

Influence of Foreign DNA Introduction and Periplasmic Expression of Recombinant Human Interleukin-2 on Hydrogen Peroxide Quantity and Catalase Activity in *Escherichia coli*

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ABSTRACT

Purpose: Oxidative stress is generated through imbalance between composing and decomposing of reactive oxygen species (ROS). This kind of stress was rarely discussed in connection with foreign protein production in *Escherichia coli*. Effect of cytoplasmic recombinant protein expression on Hydrogen peroxide concentration and catalase activity was previously reported. In comparison with cytoplasm, periplasmic space has different oxidative environment. Therefore, in present study we describe the effect of periplasmic expression of recombinant human interleukin-2 (hIL-2) on H₂O₂ concentration and catalase activity in *Escherichia coli* and their correlation with cell growth. **Methods:** Having constructed pET2hIL2 vector, periplasmic expression of hIL-2 was confirmed. Then, H₂O₂ concentration and catalase activity were determined at various ODs. Wild type and empty vector transformed cells were used as negative controls. **Results:** It was shown that H₂O₂ concentration in hIL-2 expressing cells was significantly higher than its concentration in wild type and empty vector transformed cells. Catalase activity and growth rate reduced significantly in hIL-2 expressing cells compared to empty vector transformed and wild type cells. Variation of H₂O₂ concentration and catalase activity is intensive in periplasmic hIL-2 expressing cells than empty vector containing cells. Correlation between H₂O₂ concentration elevation and catalase activity reduction with cell growth depletion are also demonstrated. **Conclusion:** Periplasmic expression of recombinant hIL-2 elevates the host cell's hydrogen peroxide concentration possibly due to reduced catalase activity which has consequent suppressive effect on growth rate.

Introduction

Prokaryotic expression systems such as *Escherichia coli* have been greatly utilized for production of recombinant proteins; however, they have not been constructed as a foreign protein producer, naturally.¹⁻³ The physiology of the host cell impeded by plasmid presence and the expression of recombinant genes and consequently cellular stress reactions are imposed.^{4,5} Metabolic load is considered as the main reason for cell growth depletion in recombinant cells.² The presence of plasmid and its replication as well as overexpression of recombinant genes are causative factors to metabolic

load.^{6,7} In these conditions, cell growth can be restricted due to the low accessibility of energy and nutrient resources, a condition identified as starvation. Accordingly, expression of many genes for amino acid biosynthesis is repressed. This starvation-like effect seems to be the chief cause for the decreased expression of the foreign gene products in high cell-density cultures.⁸

There are several approaches to improve recombinant protein production, such as selecting high level expression systems, optimizing expression conditions

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for improving protein solubility⁹ and optimization of media formulation.^{10,11} Additionally, in order to control protein production induced stresses, some gene manipulations such as metabolic genes¹²⁻¹⁵ and stress responsive genes¹⁶⁻¹⁸ have been applied in several cases. In the other side, determination of unknown inhibiting factors during foreign protein production and resolving their inhibitory effects could improve the yield of the production theoretically.

Reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are produced as normal by-products of aerobic life. Imbalance between generation and elimination of ROS promotes oxidative stress which causes lethal cell damages.¹⁹⁻²³ Oxidation of various cell constituents as DNA,²⁴ lipids and proteins,²⁵ induces fundamental changes responsible for death.^{26,27} It is established that specific oxidation of thiol groups of proteins involved in detoxification of H_2O_2 and biosynthesis pathway such as cobalamin-independent methionine synthase (MetE) is caused by H_2O_2 -induced oxidative stress. MetE is inactivated by H_2O_2 in *E. coli* which is associated with methionine limitation imposed by oxidative stress.^{28,29}

The respiratory chain can be the source of as much as 87% of the total H_2O_2 production in *E. coli*.³⁰ Most of the H_2O_2 in exponentially growing *E. coli* cultures is generated from superoxide ion ($2O_2^{\cdot-} + 2e^- + 4H^+ \rightarrow H_2O_2 + O_2$) and the generation of superoxide anion and hydrogen peroxide depends on the stage of culture development.³⁰ In *E. coli*, H_2O_2 is removed by two kinds of catalases producing H_2O and O_2 . These enzymes include hydroperoxidase I (HPI), existing during aerobic growth and transcriptionally controlled at various levels,³¹ and hydroperoxidase II (HPII),^{32,33} which is induced during stationary phase.

