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THE ROLE OF OXIDATIVE STRESS GENES AND EFFECT OF pH ON METHYLENE BLUE SENSITIZED PHOTOOXIDATION OF *ESCHERICHIA COLI*

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In this study, the survival time of wild type *E. coli* W3110 and 11 mutants was analysed with a plate count method in methylene blue added or control groups under daylight fluoroscence illumination (4950 lux) at different pH values (5.0, 6.0, 7.0, and 8.0) in phosphate buffer. As a result, while the number of bacteria did not decrease under photooxidative stress at pH 5.0 and 6.0 during a 6-hour incubation, the wild type and all mutants decreased more than 2 log. at pH 8.0, and approximately one log. at pH 7.0. It was determined that a 2 log decrease in wild type *E. coli* takes 3.7 h according to t_{99} value at pH 8, these values were 2.39 h in the *katE* mutant, 2.64 h in the *soxR* mutant, 2.67 h in the *oxyR* mutant, 2.71 h in the *sodB* mutant, 3 h in the *btuE* mutant, 3.38 h in the *zwf* mutant and 3.40 h in the *soxS* mutant, respectively ($p<0.05$). The roles of these genes were proved with complement tests. Finally, it is found that the effectiveness of photooxidative stress is in direct relation with pH, and the *katE*, *soxR*, *oxyR, sodB*, *btuE*, *zwf*, and *soxS* genes are important for the protection against this stress.

Keywords: E. coli – photooxidation – *oxyS – soxRS – katE – sodB*

INTRODUCTION

There are many environmental factors that affect the viability of bacteria in an aquatic environment. Starvation, heat, osmolarity and sunlight can be accepted as some of the factors responsible for viability of the bacteria, however, light and photooxidation caused by light are the most important factors that affect the viability of bacteria in their natural environments [6]. Photooxidative stress occurs when the light-sensitive molecules are exposed to light. They produce reactive oxygen species (ROS) [7]. ROS occurring in photooxidation attacks all types of molecules in the cells. All proteins, lipids, carbohydrates and nucleic acids are harmed by these radicals [7]. Moreover, it has been that various mutations occurred in DNA following the treatment with demonstrated methylene blue and light, as well as heredical diseases occurred in metabolic pathways [14].

For the protection of the cells against oxidative stress and the damages caused by photooxidative stress, the cells have antioxidant enzymes synthesised at a low level

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and repairing mechanisms for normal growth. As a reaction to superoxide radical ($O_2^{\bullet-}$) and H_2O_2 concentration, a number of antioxidant protein syntheses are induced. Among the genes designed by the SoxRS transcriptional factor induced by $O_2^{\bullet-}$, there are superoxide dismutase (SOD, coded by *sodA*), DNA repairing enzyme endonuclease IV (*nfo*) and isozymes resistant to $O°_2^-$ of fumarase (*fumC*) and aconitase (*acnA*). The activation of SoxRS in the cell increases the level of glucose-6-phosphate dehydrogenase (*zwf*) and decreases the level of the Fur (*fur*) receptor [5, 13]. Outer membrane protein coded by *tolC*, *acrAB* coded as drug efflux pump and MicF pressed the expression of outer membrane proteins to help eliminate the active compounds from the medium known as redox that increase the $O_2^{\bullet-}$ level [12]. Hydroperoxidase I (catalase, *katG*) controlled by OxyR transcription factor induced by H_2O_2 contains two subunits of hydroperoxide reductase (*ahpCF*), glutaredoxin I (*grxA*), glutathione reductase (*gorA*) and Fur repressor (*fur*) [21]. Besides, the protection activity against H_2O_2 which is not the under control of OxyR contains hydroperoxidase II (*katE*) and endonuclease III (*xthA*) with DNA repairing enzymes, DNA polymerase I (*polA*) and *recA* [21]. In addition, SoxRS and OxyR regulos that provide protection against harm caused by $O_2^{\bullet-}$ and H_2O_2 were demonstrated to provide important resistance against organic solvents and reactive nitrogenase [5].

