RlU ratios. Therefore, it took about 20 min for acrylamide to cause damage on this strain. Then, by passing time from 20^{th} to 80^{th} min after adding acrylamide to bacterial broth culture, ΔRLU values and RLU ratios of the strain increased, but after 100^{th} min began to decrease. In addition, there was no significant difference was observed between values at 80^{th} min and 100^{th} min ($P>0.05$). Therefore, from the moment of observing damage (passing 20 min), took 60 min to see the highest level of light emitting. It was observed that grpE promoter stayed at the highest level of activation about 20 min. No significant differences were observed between light emitting of the strain before and after adding acrylamide at 240^{th} and 1440^{th} min. Moreover, there was not any significant difference between ΔRlU and RlU ratio values after passing 240 min.

The reason of these changes could be related to the fact that when cell faced to toxic and protein damage occurred, it took time for cell to start repair mechanism (activation of *grpE*), so at the beginning light emitting was low. It took about 20 min for acrylamide to cause damage on this strain since no significant changes can be observed between ΔRLU values and RLU ratios

unite 20th min. After that, by proceeding repair mechanism and higher activation of *grpE* fusion to *lux* gene, light emitting increased. It took about 60 min for this strain to reach the highest level of ΔRLU value and RLU ratio, which means the highest level of *grpE* activation, took about 60 min. Then, most parts of damages were repaired, so activation of *grpE* as a protein damage stress responsive promoter decreased and because of the fact that grpE in this strain fusion to *lux* gene, so light emitting began to reduce. Finally, after 120th min that no significant differences was detected between light emitting before and after adding acrylamide, almost all damages repaired. Therefore, it took about 140 min for this strain to repair protein damage. This research is unique about the effect of time on *grpE-lux* fusion strain in presence of toxicants. These results are similar to the results of time effects on *recA-lux* fusion strain in presence of acrylamide except the time length of damage occurrence. Time length of occurring protein damage was less than DNA damage (20 min compared to 40 min), which lead to reach the maximum level of activation of the promoter earlier (at 80th min for *grpE* compared to 100th min for *recA*).

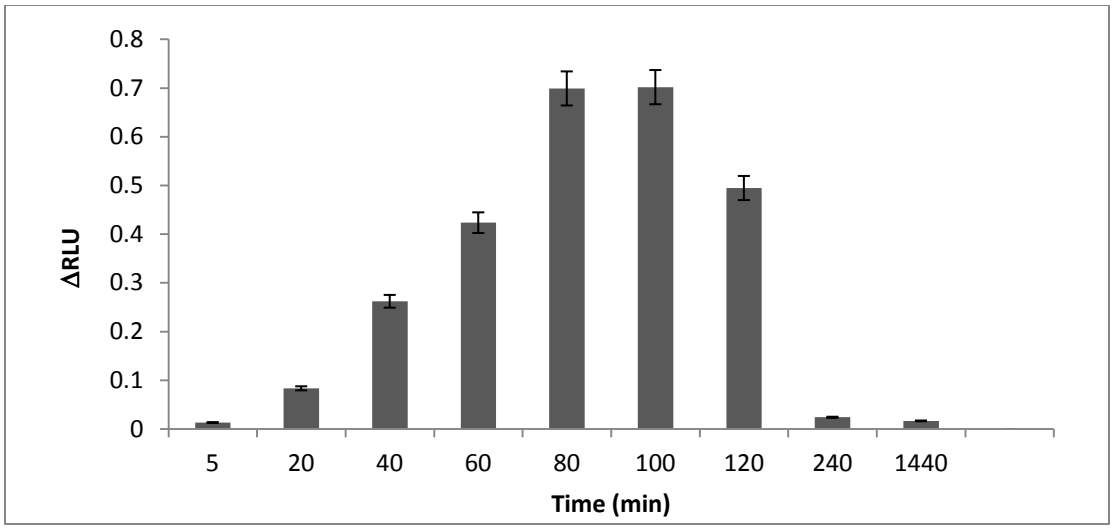

Figure 4.29 ΔRLU of *grpE-lux* fusion strain 5, 20, 40, 60, 100, 120, 240, 1440 min after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

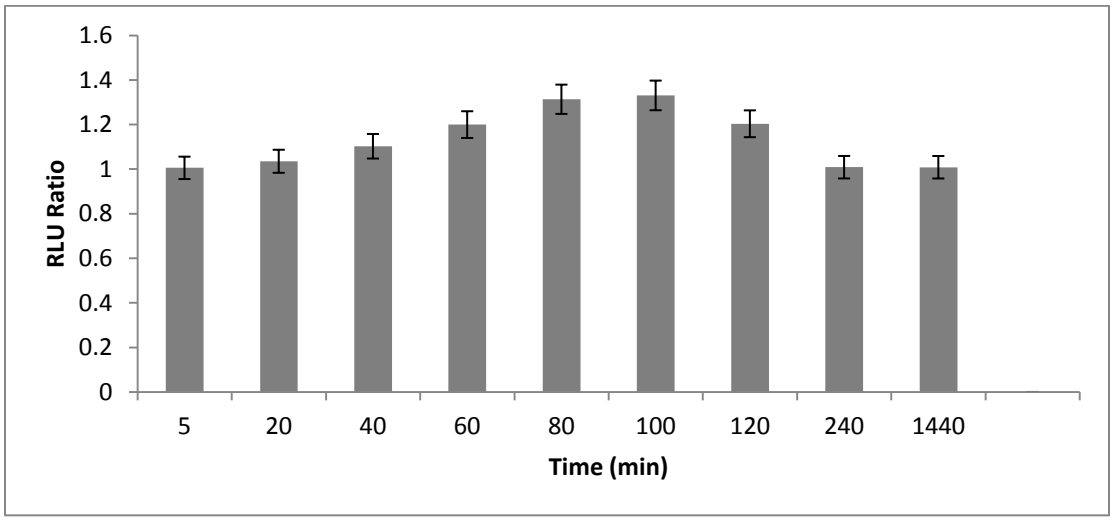

Figure 4.30 RLU ratio of *grpE-lux* fusion strain 5, 20, 40, 60, 100, 120, 240, 1440 min after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