Having synthesized in the cytoplasm, some of recombinant proteins are sent into the extracytoplasmic spaces chiefly the periplasm.^{34,35} Besides, in order to increase cell productivity and product quality, it is a common strategy to export recombinant products to the periplasm. As its oxidative environment leads to appropriate disulfide bond formation and consequent correct folding,³⁶ less degradation due to presence of fewer proteases³⁷ and the easy extraction of final proteins.³⁸ The presence of superoxide dismutases containing copper plus zinc ions (Cu, Zn-SOD)^{39,40} and KatG (HPI) enzymes metabolizing superoxide anion and hydrogen peroxide in the periplasmic space of *E. coli*, respectively, protects the environment from oxidative damage.²⁰

Reportedly, one of the impacts of starvation on *E. coli* cells is the increasing of some defense proteins responding to oxidative stress. These proteins prevent accumulation of oxidative damage in growth arrested cells.⁴¹ In addition, although, an aerobic environment seems to be preferable for *E. coli* cultivation, oxygen can easily become limited in aerobic fast-growing cultures which influence cell physiology through the accumulation of acetate.⁴² In the other side, the

presence of oxygen can oxidize electron carriers to generate hydrogen peroxide or superoxide anion, resulting in oxidative stress.⁴³ In spite of these known associations, relation of metabolic burden and oxidative stress in foreign protein producing cells has not been investigated inclusively. Recently, we studied H_2O_2 concentration and catalase activity following introduction of foreign DNA and recombinant protein expression. Our results showed a significant elevation in hydrogen peroxide concentration as the most stable component of ROS and reduction of catalase activity as an important H_2O_2 decomposer.^{44,45} This could be considered as a limiting factor in production of recombinant protein. Following our previous reports and considering the benefits of periplasmic expression of foreign proteins and consequently the high interest in production of recombinant proteins in periplasmic space, we aimed to investigate H_2O_2 concentration and catalase activity following periplasmic expression of recombinant human IL-2 as a non-enzymatic and nontoxic as well as non-functional protein for *E. coli* in the recombinant cells. Moreover, correlation between H_2O_2 concentration and catalase activity following recombinant protein expression with cell growth was studied. The special emphasis is given to the comparison of the alteration in H_2O_2 concentration and catalase activity among recombinant protein expressing, foreign DNA introduced and wild type cells.

Materials and Methods

Bacterial strains and culture media

E. coli DH5 α and BL21(DE3) strains were used as host cells for plasmid amplification and recombinant protein expression, respectively. pET-22b(+) expression vector was used for cloning of hIL-2 coding DNA. Bacteria were grown in LB (Bacto-tryptone 10 g/l, yeast extract 5 g/l, and NaCl 10 g/l) or LB agar media supplemented with ampicillin (100 μ g/ml) in shaker incubator overnight at 37 °C.

PCR amplification of hIL-2 DNA and construction of pET2hIL2 plasmid

Plasmid r-PWhIL-2B7.MA (a kind gift from Dr. Joop Gaken King's College London, London) was used as the template DNA for mature IL-2 encoding DNA amplification using polymerase chain reaction (PCR) technique. Therefore, a pair of specific forward [5'-CGC GGA TCC TGC ACC TAC TTC AAG T-3'] and reverse [5'-ACT AAG CTT TTA AGT CAG TGT TGA G-3'] primers creating *Bam*HI and *Hind*III restriction sites at 5' and 3' ends of the amplified DNA fragment, respectively, were designed based on human interleukin-2 gene sequence. The primers were supplied from Eurofins MWG Operon Company. The PCR reaction was performed to amplify DNA fragments.

