Cytoplasmic expression of recombinant interleukin-2 and interleukin-4 proteins results in hydrogen peroxide accumulation and reduction in catalase activity in *Escherichia coli*

*[1,2]Hejazi M.S., [3,4]Karimi F., [1]Mehdizadeh Aghdam E., [5]Barzegari A., [1]Farshdosti Hagh M., [1]Parvizi M., [1,6]Mahmoodi Azar L., [5]Hejazi M.A.

[1]Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, [2]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, [3]Department of Biotechnology, Faculty of Science, Maragheh University, Maragheh, [4]Pharmaceutical Nanotechnology Research Center, Tabriz University of Medical Sciences, [5]Agriculture Biotechnology Research Institute of Iran (ABRII)- Northwest & West Region, Tabriz, [6]Islamic Azad University, Science and Research Branch of Tehran, Iran

Received 4 Apr 2009; Revised 7 July 2009; Accepted 15 July 2009

ABSTRACT

Background and the purpose of the study: The Reactive oxygen species (ROS) is induced in the cells following various stresses but the effect of recombinant protein expression on ROS generation has not been studied yet. In this study, H_2O_2 concentration and catalase activity variations and their correlation with cell growth following cytoplasmic expression of human interleukin-2 (hIL-2) and mouse interleukin-4 (mIL-4) in *Escherichia coli* were investigated. Additionally, the effect of recombinant protein expression under different conditions was compared to the effect of foreign DNA introduction on these factors.

Methods: Plasmids pEThIL-2 and pETmIL-4 were used for expression of human interleukin-2 (hIL-2) and mouse interleukin-4 (mIL-4) inside the cytoplasm of the cells. Having confirmed protein expression using SDS-PAGE analysis and ELISA assay, H_2O_2 concentration and catalase activity were measured at various ODs.

Results and major conclusion: Empty vector introduction increased significantly H_2O_2 concentration of the cells. However, H_2O_2 concentration in hIL-2 and mIL-4 expressing cells was significantly higher than its concentration in empty vector transformed cells. Catalase activity was reduced in foreign DNA introduced cells. It was more lowered following expression of recombinant proteins. Results of this study revealed the relationship between foreign DNA introduction and protein expression with H_2O_2 elevation and catalase activity reduction. There was also correlation between H_2O_2 elevation and reduction in catalase activity with the cell growth depression.

Keywords: Hydrogen peroxide, catalase activity, cytoplasmic expression, *Escherichia coli*, recombinant protein

INTRODUCTION

A great number of studies have been reported on the effect of foreign DNA introduction on the host cells metabolism. Introduction and expression of foreign DNA in host organisms often changes and impairs the organism's normal metabolism. This kind of metabolism alteration arises from the metabolic load. Certain amount of cellular energy is required for the cell function and metabolism. This is while introduction of a recombinant DNA with a bigger size or with a higher copy number requires a higher amount of energy. Metabolic burden may result in cell metabolism perturbation, gene expression alteration and the relatively lower growth rate when greater amounts of energy and cell components are required especially for recombinant protein production (1−9). This effect eventually leads to reduction in the yield of protein production (10, 11). Lose of plasmid by the recombinant cells is another alteration which is caused by metabolic load (10, 12).

Reactive oxygen species (ROS) are produced in various cells from prokaryotic to human cells. ROS including H_2O_2, superoxide (O_2^-) and

Correspondence: saeidhejazi@tbzmed.ac.ir

hydroxyl radicals ($OH^{\cdot}$) are generated at high levels either by normal aerobic metabolism or exogenous factors and stresses (13−17). Superoxide anion and hydrogen peroxide give rise to production of other ROS components that are strong cytotoxic agents. Hydrogen peroxide is the most stable component of ROS. Antioxidant enzymes including catalase protect the microorganisms and biomolecules from ROS-mediated damages (18, 19).

Enormous efforts have been devoted to increase cell growth and production of recombinant proteins production from molecular cloning stage to bioprocess procedures. Identification of preventive factors helps to resolve their inhibitory effects and to improve the yield of the production. Regardless of the number of studies on ROS generation, no clear and definite relation between recombinant protein expression and ROS generation has been reported yet. Being aware of cell growth reduction in recombinant cells including *E. coli*, it was hypothesized that ROS generation could be one of the causative factors for reduced cell growth and production in recombinant cells.