A high number of molecules are known to produce radicals under the effect of sunlight in nature [6, 18]. The methods called as Photodynamic Therapy (PDT) were developed for the disinfection of water and treatment of diseases as a new antimicrobial strategy [17]. Furthermore, production of radicals which induce oxidative stress have an important role in the immune system's struggle against microorganisms [3]. In such a case, it is necessary to investigate the factors which change the effectiveness of photooxidation and the protection mechanisms against stress.

To reveal the effect of oxidative stress, the response of bacteria against different stress factors and the physiology of bacteria meanwhile struggling with stress is important to understand the effectiveness of bacteria when used in industry (such as solar disinfiction-SODIS, PDT). The objective of this study was to evaluate the influence of different pH values on the efficiency of photooxidative stress on *E. coli* and to determine which gene play an important role in photooxidative stress.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

The strains used in this study are demonstrated in Table 1. Eleven genes of *E. coli* W3110 were determined and Keio collection mutants were obtained from Japan National Genetic Center.

Table 1
Wild type *E. coli,* mutants and plasmids used in this study

Strain or plasmide	Relevant genotype	Origin or reference
W3110	Wild type	Lab Collection
JW3933	BW25113 *oxyR*::Km^r	Keio Collection[a]
JW4024	BW25113 *soxR*::Km^r	Keio Collection
JW4023	BW25113 *soxS*::Km^r	Keio Collection
JW1721	BW25113 *katE*::Km^r	Keio Collection
JW3914	BW25113 *katG*::Km^r	Keio Collection
JW3879	BW25113 *sodA*::Km^r	Keio Collection
JW1648	BW25113 *sodB*::Km^r	Keio Collection
JW1638	BW25113 *sodC*::Km^r	Keio Collection
JW1700	BW25113 *btuE*::Km^r	Keio Collection
JW0598	BW25113 *ahpC*::Km^r	Keio Collection
JW1841	BW25113 *zwf*::Km^r	Keio Collection
CO100	W3110 *oxyR*::Km^r	W3110 × P1 (JW3933) (this study)
CO101	W3110 *soxR*::Km^r	W3110 × P1 (JW4024) (this study)
CO102	W3110 *soxS*::Km^r	W3110 × P1 (JW4023) (this study)
CO103	W3110 *katE*::Km^r	W3110 × P1 (JW1721) (this study)
CO104	W3110 *katG*::Km^r	W3110 × P1 (JW3914) (this study)
CO105	W3110 *sodA*::Km^r	W3110 × P1 (JW3879) (this study)
CO106	W3110 *sodB*::Km^r	W3110 × P1 (JW1648) (this study)
CO107	W3110 *sodC*::Km^r	W3110 × P1 (JW1638) (this study)
CO108	W3110 *btuE*::Km^r	W3110 × P1 (JW1700) (this study)
CO109	W3110 *ahpC*::Km^r	W3110 × P1 (JW0598) (this study)
CO110	W3110 *zwf*::Km^r	W3110 × P1 (JW1841) (this study)
Plasmids		
B1710	*btuE*	mobile plasmid collection[b]
B3908	*sodA*	mobile plasmid collection
B1656	*sodB*	mobile plasmid collection
B4063	*soxR*	mobile plasmid collection
B4062	*soxS*	mobile plasmid collection
B1732	*katE*	mobile plasmid collection
B3961	*oxyR*	mobile plasmid collection
B1852	*Zwf*	mobile plasmid collection

[a, b]Keio collection mutants were obtained from Japan National Genetic Center.

Gene transfer with transduction

Keio collection mutants were transferred into *E. coli* W3110 with P1kc phages [11]. The obtained W3110 mutants were tested with related forward primers and the universal Km primer (Table 2). Plasmids that were obtained from Keio collection for complementation tests were isolated, transferred to obtained mutants, and checked with universal primers in PCR. Furthermore, antibiotics tests were performed.