The amplified DNA was isolated by gel electrophoresis, extracted using Qiagen gel extraction

and cloned into pTZ57R/T cloning vector. *E. coli* DH5 α cells were transformed with the cloning solution and were cultured on LB medium containing ampicillin, IPTG and X-gal. Treatment of the clones with *Bam*HI and *Hind*III enzymes resulted in the release of hIL-2 coding DNA with sticky ends. The released fragment was extracted from the gel and ligated in pET-22b(+) expression vector resulting in construction of pET2hIL2 vector (Figure 1)

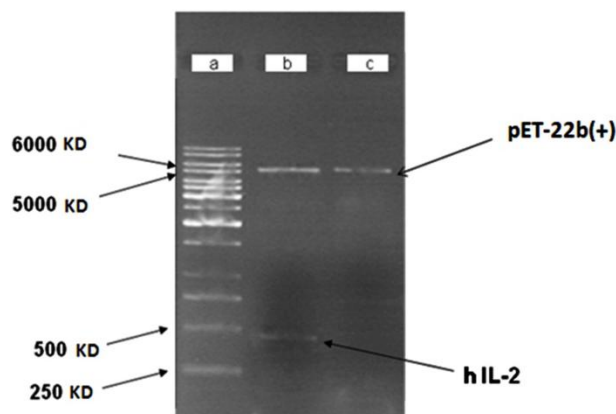


Figure 1. Agarose gel electrophoresis of pET-22b(+) and pET2hIL2 plasmids after digestion with *Bam*HI and *Hind*III restriction enzymes. a) 1kb DNA ladder, b) digested pET2hIL2 containing of hIL-2 and c) digested empty vector pET-22b(+).

Evaluation of hIL-2 expression

Having treated with IPTG (0.5 mM), cultures were incubated and then cells harvested by centrifugation at 4000 rpm. Pellets resuspended in 50 μ l of loading buffer (2X SDS gel-loading buffer) and 50 μ l of 10 mM phosphate buffer (pH 7.0) containing lysozyme. In order to extract periplasmic proteins, the solution was sonicated for 10 minutes and total proteins were isolated after the pellet resuspension in 50 μ l of loading buffer and 50 μ l of H₂O and boiled for 15 minutes. Then, SDS-PAGE electrophoresis gel (12% separating gel and 5% stacking gel) was used for evaluation of recombinant hIL-2 expression.

Growth curve

In order to draw the growth curve of the cells, optical density (OD) of the cultures was measured spectrophotometrically at 600 nm every half hour. Having adjusted the OD of overnight grown cultures at 1, the samples were diluted 1/100 (v/v) and incubated. As a means to investigate the consequence of recombinant protein expression on the growth rate, the media were treated with IPTG with final concentration of 0.5 mM at OD 0.5. As all following studies, wild type untransformed and pET-22b(+) transformed cells were used as negative controls.

Measurement of H₂O₂ concentration

Sample preparation

1.5 ml of bacterial culture was collected at ODs 0.6, 0.7, 0.8 and 1.2 by centrifugation at 13000 rpm for 10 min. The pellet was homogenized in 1.5 ml of 0.1%

(w/v) trichloroacetic acid by sonication at 22 KHz for 10 min in an ice bath. After centrifugation again at 14000 rpm for 10 min, the final supernatant was used for H₂O₂ assay.

H₂O₂ measurement

The protocol described by Velikova and colleagues⁴⁶ was used for H₂O₂ assay. 0.5 ml of the supernatant was added to the mixture of 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide and kept in dark place for 10 min. Then, the absorbance of solution was read at 390 nm and H₂O₂ concentration was calculated using the standard curve. The blank sample was made of 1 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide.

Catalase activity assay

Sample preparation for catalase activity and protein estimation

In order to measure catalase activity, suspended cells in 1 ml of buffer (50 mM phosphate buffer (pH 7.0) and 0.5 mM EDTA) were lysed in sonicator at 22 kHz in an ice bath for 10 min. After removing the pellet by centrifuging at 14000 rpm for 10 min at 4 °C, the supernatant was used to determine catalase activity.

Measurement of catalase activity

Catalase activity measurement was based on H₂O₂ decomposition assay spectrophotometrically. H₂O₂ decomposition was measured according to the absorbance difference between 0 and 5 min at 240 nm in 2 ml reaction mixture and quantified based on standard curve. The reaction mixture contained 200 μ l cell extract, 50 mM phosphate potassium buffer (pH 7.0), 0.5 mM EDTA and 10 mM hydrogen peroxide 30%. One catalase activity unit is the amount of enzyme decomposing 1.0 μ mole of hydrogen peroxide per minute at pH 7.0 and 25 °C.