This study aimed to investigate the relation between recombinant protein expression and H_2O_2 concentration as the most stable component of ROS and catalase activity as the most important enzyme in H_2O_2 detoxification. To achieve this goal, human interleukin-2 (hIL-2) and mouse interleukin-4 (mIL-4) as non-enzymatic, nontoxic and inert proteins were expressed inside the cytoplasm of *E. coli*. In this paper (a) the effect of hIL-2 and mIL-4 expression on H_2O_2 accumulation, (b) the effect of proteins expression on catalase activity, (c) comparison between the effect of recombinant protein expression and the influence of foreign DNA introduction on H_2O_2 concentration and catalase activity, (d) relation between H_2O_2 generation and catalase activity with protein expression induction and cell growth are described.

MATERIAL AND METHODS

Molecular biology reagents

Primers mIL4F (5' –ACT GGA TCC CAT ATC CAC GGA TGC GAC- 3') and mIL4R (5' –GGC AAG CTT CTA CGA GTA ATC CAT TTG CA-3'), supplied by MWG-BIOTECH, were used as forward and reverse primers for amplification of mIL-4 DNA. Luria-Bertani (LB) (Bacto-tryptone 10g/l, yeast extract 5g/l, and NaCl 10g/l with pH=7.0, all purchased from Merck-Germany) was used as culture medium, and ampicilline (100 mg/ml) was added into the medium when it was required to maintain selected pressure. Human IL-2 ELISA kit was purchased from Bender Med Systems. Retroviral based pWZLIL/4B7M expression plasmid (a kind gift from Dr. Joop Gäken King's College London, London) was used as the DNA template for PCR amplification of mature mIL-4 (composed of 120 amino acids) encoding DNA. pTZ57R/T was used as cloning vector and pET21a(+) was used as prokaryotic expression vector in which expression of DNA of interest is under the control of inducible T7 promoter (20, 21). pET21a(+) empty vector was also used as the negative control in hIL-2 and mIL-4 expression studies.

Plasmid pEThIL-2, constructed in this lab (22), was used for expression of hIL-2. This vector expresses human mature IL-2 composed of 133 amino acid residues under the control of inducible T7 promoter.

PCR amplification of mIL-4 DNA and construction of pETmIL-4 vector

PCR amplification of mIL-4 DNA was carried out using mIL4F and mIL4R primers. These primers created *Bam*HI and *Hin*dIII restriction sites at 5′ and 3′ ends of the amplified DNA, respectively. PCR amplification was performed under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 54°C for 30 sec and 72°C for 30 sec and final extension was performed at 72°C for 10 min.

Amplified DNA was cloned into pTZ57R/T cloning vector and its subcloning into pET-21a(+) expression vector was carried out using *Bam*HI and *Hin*dIII sites. All DNA manipulation procedures were conducted based on common molecular biology protocols (23).

Evaluation of recombinant hIL-2and mIL-4 expression

E. coli BL21(DE3) competent cells were transformed using heat shock technique. Bacterial transformants were cultured and isopropyl-beta-D-thiogalactoside (IPTG) as the expression inducer was added into the medium with final concentrations of 0.25 and 0.5 mM at desired ODs. The bacterial cells were harvested 2 hrs after IPTG treatment and total cytoplasmic proteins were analyzed by SDS-PAGE and ELISA methods. SDS-PAGE was performed by a modified method as described previously (12-13% separating gel, 5% stacking gel) and gels were stained with comassie brilliant blue. In this experiment, wild type (nontransformed) and non-induced related transformed cells were used as negative controls. Expression of hIL-2 was further evaluated using ELISA kit.

Growth curve

In order to draw the growth curve of the cells, the bacterial cells were cultured overnight in LB. The OD of overnight grown cultures was adjusted at 1 and then the samples were diluted to 1/100 (v/v) and incubated at the same temperature. In order to study the effect of recombinant protein expression on the cell's growth rate, the media were supplemented with IPTG with final concentration of 0.5 mM at OD 0.5. OD of cultures was measured spectrophotometrically at 600 nm every half hr. Additionally, two more experiments were conducted by diluting and adjusting the overnight culture to ODs of 0.1 and 0.2 respectively.

Cell extraction for H_2O_2 assay

For this purpose 1.5 ml of bacterial culture at ODs 0.65, 0.7 and 0.8 was centrifuged at 13000 rpm for 15 min. The pellet was resuspended in 5ml of 0.1% (w/v) trichloroacetic acid in an ice bath, sonicated at 22 kHz for 10 min and centrifuged again at 13000 rpm for 15 min. The supernatant was transferred into a new tube for H_2O_2 assay (24).