Table 2
The list of primers used in the study

Primer	Sequence
Km	CAGTCATAGCCGAATAGCCT
oxyR f	GTCAGAATGCTTGATAGGGA
soxR f	TGCCTCTTTTCAGTGTTCAG
soxS f	TCCATAAATCGCTTTACCTC
katE f	TCTGGCTGGTGGTCTATAGT
katG f	TCTCTAACGATGTGTATCGT
sodA f	CTGCTTACGCGGCATTAACA
sodB f	TTTGCTACCCTATCATACG
sodC f	TGGGGTACGACGTACCGTAA
btuE f	GTTAAAAGCAGGACGTTAGC
ahpC f	GGAAACGCATTAGCCGAATC
zwf f	ACAGTTTTCGCAAGCTCGTA
21M13	CAGGAAACAGCTATGACC
SP6	ATTTAGGTGACACTATAG

Viability experiments under photooxidative stress

E. coli W3110 and mutant strains were incubated in shaking incubator at 37 °C for 18 h at a speed of 160 rpm. Then, 1 ml of each sample was taken out and centrifuged, washed with phosphate buffer and suspended again in phosphate buffer. For viability experiments, 100 µl from this suspension was taken out and added to 50 ml phosphate buffer at different pH values. In this way, approximately 5×10^6 was provided as an initiative bacteria count. After covering the beaker with strech films, the samples were incubated under light (4950 ± 75 lux) at 24 °C. Dark control groups were covered with aluminum foil. Viability of strains under the light in different pHs were determined by the spreading plate count method. The studies were performed in a light + methylene blue medium in pH 5, 6, 7 and 8 sodium phosphate buffer. A 100 µl of methylene blue was taken from 0.1 gr ml^{-1} stock, and transferred to 50 ml phosphate buffer. Dark controls, dark + methylene blue and light controls were used as a control of photooxidation presence. The studies were carried out four times independently.

Complementation tests

After isolating the plasmids taken from the Keio collection, they were transferred to the cells of related mutants through transformation and, maintained as a powerful gene on the plasmid whose related gene is mutant in the main chromosome in the cell. Competent cells were prepared utilizing the calcium chloride method and were transformed by using 100 ng of plasmid DNA, as described elsewhere [4]. Transformants were selected on LB plates containing 100 µg of ampicillin per ml and supplemented with 50 µg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) per ml. Transformation was assessed by isolating and confirming by colony PCR the presence of the plasmid. For induction of gene on plasmid, 1 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to phosphate buffer.

Statistical analysis

Viability values were obtained by the plate count method and the values were converted to logaritmic values. t_{99} and k values were calculated by using these values. A Student *t*-test was used to see whether the changes in the results of wild type and mutants are acceptable for the analysis.

RESULTS

The survival of E. coli *under photooxidative stress in different pH value*

In this study, the effect of photooxidative stress on viability of *E. coli* and the role of pH in this effect were investigated. Control groups (L, D, D+MB) were used to evaluate results obtained by only light+methylene blue treatment. As seen in Fig. 1,

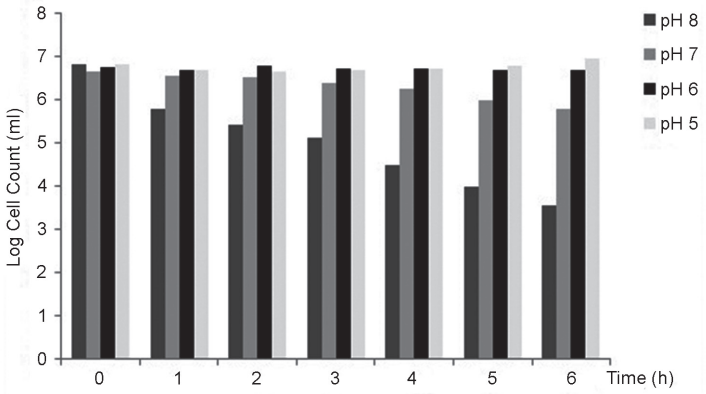

Fig. 1. The effect of photooxidative stress for the viability of *Escherichia coli* W3110 at different pH values