Total protein measurement

Total protein concentration was determined according to Bradford method⁴⁷ using bovine serum albumin as the standard. 200 μ l of cell extract was added to the solution containing 1400 μ l of 50 mM K-phosphate buffer, 0.5 mM EDTA and 400 μ l of Bradford reagent and the absorbance of reaction was measured at 595 nm between 5 to 30 min.

Statistical analyses

All data were represented as means \pm S.E.M of three or four replicates. Statistical analyses were performed using one-way analysis of variance (ANOVA). Statistical assessment of difference between mean values was performed by least significance difference (LSD) test at $p < 0.05$ using SPSS (16 version) software.

Results

Evaluation of recombinant hIL-2 expression

SDS-PAGE analysis was carried out to confirm the expression of recombinant hIL-2 protein.

Bacterial cells were induced with 0.5 mM IPTG at OD 0.5 after and incubation for 2.5 hours in shaker at 37°C. Following the total and periplasmic protein extraction and SDS- PAGE analysis, the existence of a protein band with molecular weight of about 14.5 KDa is corresponding to hIL-2 expression in the cells (Figure 2). Expression of hIL-2 protein was not observed in empty vector transformed and non-induced cells used as negative controls.

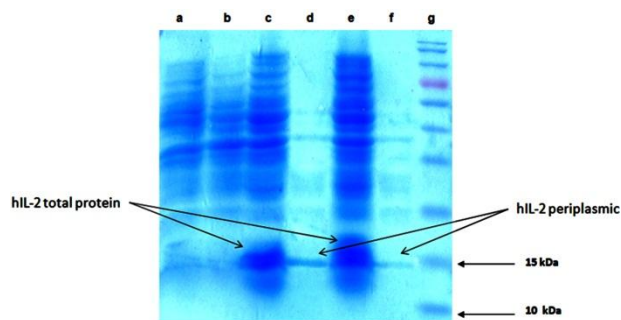


Figure 2. SDS-PAGE analysis of total and periplasmic expression of human interleukin-2. Total protein extracted from non induced pET2hIL2 transformed cells (lane a) and empty pET-22b(+) vector transformed (lane b) as negative controls. Total protein extracted from pET2hIL2 transformed cells after inducing with IPTG 0.5 mM (lane c and lane e), and periplasmic protein extraction pET2hIL2 transformed cells after inducing with IPTG 0.5 mM (lane d and lane f). Lane g is protein ladder.

Growth curve

As shown Figure 3, among three types of bacteria, wild type cells displayed faster growth than recombinant cells. It passes the lag phase and enters exponential phase faster than recombinant bacteria. pET2hIL2 as expressing cells show lower growth than others. It grew slower and passes lag phase and exponential phase later than others and also enters stationary phase earlier than pET-22b(+) and wild type cells. pET-22b(+) as empty vector transformed cells, displayed growth between wild type and expressing cells. It enters exponential phase before hIL-2 expressing cells and after wild type cells. The entrance of empty vector transformed cells into stationary phase was observed after recombinant pET2hIL2 transformed cells, but before wild type.

Measurement of H₂O₂ concentration

H₂O₂ concentration variations are represented in Figure 4. Comparison of bacterial cells H₂O₂ content indicates a significant increase at the amount of H₂O₂ between wild type and hIL-2 expressing cells at each OD. According to Figure 4 increase of H₂O₂ content was observed from OD: 0.6 to OD: 0.7 at all cell types. A noticeable difference between OD: 0.7 and OD: 0.8 was not existed in H₂O₂ quantities, but decrease of H₂O₂ was distinguished at OD: 1.2 in all bacteria. Peak of H₂O₂ amount was $19.43 \pm 1 \mu\text{M}$ at OD: 0.7 related to hIL-2 expressing cells. At the same optical density, pET-22b(+) empty vector transformed cells and wild type cells showed $11.80 \pm 0.65 \mu\text{M}$ and $9.09 \pm 1.11 \mu\text{M}$