4.3.2.4 Cell age effects

In order to assess the effect of cell age, 1000 µg/L of acrylamide was added to the strain cultures, which were kept for different period of time in refrigrator. As seen in figures 4.31 and 4.32 no significant difference in ΔRLU values and RLU ratios of *recA*- *lux* fusion strain up to day 14th was observed (P>0.05) and only after that the values showed decrease. Therefore, this bacterial broth culture could be used efficiently up to 2 weeks exactly the same efficiency which was observed for *recA*-*lux* fusion strain. This also can be a great advantage of this biosensor for its future application in industrial field

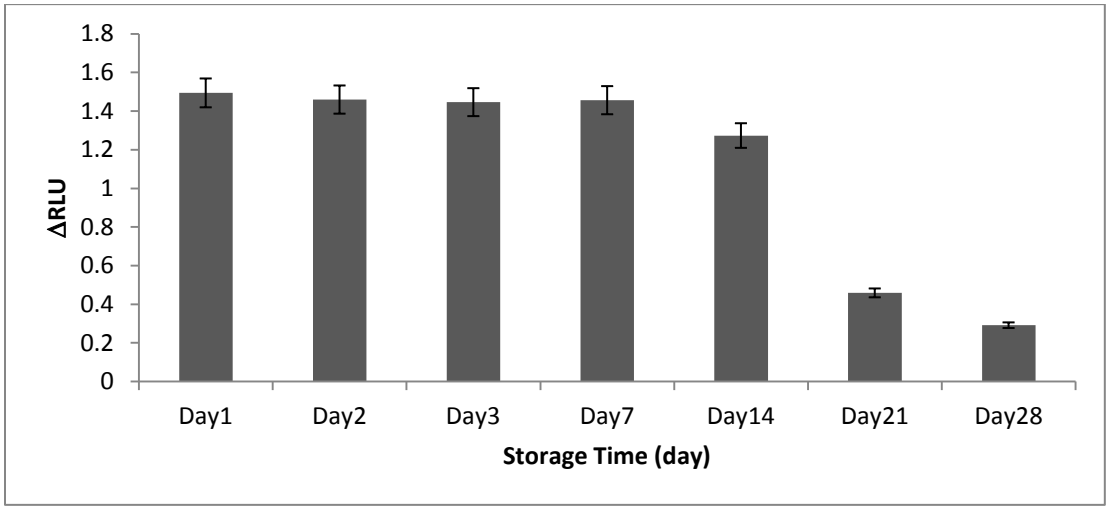

Figure 4.31 ΔRLU of *grpE-lux* fusion strain after adding 1000 µg/L acrylamide to the culture, which was stored for different period. Shown are mean ± S.E.M. (n=3).

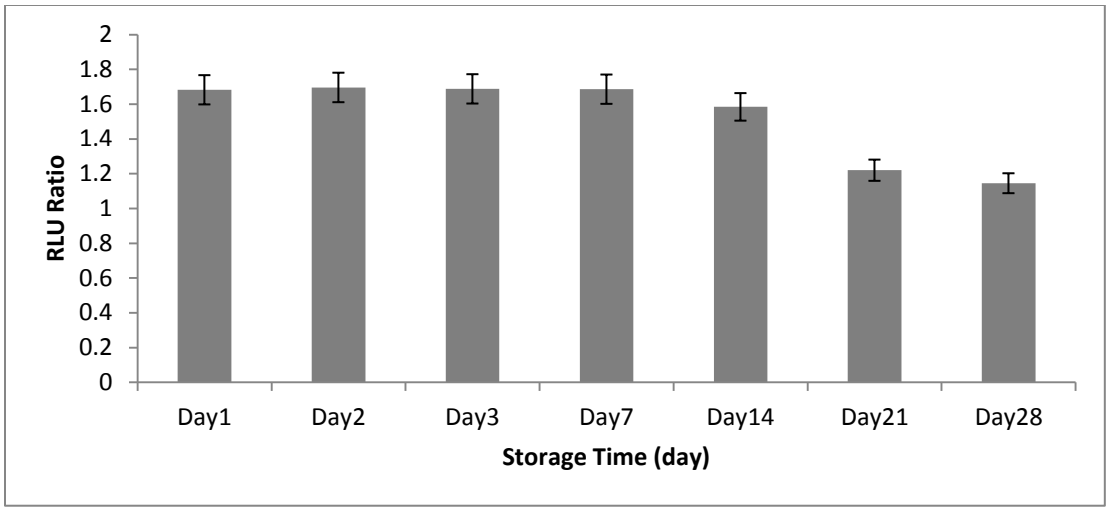

Figure 4.32 RLU ratio of *grpE lux-fusion* strain after adding 1000 µg/L acrylamide to the culture, which was stored for different period. Shown are mean ± S.E.M. (n=3).

4.3.2.5 Light effects

To assess the effect of light on light emitting of *grpE-lux* fusion strain in presence of acrylamide, ΔRLU values and RLU ratios of culture containing1000 µg/L were measured after keeping in 3 different conditions: dark room, light room (under bio cabinet), and directly put under lamp (with 10 cm distance to lamp). As seen in Figures 4.33, 4.34, and statistics showed in presence of acrylamide, light did not have significant effect on ΔRLU and RLU ratio of *grpE- lux* fusion strain of *E. coli* when comparing to ΔRLU value and RLU ratio of this strain at dark condition. Only under direct light with close distance to sample, ΔRLU was too low, which probably related to deactivation of acrylamide. Therefore, light cannot have any effect on the results of experiments (done under bio cabinet). These results are the same as the results of *recA-lux* fusion strain.