Table 3
Survival of wild type *E. coli* W3110 and mutants under photooxidative stress at pH 5 and 6

pH 5	L+MB				L				D+MM				D			
	0.h	2.h	4.h	6.h	0	2	4	6	0	2	4	6	0	2	4	6
W3110	6.84	6.92	6.83	6.68	6.84	6.73	6.72	6.75	6.84	6.63	6.66	6.76	6.84	6.61	6.6	6.59
oxyR	6.72	6.65	6.52	6.67	6.72	6.76	6.69	6.71	6.72	6.89	6.76	6.83	6.72	6.78	6.81	6.75
soxS	6.63	6.72	6.73	6.80	6.63	6.70	6.56	6.80	6.63	6.65	6.73	6.98	6.63	6.8	6.73	6.80
soxR	6.79	6.72	6.66	6.83	6.79	6.65	6.57	6.90	6.79	6.73	6.65	6.89	6.79	6.78	6.53	6.71
katE	6.39	6.6	6.53	6.56	6.39	6.52	6.43	6.60	6.39	6.34	6.45	6.58	6.39	6.36	6.41	6.43
katG	6.72	6.67	6.57	6.64	6.72	6.58	6.48	6.75	6.72	6.57	6.45	6.51	6.72	6.54	6.41	6.68
ahpC	6.66	6.56	6.53	6.41	6.66	6.52	6.52	6.54	6.66	6.51	6.50	6.48	6.66	6.4	6.48	6.64
btuE	6.60	6.9	6.61	6.56	6.60	6.77	6.51	6.51	6.60	6.63	6.56	6.59	6.60	6.58	6.46	6.62
sodC	6.77	6.67	6.68	6.88	6.77	6.65	6.74	6.85	6.77	6.6	6.60	6.89	6.77	6.58	6.58	6.87
sodA	6.91	6.77	6.71	6.81	6.91	6.71	6.69	6.78	6.91	6.83	6.82	6.98	6.91	6.67	6.83	6.74
sodB	6.57	6.53	6.42	6.78	6.57	6.34	6.45	6.87	6.57	6.34	6.59	6.89	6.57	6.54	6.54	6.79
zwf	6.82	6.68	6.57	6.68	6.82	6.56	6.59	6.71	6.82	6.63	6.62	6.81	6.82	6.62	6.67	6.61

Table 3 (cont.)

pH 6	L+MB				L				D+MM				D			
	0.h	2.h	4.h	6.h	0	2	4	6	0	2	4	6	0	2	4	6
W3110	6.75	6.68	6.74	6.96	6.75	6.67	6.90	6.84	6.75	6.74	6.81	6.92	6.75	6.87	6.89	6.90
oxyR	6.53	6.54	6.48	6.56	6.53	6.34	6.57	6.81	6.53	6.29	6.40	6.40	6.53	6.36	6.40	6.50
soxS	6.60	6.77	6.70	6.69	6.60	6.70	6.60	6.53	6.40	6.42	6.40	6.58	6.40	6.36	6.30	6.40
soxR	6.52	6.70	6.84	6.67	6.52	6.40	6.67	6.51	6.52	6.34	6.61	6.63	6.52	6.48	6.48	6.32
katE	6.64	6.49	6.79	6.71	6.64	6.79	6.60	6.54	6.44	6.66	6.67	6.65	6.44	6.48	6.34	6.65
katG	6.71	6.85	6.85	6.90	6.71	6.76	6.58	6.79	6.71	6.83	6.84	6.48	6.71	6.52	6.79	6.80
ahpC	6.70	6.83	6.67	6.66	6.70	6.60	6.70	6.50	6.70	6.55	6.74	6.70	6.70	6.65	6.66	6.74
btuE	6.59	6.56	6.60	6.76	6.49	6.36	6.41	6.85	6.49	6.46	6.80	6.60	6.49	6.67	6.80	6.60
sodC	6.55	6.75	6.62	6.64	6.55	6.45	6.36	6.87	6.55	6.51	6.52	6.79	6.55	6.52	6.30	6.40
sodA	6.58	6.36	6.64	6.46	6.58	6.53	6.45	6.58	6.58	6.45	6.43	6.48	6.58	6.41	6.26	6.38
sodB	6.47	6.34	6.49	6.18	6.47	6.40	6.32	6.54	6.47	6.00	6.58	6.34	6.47	6.40	6.38	6.28
zwf	6.69	6.76	6.81	6.71	6.69	6.60	6.72	6.60	6.69	6.68	6.72	6.52	6.69	6.65	6.71	6.65