H₂O₂ content respectively. Lowest amount of H₂O₂ was measured as $2.56 \pm 0.87 \mu\text{M}$ and $2.26 \pm 0.92 \mu\text{M}$ at OD: 0.6 and 1.2 in wild type cells. At ODs 0.6, 0.8 and 1.2, the highest amount of H₂O₂ was also measured in recombinant hIL-2 expressing cells as $13.61 \pm 0.69 \mu\text{M}$, $13.76 \pm 1.13 \mu\text{M}$ and $7.13 \pm 0.28 \mu\text{M}$ respectively. The amounts of H₂O₂ were $8.94 \pm 1.13 \mu\text{M}$, $11.80 \pm 0.65 \mu\text{M}$, $8.34 \pm 0.66 \mu\text{M}$ and $3.64 \pm 1.02 \mu\text{M}$ from OD 0.6 to 1.2 in pET-22b(+) empty vector transformed cells, approximately, the quantities between wild type and expressing cells. Finally, comparison of H₂O₂ concentration among different types of bacterial cells at the all ODs showed a significant increase on H₂O₂ amount from wild type to pET2hIL2 expressing cells.

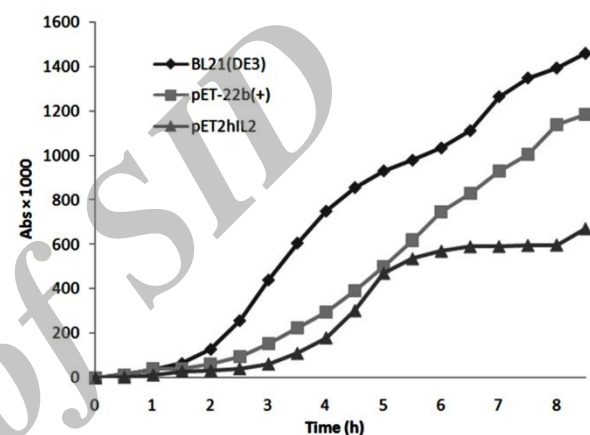


Figure 3. Growth curve of wild type and recombinant *E. coli* cells (pET-22b(+), pET2hIL2) for 8 hours. Optical density was measured every 30 minutes and induction of the cells was done with IPTG (0.5 mM) at OD: 0.5 by 0.5 mM IPTG.

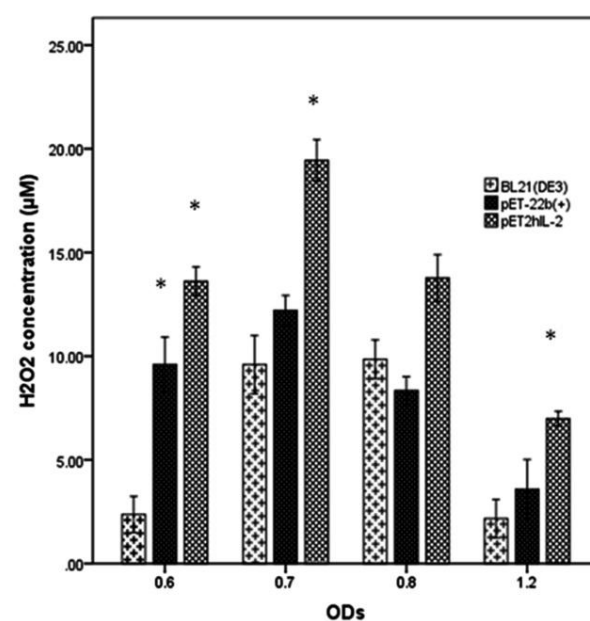


Figure 4. H₂O₂ concentration of wild type (BL21(DE3)) and recombinant *E. coli* cells (pET-22b(+), pET2hIL2) at OD 0.6, 0.7 0.8 and 1.2. Significant increase in H₂O₂ concentration is observed from BL21(DE3) to recombinant cells which is shown by "*" ($p < 0.05$). All data were represented as means \pm S.E.M of three or four replicates.