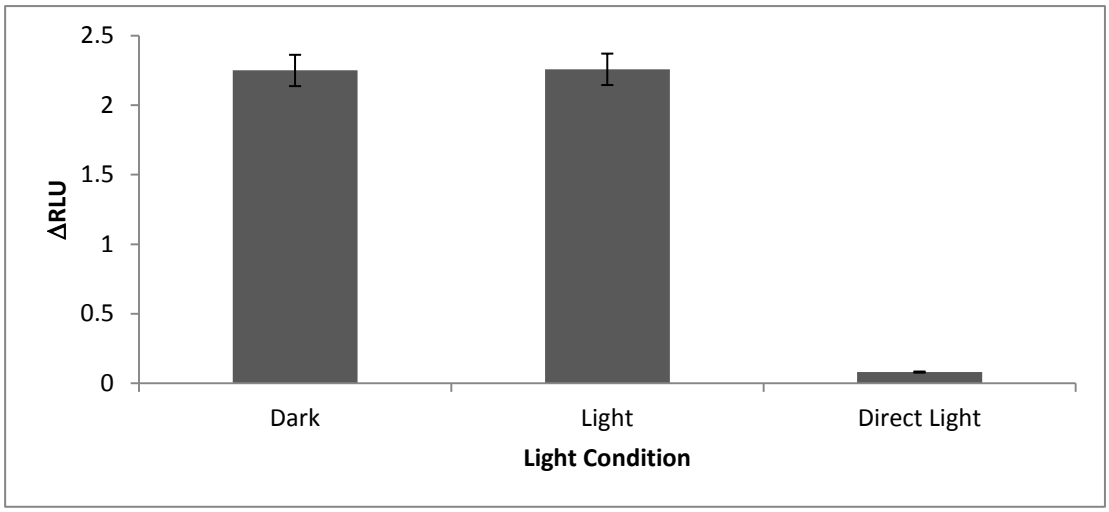

Figure 4.33 ΔRLU of *grpE-lux* fusion strain in different light condition after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

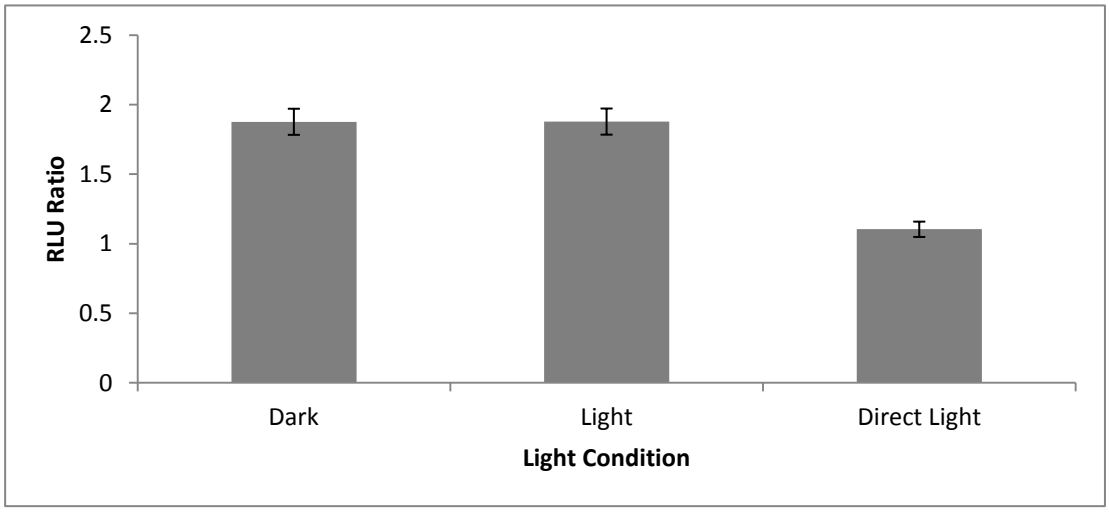

Figure 4.34 RLU ratio of *grpE-lux* fusion strain in different light condition after adding 1000 µg/L acrylamide. Shown are mean ± S.E.M. (n=3).

4.4 Conclusion

4.4.1 The *recA-lux* fusion strain

Overall, DPD2222 strain of luminous *E. coli*, which contains DNA damage responsive promoter, has the ability to show detectable optical dose-responsive response in a wide range of acrylamide concentrations (1-10000 µg/L) in real time. Moreover, it took about 40 min for acrylamide to cause damage on *recA-lux* fusion strain and 60 min for recA promoter to reach its maximum activation level. Therefore, the highest light emitting and response of the strain were detectable 100 min after adding acrylamide to the culture. In addition, this study had a new approach about storage of *recA- lux* fusion strain for detecting acrylamide since it was showed that the culture of this strain could be used efficiently up to two weeks, which can have time- saving and economical benefits in food industries. It was also revealed by this study that researchers in laboratory conditions did not need to have any concerns about the effects of light on the response of the strain to acrylamide. Furthermore, one of the important unique approaches of this study was the effects of temperature on *recA-lux* fusion strain; At 37°C, acrylamide could have lethal effects on *recA-lux* fusion of *E. coli* at lower concentrations compared to ambient temperature (1000 µg/L compared to 10000 µg/L) since irreparable damage of DNA occurred at lower concentration of acrylamide. Moreover, for doses of acrylamide, at which DNA damage could be repaired, the repair process took longer time at 37 °C compared to room temperature. As 37 °C is human body temperature, these approaches can help future research on acrylamide effects on human cells and DNA damage. Therefore, given all advantages and abilities of *recA- lux* fusion strain of *E. coli*, this strain can

have a considerable potential to be used as a suitable biosensing element for detecting acrylamide in food.