L+MB: Light with methylene blue (MB), L: Light control without MB, D+MB: Dark control with MB, D: Dark control without MB.

Table 4

Survival of wild type *E. coli* and mutants under photooxidative stress at pH 7 and 8

pH 7	L+MB				L				D+MM				D			
	0.h	2.h	4.h	6.h	0	2	4	6	0	2	4	6	0	2	4	6
W3110	6.70	6.51	6.27	5.89	6.66	6.60	6.55	6.53	6.64	6.68	6.59	6.56	6.73	6.51	6.63	6.57
oxyR	6.59	6.38	6.00	5.60	6.75	6.52	6.38	6.35	6.52	6.37	6.47	6.40	6.49	6.40	6.48	6.44
soxS	6.49	6.30	5.99	5.16	6.74	6.57	6.59	6.59	6.53	6.45	6.48	6.45	6.72	6.64	6.61	6.56
soxR	6.74	6.49	6.29	5.70	6.76	6.65	6.59	6.55	6.70	6.67	6.53	6.60	6.52	6.63	6.45	6.63
katE	6.68	6.22	5.90	5.33	6.63	6.42	6.30	6.17	6.53	6.44	6.33	6.39	6.62	6.31	6.46	6.34
katG	6.59	6.38	6.21	5.21	6.58	6.51	6.46	6.40	6.69	6.64	6.63	6.62	6.81	6.70	6.77	6.76
ahpC	6.63	6.31	6.07	5.79	6.55	6.45	6.64	6.45	6.86	6.58	6.58	6.57	6.65	6.34	6.53	6.46
btuE	6.50	6.37	6.01	5.73	6.61	6.50	6.47	6.59	6.64	6.61	6.52	6.59	6.60	6.55	6.41	6.48
sodC	6.47	6.09	5.70	5.34	6.59	6.50	6.41	6.44	6.51	6.48	6.51	6.46	6.47	6.53	6.34	6.41
sodA	6.70	6.57	6.13	5.82	6.74	6.71	6.49	6.40	6.53	6.65	6.52	6.45	6.69	6.63	6.59	6.49
sodB	6.50	6.37	5.64	5.08	6.32	6.37	6.24	6.18	6.54	6.35	6.27	6.29	6.65	6.55	6.30	6.20
zwf	6.73	6.42	6.26	5.83	6.68	6.54	6.56	6.66	6.62	6.54	6.54	6.52	6.82	6.60	6.57	6.57

Table 4 (cont.)