Catalase activity assay

Catalase is an endogenous antioxidant enzyme present in all aerobic cells and removes toxic H_2O_2 molecule from the cell by converting it into H_2O molecule. One catalase unit is the amount of enzyme decomposing 1.0 μ mole of hydrogen peroxide per minute at pH 7.0 and 25 °C. As shown in Figure 5, catalase activity was decreased significantly from 11.92 ± 0.72 $Umg^{-1}min^{-1}$ in wild type cells to 9.97 ± 0.61 $Umg^{-1}min^{-1}$ and 5.97 ± 0.78 $Umg^{-1}min^{-1}$ respectively in empty vector transformed cells and expressing cells at OD: 0.6. Wild type cells had maximum catalase activity compared to recombinant bacteria as 16.86 ± 0.66 $Umg^{-1}min^{-1}$ and 17.38 ± 0.76 $Umg^{-1}min^{-1}$ especially at OD: 0.7 and 0.8. Empty vector transformed cells displayed high catalase activity than pET2hIL2 producing cells and low catalase activity than BL21 (DE3) wild type cells at ODs 0.6, 0.7, 0.8 and 1.2 as 9.97 ± 0.61 $Umg^{-1}min^{-1}$, 12.05 ± 0.59 $Umg^{-1}min^{-1}$, 12.68 ± 1.04 and 9.85 ± 0.67 $Umg^{-1}min^{-1}$. The measured catalase activity at all ODs (0.6, 0.7, 0.8 and 1.2) was demonstrated the noticeable decrease from wild type to pET-22b(+) empty vector transformed cells and pET2hIL2 expressing cells. Finally, catalase activity among different types of bacteria at all ODs showed a significant decrease on the level of catalase activity from wild type to pET2hIL2 expressing cells and also, high level of catalase activity was shown at OD: 0.7 and 0.8 related to wild type cells.

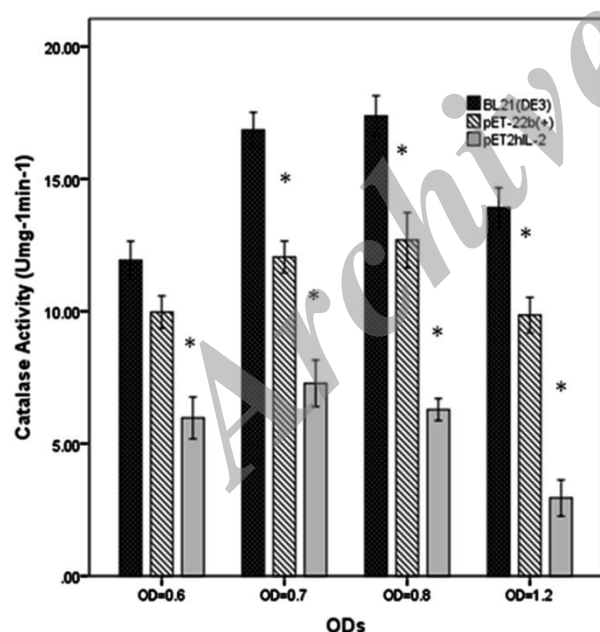


Figure 5. Catalase activity of wild type and recombinant *E. coli* cells (pET-22b(+), pET2hIL2) at OD 0.6, 0.7, 0.8 and 1.2. Significant decrease in catalase activity is distinguished among wild type cells and recombinant *E. coli* cells which are shown by *** ($p < 0.05$). All data were represented as means \pm S.E.M of three or four replicates.

Discussion

Reaching high levels of recombinant protein production impairs the metabolism of host cell⁴⁸ even if the protein itself is non toxic or has no obvious biological activity

on the cell.^{2,3} These challenges often occur in the place where recombinant proteins locate. All recombinant proteins are first produced and mostly sited in the cytoplasm.² However, in *E. coli*, foreign gene products can be placed in various intracellular compartments, such as the cytoplasm, inner membrane, periplasm, and outer member⁴⁹ or can be secreted extracellularly.⁵⁰