4.4.2 The *grpE-lux* fusion strain

In general, DPD2234 strain of *E. coli*, which contains protein damage responsive promoter like DPD2222 had ability to show detectable optical dose responsive respond in a wide range of acrylamide concentrations (1-10000 µg/L) in real time. Furthermore, it took acrylamide about 20 min to cause protein damage on *grpE*-lux fusion strain, which was less than the time needed to cause DNA damage since significant increase in light emitting of *recA-lux* fusion strain was observed after 40 min. Therefore, in presence of acrylamide, protein damage occurred earlier than DNA damage. On the other hand, it took about 60 min for grpE promoter to reach the maximum level of its activation, the same as recA. The highest light emitting of *grpE-lux* fusion could be observed 80 min after adding acrylamide, and then it stayed at the same level for 20 min. In addition, it was revealed that researcher did not need to have any concerns about the effect of light on respond of *grpE-lux* fusion strain to acrylamide in laboratory conditions, which was the same as *recA-lux* fusion strain. Moreover, the *grpE-lux* fusion strain culture (similar to that of *recA-lux* fusion culture) had the advantage of being efficiently usable for up to two weeks. Finally, it could be concluded that in presence of acrylamide, abilities and optimum conditions of *grpE-lux* fusion strain of *E. coli* were very similar to *recA-lux* fusion strain except that the relative light emitting of DPD2234 was lower than that of *recA-lux* fusion strain. Therefore, *grpE-lux* fusion strain can also have the potential to be used as a biosensing element to detect acrylamide in food.

Chapter 5: Elucidation of toxicity by understanding correspondent cell repair mechanisms

5.1 Introduction

Since the responses of an organism to chemical insult typically include repair mechanisms specific for the damage incurred, these response can reveal molecular modes of toxicity. Numerous such damage induced stress responses are common throughout biology. For instance, the heat shock response, which results in elevated expression of proteins such as molecular chaperones and proteases, is induced in essentially all organisms by stresses that result in the accumulation of non-native proteins in the cell (Morimoto et al., 1994). DNA damage induced stress responses are also prevalent in biological systems. In E. *coli*, the SOS response, which results in elevated levels of proteins specific for repair of DNA lesions, is induced by DNA damage (Walker 1996). Consequently, induction of the SOS response in *E. coli* serves as a biological indicator of DNA damage caused by chemicals and other treatments. Accordingly, induction of the SOS response in *E. coli* correlates well with carcinogenicity in mammals (Quillardet, 1993). Thus, the bacterial heat shock, SOS and other stress responses have ability to be good models to detect and discover modes of chemical toxicity. Therefore, in this section, two variants of the Comet assay method were used to assess DNA damage caused by acrylamide.

5.2 Materials & Methods

5.2.1 Neutral comet assay

In order to assess the type of DNA damage caused by acrylamide on *recA-lux* fusion cells, neutral comet assay, which is a sensitive, reliable method to detect double-stranded DNA break was used as follow.

5.2.1.2 Sample preparation

First, the dilution of fresh *recA- lux* fusion strain culture containing 1×10^5 cells/ml was prepared. Then, cell centrifuged (at 13000-16000×g) for 2 min and the supernatant was discarded. After that, the cell washed with 1×PBS and the supernatant discarded.

5.2.1.3 Slide preparation

Immediately, before the use of clean microscope slides (Trevigen®) they were dripped briefly in 1% agarose in solution (prepared with sterile H2O). Then, precoated slides were placed in an incubator at 60–70°C for approximately 15 min to complete evaporation of agarose. Therefore, a thin coating layer was form, which can facilitate adhesion of the stratified agarose microgel for the following steps.

5.2.1.4 Microgel formation and processing

First, in order to form a suitable microgel in which to embed cells, agarose suspensions were pipetted onto the clear window region of the slide surface before the addition of a 22 mm×50 mm coverglass. After incubating the slide at 4°C for 10 min to allow the initial layer of agarose to cool, the covers lip was removed and a subsequent agarose layer was added in the same manner. The first layer consisted of 200 μL of 0.5% agarose (SR 3:1 blend, Apex BioResearch Products, Research

Triangle Park, NC) prepared in 0.1× PBS or 0.85% NaCl and maintained at 55–60°C. For the second layer, 2 μL of exposed cells was mixed thoroughly with 200 μL of the same 0.5% agarose solution and 100 μL of this mixture was transferred to the slide. A third layer was comprised of the 0.5% agarose solution containing 5 μg/mL RNase A (5 Prime, Gaithersburg, MD), 1 mg/mL lysozyme (Sigma, St. Louis, MO), and 0.25% sodiumN-lauroyl sarcosine. Slides were refrigerated for 10 min at 4 °C and incubated for 30 min at 37 °C. Embedded cells were then lysed by immersing slides in a solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% sodium lauroyl sarcosine, and 1% Triton X-100 for 1 hour at room temperature. Triton X-100 was added fresh to the lysis solution by stirring for at least 10 min at room temperature. Following lysis, slides were immersed in an enzyme digestion solution prepared with 2.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.4, and 0.5–0.6 mg/mL of proteinase K (Ambion, Austin, TX) for 2 h at 37°C. Lysis and enzyme digestion steps were carried out in opaque transporters to prevent additional strand fragmentation due to light exposure.

5.2.1.5 Electrophoresis and slide processing

After enzyme digestion, slides were transferred to an opaque electrophoresis tank (Cleaver Scientific, Rugby, UK) and equilibrated for 20 min in buffer containing 300 mM sodium acetate and 100 mM Tris, pH 9. Slides were electrophoresed in this buffer for 50 min at 12 V. Then, after electrophoresis, slides were sequentially immersed in 1 M ammonium acetate prepared in ethanol for 20 min, absolute ethanol for 30 min, and 70% ethanol for 10 min. Then, slides were allowed to dry in ambient air for complete evaporation of the microgel overnight.