pH 8	L+MB				L				D+MB				D			
	0.h	2.h	4.h	6.h	0	2	4	6	0	2	4	6	0	2	4	6
W3110	6.82	5.42	4.48	3.57	6.81	6.72	6.87	6.51	6.58	6.62	6.57	6.58	6.65	6.74	6.23	6.53
oxyR	6.69	4.96	3.71	2.19	6.71	6.62	6.59	6.32	6.83	6.74	6.75	6.32	6.65	6.62	6.65	6.34
soxS	6.68	5.31	4.55	3.16	6.70	6.53	6.68	6.54	6.60	6.46	6.64	6.65	6.54	6.46	6.43	6.48
soxR	6.59	4.45	3.48	2.05	6.53	6.36	6.32	6.36	6.57	6.41	6.49	6.36	6.54	6.54	6.49	6.58
katE	6.45	3.25	2.36	1.50	6.48	6.26	6.34	6.51	6.49	6.38	6.43	6.51	6.52	6.26	6.28	6.18
katG	6.37	4.67	3.79	3.12	6.30	6.36	6.40	6.67	6.28	6.48	6.40	6.83	6.30	6.54	6.30	6.65
ahpC	6.65	5.31	4.51	3.91	6.65	6.53	6.72	6.63	6.65	6.72	6.70	6.78	6.68	6.62	6.60	6.69
btuE	6.67	5.01	3.78	2.67	6.49	6.18	6.34	6.54	6.60	6.57	6.56	6.65	6.38	6.40	6.36	6.46
sodC	6.46	5.11	4.00	3.24	6.31	6.34	6.43	6.28	6.34	6.37	6.30	6.2	6.28	6.31	6.38	6.32
sodA	6.73	5.17	4.25	3.37	6.40	6.32	6.46	6.48	6.25	6.30	6.43	6.34	6.35	6.28	6.28	6.43
sodB	6.62	4.88	3.35	2.19	6.38	6.25	6.31	6.21	6.25	6.20	6.37	6.23	6.23	6.30	6.25	6.19
zwf	6.67	4.53	3.69	3.12	6.53	6.51	6.51	6.60	6.74	6.62	6.50	6.74	6.51	6.51	6.40	6.50

L+MB: Light with methylene blue. L: Light control without MB. D+MB: Dark control with methylene blue. D: Dark control without MB.

the number of wild type *E. coli* decreased in light+MB medium at pH 7 and 8 whereas no decrease occurred at other pH values (pH 5 and 6) in the control groups (Tables 3, 4, W3110). The count of *E. coli* W3110 at pH 8 decreased from 6.72 log to 3.57 log however it decreased from 6.70 to 5.89 at pH 7. As a result, photooxidative stress was very effective at alkaline pH.

After these results, mutants and viability experiments were performed to understand which antioxidant genes play an important role under photooxidative stress.

The effect of photooxidative stress on mutants at different pH values

After determining the effects of photooxidative stress at an alkaline pH, it was investigated what gene was involved in these effects *oxyR*, *soxR, soxS, katE, katG, ahpC, btuE, sodA, sodB, sodC* and *zwf* genes were studied. Analysing Tables 3–5, it can be seen that photooxidative stress had no effect at pH 5 and pH 6 during a 6-hour incubation (Table 3). Besides, there was no decrease observed at pH 5 and 6 in the same period for both wild type and in mutants in the control samples (dark, light and dark+MB samples). As a conclusion, it can be stated that wild type *E. coli* was not affected by photooxidative stress at pH 5 and pH 6 and, the mutant genes related to oxidative stress did not have any role in the viability.

At pH 7 (Table 4), it was indicated that the photooxidative stress affected wild type *E. coli* and mutants. Whereas in wild type *E. coli* and *oxyR, soxR, ahpC, btuE, zwf, sodC* and *sodA* mutants decreased by approximately one log during a 6-hour incubation. It was seen that *sodB* decreased 1.42 log, *katG* 1.38 log, *soxS* 1.33 log, *katE* 1.30 log, respectively. Hence, when pH arose from an acidic value to a neutral value, the effect of photooxidative stress increased. As there was no decrease in the control groups (L, D, D+MB) this showed that this stress was caused directly by the photooxidative stress. In the experiments carried out in pH 8 phosphate buffer (Table 4), it was clearly realized that all wild types *E. coli* W3110 and mutants were affected by photooxidative stress. From the genes investigated, *katE, oxyR, soxR, btuE, sodB, soxS* and *zwf* proved to play an important role in photooxidative stress.