Aerobic microorganisms are always under the risk of oxidative stress following imbalance between generation and detoxifying of ROS.⁵¹ Nevertheless, *E. coli* and other cells are equipped with several antioxidant enzymes against consequent oxidative damage. For instance, *E. coli* has two forms of superoxide dismutase (MnSOD and FeSOD)⁴⁰ and two forms of catalase (HPI and HPII).³³ SOD decomposes the superoxide radical into hydrogen peroxide and oxygen, and catalase catalyses hydrogen peroxide into molecular oxygen and water.⁵²

Considering recombinant protein expression as a kind of challenge in the host cells, ROS generation could be one of the causative factors for reduced cell growth and protein production in recombinant cells. This hypothesis was proved in our previous study on cytoplasmic hIL-2 and mouse interleukin-4 (mIL-4) expressing cells. The present study aimed to evaluate ROS generation and cell growth rate following of recombinant protein expression in periplasm, as a location with different aspects of oxidation properties.

The best conditions for recombinant protein production such as bacterial hosts, expression systems and products' purification are so important.⁵³⁻⁵⁵ We have already reported H_2O_2 generation following expression of recombinant proteins in *E. coli*.^{44,45} In this study the effect of periplasmic expression of hIL-2 on H_2O_2 concentration and catalase activity was investigated. To achieve this goal variation in H_2O_2 concentration and catalase activity were examined in wild type and recombinant cells containing pET-22b(+) and pET2hIL2 vectors. Both transformed cells (pET-22b(+) and pET2hIL2) are ampicillin resistant cells, but pET2hIL2 expressing cells has an extra sequence of hIL-2 gene compared to empty vector pET-22b(+). Assuming that the only difference between pET2hIL2 and pET-22b(+) transformed cells is hIL-2 protein production, the various changes in H_2O_2 concentration and catalase activity in hIL-2 expressing cells and empty vector transformed cells is attributed to absolute effect of the recombinant protein expression. Recombinant cells and wild type cells were also compared in terms of growth rate.

Growth curve analysis (Figure 3) showed the fastest and the highest growth rate for wild type cells. Wild type cells passed lag phase and entered exponential phase faster than recombinant cells. Among recombinant cells, periplasmic protein expressing cells grew slower than wild type cells and empty vector harboring cells. Therefore, empty vector transformed cells had a growth rate between wild type cells and hIL-2 expressing cells. The elevated H_2O_2

concentration was observed in recombinant cells compared to wild type cells. Between transformed cells, H₂O₂ concentration was increased significantly in periplasmic hIL-2 expressing cells compared to empty vector harboring cells (Figure 4). These variations derive from the presence of foreign DNA and recombinant protein expression in recombinant bacterial cells. In contrast to the increase of H₂O₂ concentration, reduction of catalase activity was observed in recombinant cells. Catalase activity was decreased significantly in recombinant cells compared to wild type cells. Maximum catalase activity was observed in wild type cells. Recombinant periplasmic hIL-2 expressing cells had the lowest catalase activity and empty vector pET-22b(+) harboring cells showed quantities between expressing cells and wild type cells, like other comparative instances (Figure 5). The results obtained from the present study show that entrance of foreign DNA in host cells reduces growth rate of recombinant bacteria which is in accordance with H₂O₂ accumulation and catalase activity decline. Variation of H₂O₂ concentration and catalase activity is intensive in periplasmic hIL-2 expressing cells than empty vector containing cells. These results revealed a correlation between expression of recombinant hIL-2 protein and changes in H₂O₂ amount and catalase activity. Our findings suggest complementary studies to elucidate the effect of ROS elimination on the improvement of growth rate and recombinant protein production in recombinant cells.

Conclusion

It is concluded that periplasmic hIL-2 expression affects the host cell's hydrogen peroxide concentration possibly due to reduced catalase activity. These effects results in suppression of the growth rate of the recombinant cells meaning that in addition to metabolic load, "H₂O₂ upshift stress resulted from hIL-2 expression" could be considered as a reason for cell growth repression. Additionally, comparison of recombinant hIL-2 expression and introduction of DNA (empty plasmid) into the cells showed that influence of recombinant hIL-2 expression on the H₂O₂ concentration elevation and catalase activity reduction is more than the effects of empty vector introduction.

Conflict of Interest

The authors report no conflicts of interest.

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