H_2O_2 measurement

H_2O_2 level was determined according to the protocol described by Velikova and colleagues (24). To a tube containing 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide was added 0.5 ml of supernatant. The absorbance of the supernatant was measured immediately at 390 nm. The blank was run without the sample extract. The amount of H_2O_2 was calculated from the standard curve which was made earlier with known concentrations of H_2O_2.

Cell extraction for protein estimation and catalase activity assay

Catalase activity was measured at ODs of 0.6 and 0.7. Cell-free extracts were prepared by lysing the cells using sonication at 22 kHz in an ice bath for 10 min. Cell debris were removed by centrifugation at 14000 rpm for 15 min (25) and protein concentration was determined by Bradford method (26). Catalase activity was assayed on the basis of H_2O_2 decomposition (extinction coefficient of 39.4 mM cm^{-1}) through measurement of the decrease in absorbance at 240 nm. Hydrogen peroxide consumption was assayed in 2 ml of solution containing 50 mM potassium-phosphate buffer (pH 7.5), 0.5 mM EDTA, 10 mM hydrogen peroxide, and 20 µl of the sample extract. The blank was prepared without the extract. One unit of catalase activity was defined as the amount of supernatant protein that utilizes 1 µmol of hydrogen peroxide per minute under the employed conditions ($Umg^{-1}min^{-1}$) (27).

Growth rate and H_2O_2 determination in different concentrations of IPTG

To study the effect of different concentration of IPTG on the cell's growth rate and H_2O_2 level, wild type *E. coli* BL21(DE3) and transformed BL21(DE3) cells harboring pETmIL-4 were induced with IPTG with final concentrations of 0.05, 0.01, 0.25, 0.5, 1 mM at OD 0.5 in different tubes. H_2O_2 assay was carried out at OD 0.7 as explained above.

Statistical analyses

All data were represented as means ± 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 software.

RESULTS

Construction of pETmIL-4 plasmid

Mouse mature IL-4 encoding DNA was amplified using PCR technique and the amplification result was evaluated using gel electrophoresis. The presence of a DNA band with about 360 nucleotides size confirmed the amplification of mIL-4 encoding DNA. The mIL-4 encoding DNA was cloned into pTZ57R/T vector and then subcloned into pET21a(+) using *Hin*dIII and *Bam*HI sites. The success of cloning was evaluated by double digestion of the resultant plasmids with *Hin*dIII and *Bam*HI enzymes. The release of a DNA band with about 360 nucleotides represented the presence of mIL-4 encoding DNA inside the vector (Fig. 1). The constructed plasmid was called pETmIL-4, in which IL-4 DNA is driven by inducible T7 promoter.

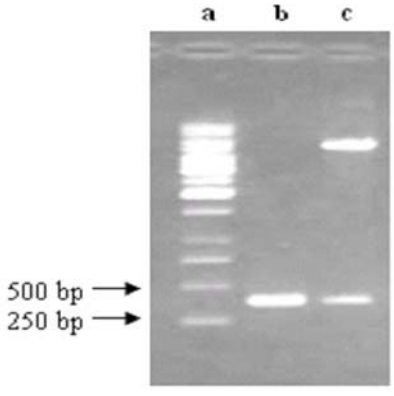

Figure 1. Agarose gel electrophoresis of PCR amplification of mIL-4 encoding DNA and digestion of constructed pETmIL4 plasmid: Lane **a)** 1Kb DNA ladder; lane **b)** PCR product; lane **c)** digestion of pETmIL4 plasmid with BamHI and HindIII restriction enzymes.