5.2.1.6 Staining and visualization

Before staining, slides were pretreated with a freshly prepared solution of 5% DMSO and 10 mM NaH2PO4. Pre-treatment comprised of pipetting 50 μL of the solution in a dropwise fashion over the clear window. During the time that slides were still wet, DNA was stained with 50 μL of 1 μ M YOYO-1 (Molecular Probes, Eugene, OR) in 5% DMSO. Then, they were visualized using an Axioskop 40 fluorescent microscope (Zeiss, Göttingen, Germany) at 40× magnification (Achroplan and EC Plan-Neofluar objectives) with the appropriate filter set for YOYO-1 (excitation 491 nm and emission 509 nm) (Solanky and Haydel, 2012).

5.2.2 Alkaline Comet assay

Since neutral comet assay allows the detection of only DNA double strand breaks, in 1988 Singh et al used alkaline comet assay, which can even detect single strand breaks (Dhawan, 2009). In a neutral variant, the molecule of DNA is preserved as a double stranded structure that leads to uncovering of double stranded DNA breaks (Yasuhara et al., 2003). In the alkaline variant of the method, the denaturing step allows revealing simultaneously double and single- stranded DNA breaks (Moller, 2006). Sample and slide preparation were the same as 5.2.1.

5.2.2.2 Solution preparation

Lysis solution was prepared at 4 °C for at least 20 min before use. In addition, LMA agarose was melted in a beaker of boiling water for 5 min, with cap loosen and bottle was placed in 37 °C water bath for at least 20 min to cool. Agarose temperature is critical to prevent heat shock of cells.

5.2.2.3 Microgel formation and processing

Cells were combined with molten agarose (at 37 °C) at ratio of 1: 10 (v/v) and immediately they had to be transferred by pipette 20µl on comet slide (Trevigen®). Also, side of pipette tip was used to spread agarose/cells over sample area to ensure complete coverage of the samples area. Moreover, for spreading samples more evenly, the slides were warmed at 37 °C before use. After that, they were placed at 4 °C in the dark (refrigerator) for 1 hour when, a 0.5mm clear ring appeared. After that, slides were immersed in 4 °C lysis solution and incubated overnight. Next day, excess buffer was drained from slides and immersed in freshly prepared Alkaline Unwinding (PH>13) solution for 1 hour at 4 °C. We added 850 ml electrophoresis solution and placed slides in electrophoresis slide tray and covered with slide tray overlay and the power of 21 volts applied for 30 min. Then, excess electrophoresis solution gently drained and immersed twice in deionized water 30 min each, in 70% ethanol for 20 min. Finally, samples were dried at 37 °C overnight.

5.3.2.4 Staining and visualization

First, 100 µL of DAPI was placed onto each circle of died agarose and stained 30 min dark at room temperature. Then, slides were tapped to remove excess DAPI solution and rinsed briefly in water. After all, we saw slides by epifluoresence microscopy (Trevigen, 2013).

5.3 Results & Discussion

In healthy cells, the fluorescence is confined to the nucleoid (comprised of high molecular weight DNA): undamaged DNA is supercoiled and thus, does not migrate very far out of the nucleoid under the influence of an electric current. Whereas in cells that have accrued DNA damage and lost supercoiling, migrating fragments (comet tail) from the nucleoid (comet head) are observed. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage (Yasuhara et al., 2003; Moller, 2006).

5.3.1 Neutral comet assay

5.3.1.1 Ambient Temperature

Neutral comet assay was used for detecting DNA damage in *recA- lux* fusion cultures, after adding 100, 1000, 5000, and 10,000 µg/L concentration of acrylamide and keeping 100 min at room temperature. As seen in figure 5.1, no damage was observed up to adding 10000 µg/L concentration of acrylamide. Therefore, if these results are compared with those of 4.3.1.1 (Figure 4.1 and Figure 4.2), it can be concluded that at the concentrations of acrylamide at which cell dampening occurred, double strand breaks were detected.

Therefore, current results confirm previous research, which motioned that *E. coli* bacteria could not repair double- stranded DNA breaks (Ginsberg and Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Ginsberg and Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Krasin and Hutchinson, 1977; Ulmer, 1979; Wang and Smith, 1986; Sedelnikova et al., 2002).

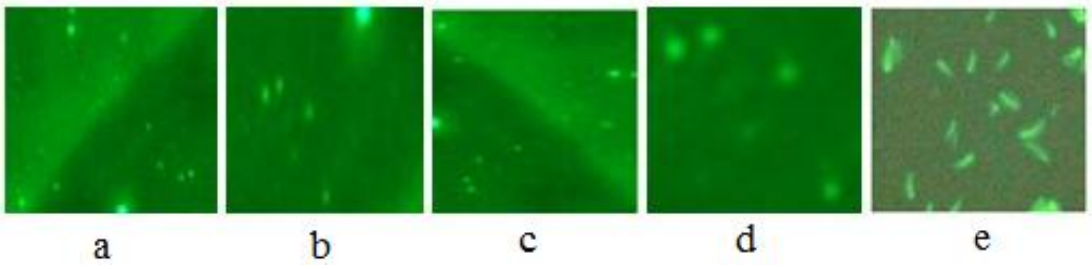

Figure 5.1 Representative images acquired from neutral comet assays following *recA* promoter *lux* fusion strain of *E. coli* exposed to acrylamide for 100 min at ambient temperature. (a) Control sample. (b) Sample containing 100 µg/L acrylamide. (c) Sample containing 1000µg/L acrylamide. (d) Sample containing 5000µg/L acrylamide. (e) Sample containing 10,000µg/L acrylamide.