Complementation tests were performed to analyze the effect of mutations. Performing these tests, the gene which became mutant was transferred to plasmid and this plasmid was transformed into the mutant. In such a way, despite that the related gene in the chromosome was mutant, this related gene was in the plasmid and its expression was controlled by IPTG. As a result of these studies, the viability results of strains that were complemented were similar to the wild type and some of them were more viable (Table 5). In the complementary tests, genes which were under photooxidative stress in pH 8, had their t_{99} values restored to 3.78 h for *katE* gene, 4.06 h for *soxR*, 4.07 h for *oxyR*, 4.2 h for *sodB*. Thus the advantageous roles of these genes were proved. It can be said that when the genes on the plasmid were highly induced with IPTG, the complement strains were better according to the wild type and they protected the cell.

Table 5
T_{99} and k value of mutants and complementation tests under photooxidative stress at pH 8

Strain	h (log.)	6. h (log.)	k	T_{99} (h)
E. coli W3110 wild type	6.82	*3.57*	–0.541	3.70
E. coli W3110 wild type (ıptg control)	6.61	3.42	–0.531	3.76
E. coli W3110 *katE*::km	6.45	*1.50**	–0.833	*2.39*
E. coli W3110 *katE*::km pNT3::*katE*	6.81	3.63	–0.529	3.78
E. coli W3110 *soxR*::km	6.59	*2.05**	–0.756	*2.64*
E. coli W3110 *soxR*::km pNT3::*soxR*	6.58	3.80	–0.395	4.06
E. coli W3110 *oxyR*::km	6.69	*2.19**	–0.749	*2.67*
E. coli W3110 *oxyR*::km pNT3::*oxyR*	6.60	3.65	–0.491	4.07
E. coli W3110 *sodB*::km	6.62	*2.19**	*–0.738*	*2.71*
E. coli W3110 *sodB*::km pNT3::*sodB*	6.58	3.67	–0.435	4.20
E. coli W3110 *btuE*::km	6.67	*2.67**	*–0.666*	*3.00*
E. coli W3110 *btuE*::km pNT3:: *btuE*	6.52	4.04	–0.414	4.83
E. coli W3110 *zwf*::km	6.67	*3.12**	*–0.592*	*3.38*
E. coli W3110 *zwf*::km pNT3:: *zwf*	6.34	3.70	–0.441	4.54
E. coli W3110 *soxS*::km	6.68	*3.16**	–0.587	3.40
E. coli W3110 *soxS*::km pNT3:: *soxS*	6.67	4.11	–0.425	4.70
E. coli W3110 *sodA*::km	6.73	*3.37*	*–0.559*	*3.58*
E. coli W3110 *sodC*::km	6.46	*3.24*	*–0.536*	*3.73*
E. coli W3110 *katG*::km	6.37	*3.17*	–0.542	3.69
E. coli W3110 *ahpC*::km	6.65	*3.71*	*–0.456*	*3.82*

*Singificant values ($p < 0.05$).

DISCUSSION

The survival of *E. coli* under different stress conditions requires activation of specific genes at sufficient levels. This synthesis is controlled by multiple regulatory mechanisms. An understanding of the interplay between these multiple regulatory mechanisms would provide insight into the survival of *E. coli* in natural environments. The cytotoxicity of illuminated photosensitizing agents in the presence of oxygen has been recognized since the beginning of the century. It is commonly referred to as the photodynamic effect [17]. Photooxidative stress is one of the stresses which can be seen in nature and in the immune system which the microorganisms must overcome. There is little knowledge about how and under what conditions the effect of this stress changes. It should contribute to both public health and bacterial molecular mechanisms through studying the protective mechanisms and the effective factors on the effectiveness of photooxidative stress.

At the end of the present studies, it was recognised that pH plays an important role in the presence of photooxidative stress. Fatal effect of photooxidative stress increased in *E. coli* at a neutral pH and alkaline pH levels whereas it had no effect at an acidic pH.