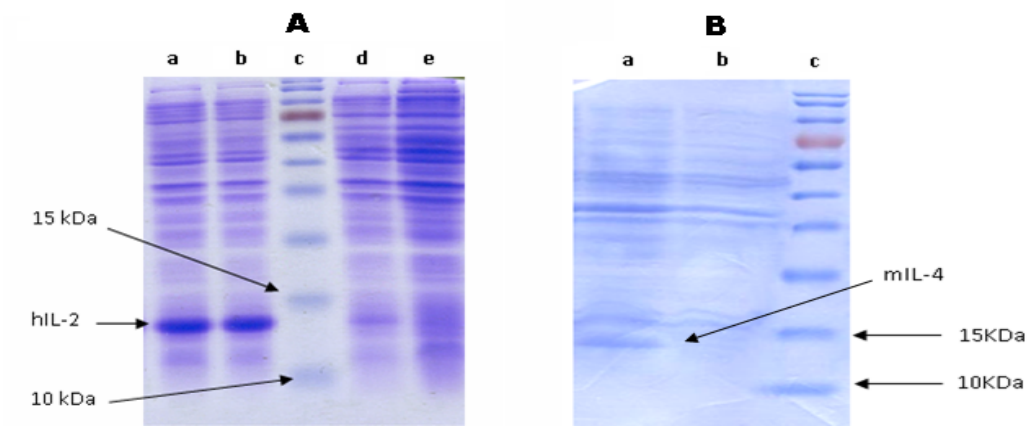

Figure 2. (A) SDS-PAGE analysis for hIL-2 expression. Lanes **a** and **b** show hIL-2 protein band from pEThIL-2 transformed cells treated with 0.25 and 0.5 mM IPTG, respectively; lane **c** is protein marker; lanes d and e demonstrate cytoplasmic proteins of non-induced pEThIL-2 transformed and wild type cells, respectively. **(B)** SDS-PAGE analysis for mIL-4 expression. Lane **a** shows mIL-4 protein band revealing cytoplasmic expression of mIL-4 in IPTG treated pETmIL-4 cells; lane **b** is extracted protein analysis of wild type cells; lane **c** is protein marker.

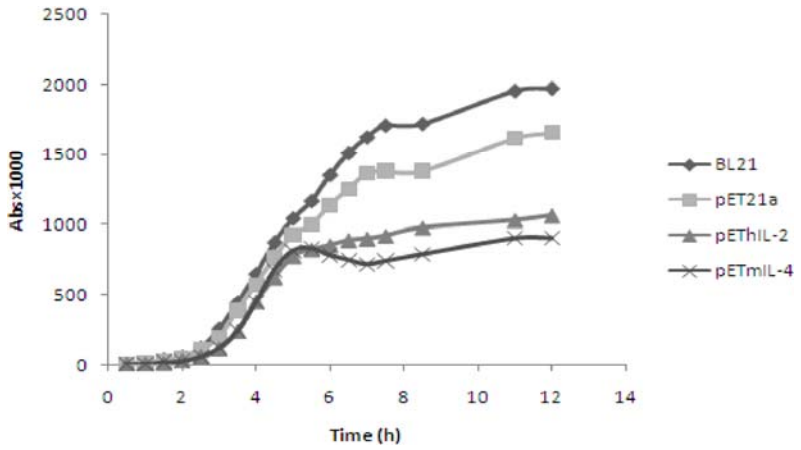

Figure 3. Growth curve of wild type and recombinant *E. coli* cells for 12 hours. Cells were induced by 0.5 mM IPTG at OD 0.5.

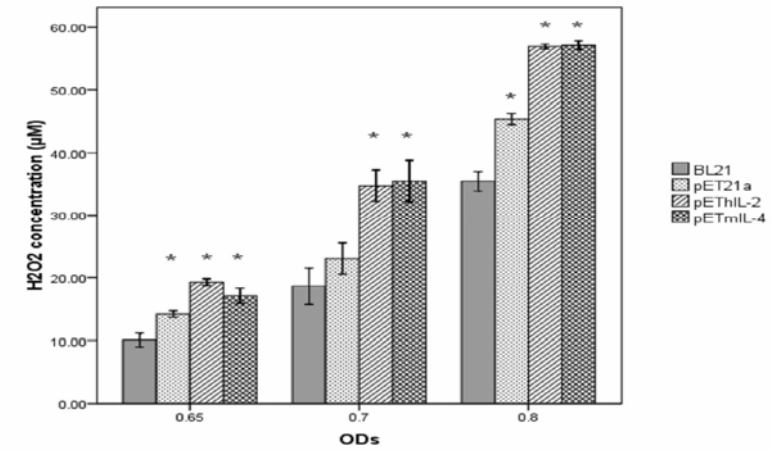

Figure 4. H_2O_2 concentration of wild type and recombinant *E. coli* cells at OD 0.65, 0.7 and 0.8. "*" represents significant difference in H_2O_2 concentration compared to the wild type and empty vector harboring cells at the same OD ($p<0.05$).