To detect the effect of acrylamide exposure time on occurring double stranded DNA breaks, at concentration of acrylamide at which double stranded DNA breaks could be observed, neural comet assay was done for samples after 60, 80, and 1440 min of exposure to acrylamide at room temperature. As seen in figure 5.2, after 60 min no damage was observed. After 80 min, damages could be detected only for some cells. Finally, after 1440 min or 24 hours the damages were the same as what were observed in figure 5.1. Therefore, DNA damage of *recA*-*lux* fusion strain was less severe after exposing to acrylamide for 80 min compared to 100 min. In addition, DNA damage could not be repaired since the damages after, 100 and 1440 min (24 hours) were the same.

Thus, these results are also consistent with the results of previous studies, which showed *E. coli* double stranded DNA breaks are unrepairable (Ginsberg and

Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Ginsberg and Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Krasin and Hutchinson, 1977; Ulmer, 1979; Wang and Smith, 1986; Sedelnikova et al., 2002).

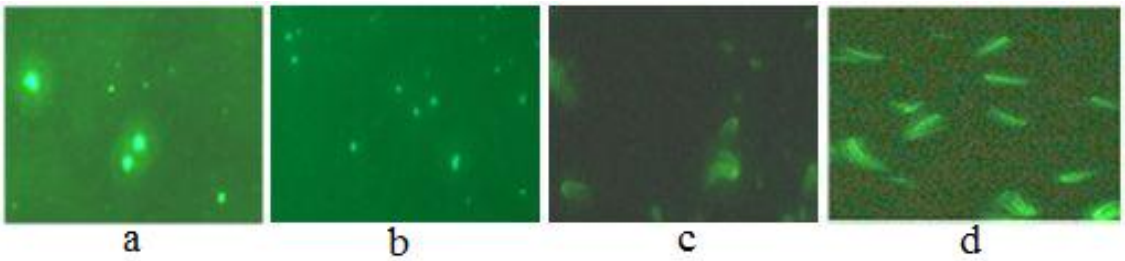

Figure 5.2 Representative images acquired from neutral comet assays following *recA* promoter *lux* fusion strain of *E. coli* exposure to 10,000µg/L acrylamide at ambient temperature. (a) Control sample. (b) Sample exposed to acrylamide for 60 min. (c) Sample exposed to acrylamide for 80 min (d) Sample exposed to acrylamide for 1440 min (24 hours).

5.3.1.1 Body Temperature

Moreover, in this study comet assay was used for detecting DNA damage in samples containing 100, 500, 1000, 10,000 µg/L of acrylamide concentrations and keeping 100 min at 37 ºC. This time, as seen in figure 5.3, DNA damage was observed after adding 1000 µg/L of acrylamide and no damage was observed before this concentration. Therefore, double stranded DNA breaks occurred at lower concentrations of acrylamide at 37 ºC compared to ambient temperature (1000 µg/L compared to 10,000 µg/L), which confirm results of 4.3.1.6.1 of this study (Figure 4.15 and Figure 4.16) that showed more severe effect of acrylamide on *recA*- *lux* fusion strains at body temperature compared to ambient temperature. In addition, it was observed that at 37 ºC whenever cell dampening occurred (Figure 4.15 and

Figure 4.16) double stranded breaks of the strain were detected. Thus, these results showed that *E. coli* double stranded DNA breaks were unrepairable the same as what was revealed by previous studies (Ginsberg and Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Ginsberg and Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Krasin and Hutchinson, 1977; Ulmer, 1979; Wang and Smith, 1986; Sedelnikova et al., 2002).

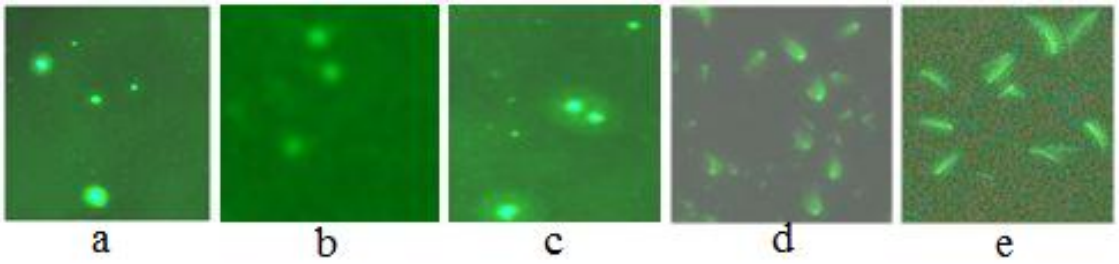

Figure 5.3 Representative images acquired from neutral comet assays following *recA* promoter *lux* fusion strain of *E. coli* exposed to acrylamide for 100 min at 37°C. (a) Control sample. (b) Sample containing 100 µg/L acrylamide. (c) Sample containing 500 µg/L acrylamide. (d) Sample containing 1000 µg/L acrylamide. (e) Sample containing 10,000 µg/L acrylamide.

In order to detect the effect of acrylamide exposure time on occurring double stranded DNA breaks, at concentrations of acrylamide at which double stranded DNA breaks could be detected, neural comet assay was done after 60, 80, and 1440 min that samples exposed to acrylamide at room temperature. As seen in figure 5.4 it can be concluded that the same as what were observed at ambient temperature (Figure 5.2), after 60 min no damage was detected. After 80 min, damages were observed for some cells and finally after 1440 min or 24 hours the damages were the same as what had been observed after 100 min in the previous part (Figure 5.3).