In previous studies, using methylene blue and light sources, most of the changes in DNA treated with MB occurred in deoxyguanosine residuals [16]. Oxidative stress response in *E. coli* is accompanied by the activation of two regulons. These regulons are *soxRS* and *oxyRS* [20]. The SoxR regulon in *E. coli* plays important roles in protection against superoxide and nitric oxide stresses [1]. In our study, two regulons are in accordance with the literature and are accepted as highly important in the viability under photooxidative stress. Dye-mediated photooxidations are proceeded by two different pathways [17]. In the first pathway, excited triplet state dyes react directly with an oxidizable substrate. Electron or hydrogen atom transfer generates a semi-oxidized substrate radical and a semi-reduced dye radical. Subsequent reactions of both species with oxygen yield oxidized and modified substrates, regenerated ground state dye, oxygen radicals, and hydrogen peroxide (H_2O_2). In the second pathway, excited state dye triplets are quenched directly by molecular oxygen, yielding ground state dye and a singlet oxygen ($^1O_2$). Photodamage is caused by reactions of singlet oxygen with amino acids, nucleotides, and lipids. Under conditions of high oxygen concentration and in the absence of strong reducing agents, this second pathway is favored by most photosensitizing compounds. The underlying mechanism of the photodynamic effect *in vivo* remains poorly understood. When sensitizer dyes, such as methylene blue or rose bengal, are treated with light, it was seen that a singlet oxygen generally occurred [10]. However, different factors may affect the effectiveness of oxidative stress.

Many changes occur in cells because of phooxidation. This stress causes cells to enter viable but non-culturable state (VBNC) [8], decrease porin protein synthesis [15] and decrease the synthesis of oxidative stress enzymes such as in a seawater medium [9]. In our study, *katE* mutant was mostly affected under photooxidative stress among the studied eleven gene mutants. This gene is controlled by *rpoS*. *rpoS* provides the control of *katE* that plays a role in oxidative stress as in many cases of *E. coli* at stationary phases in starvation stress as in phosphate buffer [19]. The importance of this gene can be related to the exposed photooxidative stress at stationary phase. *katG* is controlled by OxyR. In our study, it was seen that the *katG* mutant was not different from the wild type *E. coli* and it was determined that *katE* was more important than *katG*. We can say that the *katG* gene were not charged in the phosphate buffer as it was induced when it was in a logarithmic phase. As *oxyR* and *soxR* regulator genes were mutant, they gained important sensitivity compared to wild type *E. coli* under photooxidative stress. Kim et al. [10] claimed that the *oxyR* mutant was highly sensitive when exposed to methylene blue and visible light led to a singlet oxygen. Our study proved that the results are in accordance with the literature data. However, the *katE* mutant was more sensitive than the *oxyR* mutant. Also, the *soxR* mutant showed the same similarity with the *oxyR* mutant. Our study demonstrated that *sodB* was more important than *sodA* and *sodC* among the SOD enzymes which

are vital for viability under photooxidative stress. *sodB* is known as superoxide dismutase including Fe. When *sodA* coded Mn-SOD in *E. coli*, *sodC* coded Cu/Zn-SOD. These results suggest that *katE, oxyR, soxR* and *sodB* genes play a more important protective role than other genes in singlet oxygen mediated damage. It indicates that the *katE* mutant may be affected by the regulator more than *oxyR*. Such a case shows that there can be other factors that control the expression of the *katE* gene. Among the studied genes*, btuE, zwf* and *soxS* genes are not as statistically important as *katE*, *oxyR*, *soxR* and *sodB*. The role of genes are proved correctly with the important genes complementation tests. The roles of *sodA, sodC*, *katG* and *ahpC* mutations were statistically not significant regarding the viability under photooxidative stress.

In our study, photooxidation with methylene blue did not have any effect at pH 5 and pH 6, whereas it had effects at pH 7 and especially at pH 8. It shows that photooxidation is more effective at an alkaline pH. Besides *oxyR, soxR* and *sodB* genes in *E. coli*, the most important gene for the viability under photooxidative stres at alkaline pH is *katE*.

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