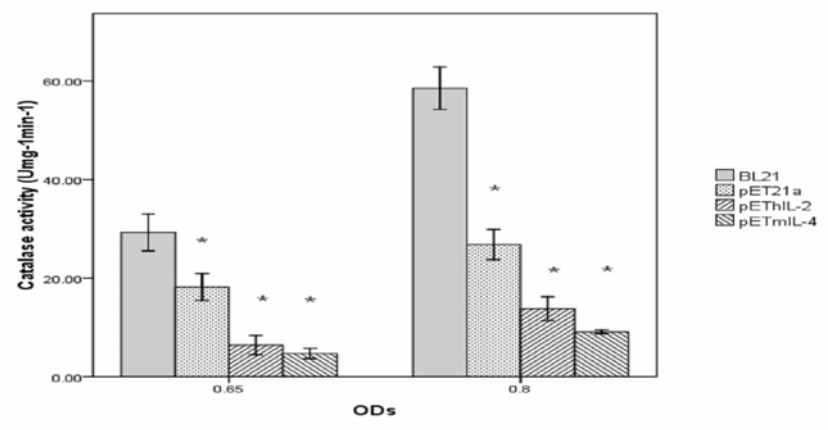

Figure 5. Catalase activity of wild type and recombinant *E. coli* cells at OD 0.7 and 0.8.
"*" shows significant reduction of catalase activity compared to the wild type and empty vector harboring cells at the same OD ($p<0.05$).

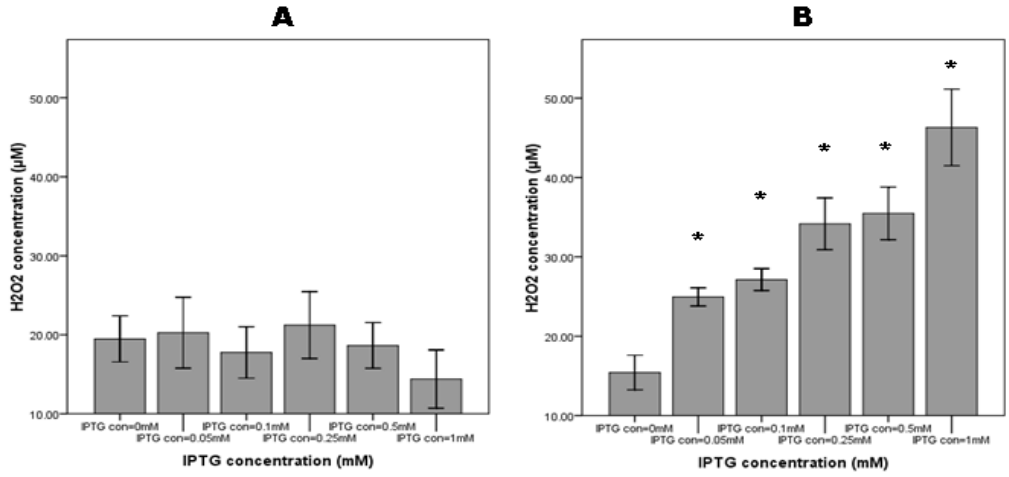

Figure 6. Effect of different concentrations of IPTG on H_2O_2 concentration in: (**A**) wild type *E. coli* cells and (**B**) pETmIL-4 transformed cells at OD 0.7. "*" indicates significant difference in the amount of H_2O_2 concentration compared to the cells that were not induced with IPTG ($p<0.05$).

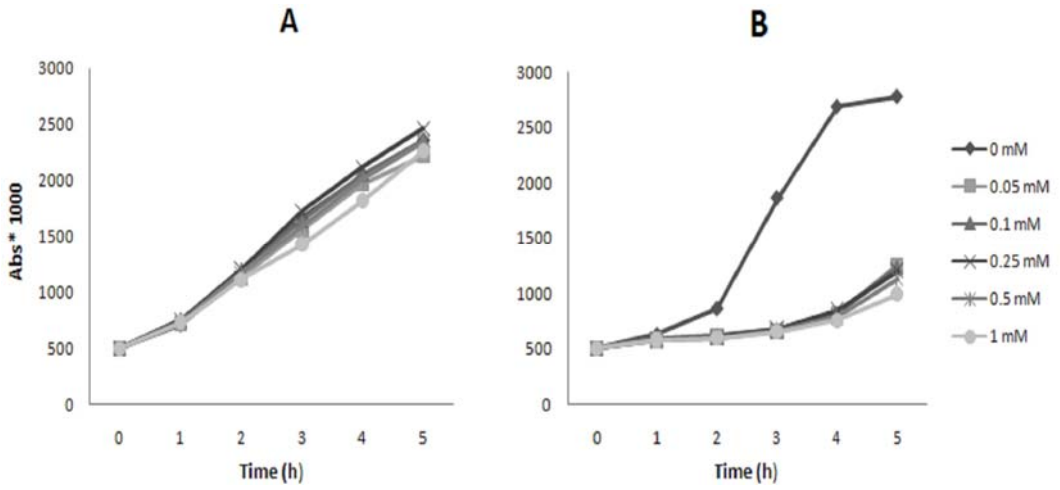

Figure 7. Effect of different concentrations of IPTG on the growth rate of: (**A**) wild type *E. coli* and (**B**) recombinant *E. coli* cells harboring pETmIL-4.