Thus, DNA damage of the strain was more severe after 100 min exposing to acrylamide compared to 80 min. Moreover, *recA-lux* fusion strain could not repair the damages since the same damages were detected after 24 hour. Therefore, it was revealed that double-stranded DNA break of *E. coli* was not repairable the same as the results of previous research (Ginsberg and Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Ginsberg and Webster, 1969; Bonura et al., 1975; Sedgwic, 1976; Krasin and Hutchinson, 1977; Ulmer, 1979; Wang and Smith, 1986; Sedelnikova etal., 2002).

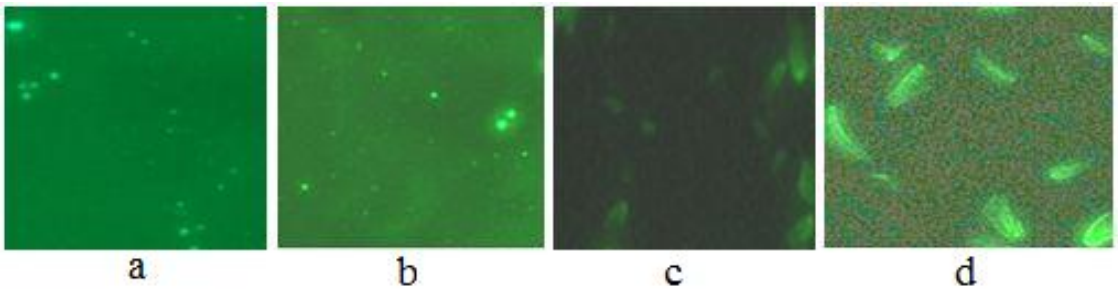

Figure 5.4 Representative images acquired from neutral comet assays following *recA* promoter *lux* fusion strain of *E. coli* exposure to 1000µg/L acrylamide at 37°C. (a) Control sample. (b) Sample exposed to acrylamide for 60 min. (c) Sample exposed to acrylamide for 80 min. (d) Sample exposed to acrylamide for 1440 min (24 hours).

5.3.2 Alkaline Comet assay

5.3.2.1 Ambient Temperature

For all concentrations of acrylamide that DNA damage in *recA*- *lux* fusion strain at ambient temperature was not detected by using neutral comet assay method, no DNA damage was observed by applying alkaline comet assay either (Figure 5.5). Since, alkaline comet assay can show single- stranded DNA breaks, no single-stranded DNA break occurred in samples containing acrylamide concentrations below 10,000µg/L.

Therefore, it can be concluded that no single-stranded break could occur on *recA*- *lux* fusion in presence of acrylamide or probable damages might be below the limit of sensitivity of the method, which is approximately 50 strand breaks (Olive and Banath, 2006).

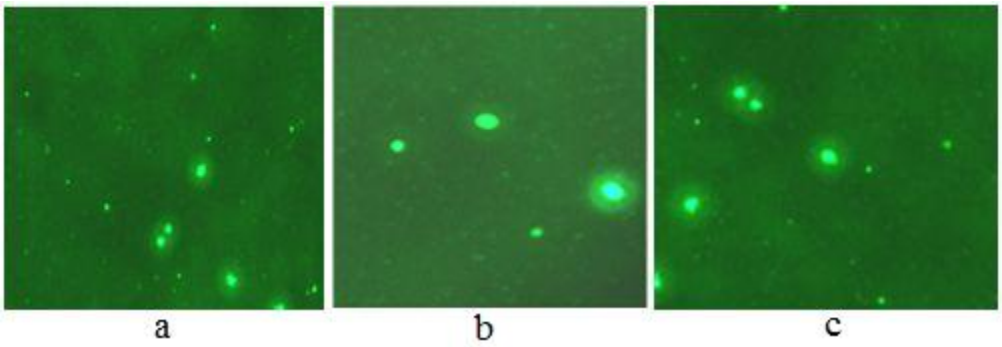

Figure 5.5 Representative images acquired from alkaline comet assays following *recA* promoter *lux* fusion strain of *E. coli* exposure to acrylamide for 100 min at ambient temperature. (a) Control sample. (b) Sample containing 1000 µg/L acrylamide. (c) Sample containing 5000µg/L acrylamide.

5.3.2.2 Body Temperature

Moreover, at 37°C, all samples containing concentrations of acrylamide, which did not show DNA damage in *recA- lux* fusion strain by applying neutral comet assay method, no DNA damage was observed by alkaline comet assay either (Figure 5.6).

Therefore, no single-stranded DNA break was detected in samples containing concentrations of acrylamide below 1000µg/L. Since, alkaline comet assay has ability to detect single- stranded DNA breaks, it can be realized that the same as what was observed at ambient temperature for the samples without any double-stranded break, no single-stranded break was observed either. Therefore, there might be two reasons for observing an increase in light emitting of the strain. First, it is possible that single-stranded DNA break down occurred in some cells. This number was below the limit of sensitivity of comet assay, which is 50 breaks (Olive and Banath, 2006). Second, although recA has a key role in SOS response, groups of more than forty unlinked genes that are controlled by the LexA repressor are involved in SOS regulon, and their expression is induced to high levels following exposure to DNA-damaging agents (Courcelle et al., 2001). These genes encoded proteins, which participate in all aspects of DNA metabolism, acting to repair DNA damage, activate transcription and transport and facilitate the error-prone, recombinational repair of DNA (Ogawa et al., 1979; Roberts et al., 1979). Therefore, if acrylamide caused base modification damage on DNA that might cause changes or mutation in any of these forty genes, it could start the SOS response of the cell and activation of recA. This could cause an increase in light emitting of *recA-lux* fusion strain. Also, previous studies showed that

mutations in UvrD gene led to expression of the SOS response, and consequently activation of recA in the absence of DNA breaks (Nickoloff and Hoekstra, 1998).

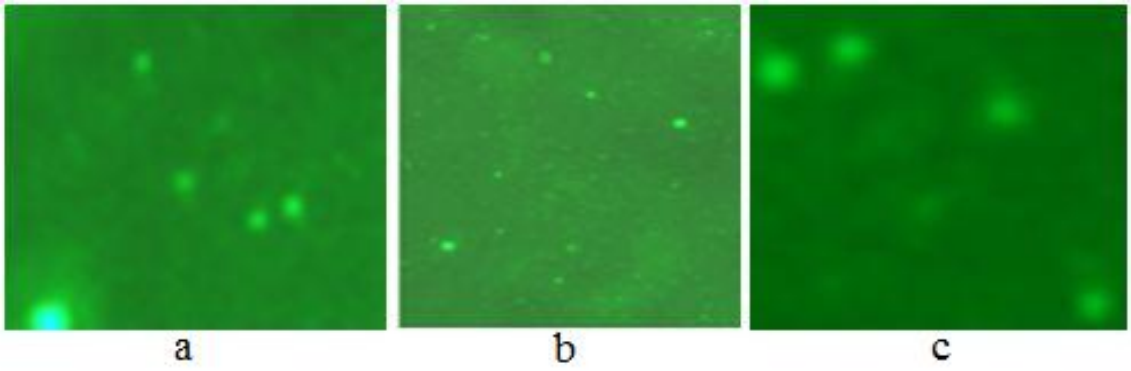

Figure 5.6 Representative images acquired from alkaline comet assays following *recA* promoter *lux* fusion strain of *E. coli* exposure to acrylamide for 100 min at 37°C. (a) Control sample. (b) Sample containing 100 µg/L acrylamide. (c) Sample containing 500 µg/L acrylamide.

5.4 Conclusion

Overall, it can be concluded that at 37°C double-stranded DNA break, which is the most severe type of DNA damage and cannot be repaired by *E. coli* cells, occurred in DPD222 strain in presence of lower amount of acrylamide compared to ambient temperature (1000 compared to10,000 µg/L). When light emitting of DPD222 , a genetically modified strain of *E. coli*, (in which DNA damage stress responsive promoter, recA, fusion to lux genes) increase (RLU ratio >1 and ΔRLU value > 0) in presence of any chemical, it indicates that the chemical caused some type of DNA damage, and recA was activated in order to repair such damages. On the other hand, when light emitting of the strain decreases (RLU ratio <1 and ΔRLU value <0) in presence of any chemical, it means that the damage level was more than

the ability of cell to repair it so cell dampening had occurred. As a result, if at any concentrations of acrylamide a decrease in light emitting of DPD222 strain is measured, it can be expected to detect the unrepairable level of DNA damage, which is double-stranded DNA break. Thus, in present study, as was expected, at the concentrations of acrylamide >10,000 µg/L at ambient temperature, and >1000 µg/L at 37°C that RLU ratios were below 1 and ΔRLU values are negative, double-stranded DNA breaks were observed. At concentrations of acrylamide below 10,000 µg/L at ambient temperature and below 1000 µg/L at 37°C, an increase in light emitting of the strain was measured (RLU ratio >1 and ΔRLU value >0). Therefore, some kind of repairable DNA damage had to have occurred in cells although, no single-stranded DNA damage (the most sever repairable DNA damage) was detected by alkaline comet assay at those concentrations. Therefore, at concentrations with a detectable increase in light emitting of the strain that showed no single-stranded DNA break, two possible theories can be proposed. First, it is possible that single-stranded DNA breaks occurred only in some cells. The numbers of these breaks were below the limit of sensitivity of comet assay. Second, the possible base modification damage of DNA might cause mutation in any of the forty genes (involved in SOS response).This might be the reason of detecting light emitting of the cells. The methods to detect base modification and mutation are very expensive and the interpretation of their results can be a new project by itself.

Chapter 6: Conclusions and Recommendations

All of the five genetically engineered strains containing selected stress-responsive *E. coli* promoters fusion to the *luxCDABE,* which *included : recA-lux* (in the SOS regulon), *grpE-lux* (in the heat shock regulon), *inaA-lux* (internal acidification responsive and in the Sox and Mar regulon), *yciG-lux* (in the ∂^s-dependent stress response regulon), and *o513-lux* (in the ∂^s independent, stationary phase inducible stimulon) were capable to show optical dose responses in presence of wide range of acrylamide concentrations. Therefore, all of these strains have the potential to be used as optical microbial biosensors in order to detect acrylamide in food. Among them, the best responses were related to *recA-lux* fusion strain followed by *grpE-lux* fusion strain. Therefore, these two strains could be optimal candidates to be used as novel biosensing elements for detection of acrylamide in food. Furthermore, current research showed that the optimum conditions for *recA-lux* fusion and *grpE-lux* strains to detect acrylamide were similar to each other. These strains can be used as a panel, which can increase the reliability of the test results. Moreover, this study revealed that acrylamide levels as low as 1 µg/L, (the average concentration which usually can be found in high starch heated foods such as French fries), could be detected by using these two strains. The findings also revealed that these two strains could have several advantages including: usability of the cultures for up to two weeks, yielding best response after 100 min of exposure to acrylamide, and none-responding to natural (regular laboratory conditions) light. In addition, another unique finding of present study was that acrylamide at 37°C caused unrepairable DNA damage on *recA-lux* fusion cells at lower concentrations compared to ambient

temperature (1000µg/L compared to 10,000µg/L), which in turn led to cell dampening. Furthermore, it was indicated that it took longer time for cells to recover from repairable DNA damages at 37 °C compared to ambient temperature (300min compared to 240 min). It is highly recommended that future studies focus on figuring out the exact types of probable DNA damages caused by acrylamide on *recA-lux* fusion strain of *E. coli* through detecting any mutation that might occur in any of the forty genes, participating in SOS response. Additionally, it can be very useful to undertake further research on probable effects of food ingredients on light emitting of *recA-lux* fusion and *grpE-lux* fusion strains.

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