Recombinant hIL-2 and mIL-4 expression analysis
As illustrated in Fig. 2A, the presence of a sharp protein band with about 14.5 kDa weight corresponding to human IL-2 in induced recombinant bacteria and the absence of this band in wild type bacteria confirmed the expression of the recombinant protein in induced bacteria. Expression of hIL-2 was further confirmed quantitatively by ELISA (data not shown). Expression of mouse IL-4 was also confirmed by SDS-PAGE analysis (Fig. 2B).

Growth curve
Fig. 3 represents the effect of DNA introduction and recombinant proteins (hIL-2 and mIL-4) expression on *E. coli* growth rate. As shown in Fig. 3, the wild type cells entered exponential phase before recombinant cells, i.e. empty vector transformed, hIL-2 and mIL-4 expressing cells. Based on exponential phase, three different growth rates were identified. Wild type cells displayed the fastest growth with a doubling time of 32 min, while hIL-2 and mIL-4 expressing cells had the lowest growth rate with a doubling time of 40 min. pET21a(+) empty vector transformed cells grew slower than wild type cells and faster than hIL-2 and mIL-4 expressing cells. Doubling time of empty vector transformed cells was 35 min. Empty vector transformed cells entered the stationary phase after hIL-2 and mIL-4 expressing cells. The entrance of wild type cells into stationary phase was observed after the other three cell types. Accordingly, the wild type cells showed the most concentrated, the empty vector transformed cells illustrated moderate and recombinant proteins expression cells showed the lowest cell densities. The similar comparative results were obtained from experiments carried out by starting OD of 0.1 and 0.2 as explained at section 2.4 (data not shown).

H_2O_2 concentration assay
As it is shown in Fig. 4, the amount of H_2O_2 concentration was increased significantly ($p<0.05$) from 10.11 ±1.88 µM in wild type cells to 14.21 ± 0.89 µM in the cells harboring empty pET21a(+) vector at OD 0.65. However, the highest amount of H_2O_2 was measured in recombinant hIL-2 and mIL-4 expressing cells as 19.28 ± 0.89 and 17.13±2.05 µM, respectively. H_2O_2 concentration in these cells was significantly higher than that of empty vector transformed cell. At ODs 0.7 and 0.8, the level of H_2O_2 concentrations in wild type cells were 18.64 ± 2.90 and 35.47 ± 2.68 µM, respectively, which elevated to 23.03 ± 2.50 and 45.43 ± 1.55 µM ($p<0.05$) in empty vector transformed cells. Moreover, concentration of H_2O_2 in hIL-2 and mIL-4 expressing cells were 34.74 ± 2.52 and 35.47 ± 3.32 µM at OD 0.7, respectively. At OD 0.8, H_2O_2 concentrations of hIL-2 and mIL-4 expressing cells were 56.94 ± 0.67 and 57.13±1.17 µM, respectively, which were significantly higher than those of wild type and empty vector transformed cells.

Catalase activity assay
According to Fig. 5, catalase activity in wild type cells was 29.26 ± 3.69 $Umg^{-1}min^{-1}$, which decreased signifacntly to 18.21 ± 2.73 $Umg^{-1}min^{-1}$ in empty vector transformed cells at OD of 0.7. Catalase activity was further decreased significantly at OD 0.7 to 6.42 ± 1.95 and 4.73 ± 1.05 $Umg^{-1}min^{-1}$ in hIL-2 and mIL-4 producing cells, respectively. The same concept of catalase activity was observed at OD 0.8. Wild type cells had the highest level of catalase activity of 58.51 ± 4.29 $Umg^{-1}min^{-1}$, which decreased significantly to 26.82 ± 3.07 $Umg^{-1}min^{-1}$ in empty vector transformed cells. Expressing hIL-2 and mIL-4 cells displayed the lowest activity of catalase. Catalase activity in hIL-2 and mIL-4 expressing cells was 13.81 ± 2.4 and 9.11 ± 0.36 $Umg^{-1}min^{-1}$, respectively.

Growth curve and H_2O_2 level at different IPTG concentrations
Results showed an increase in H_2O_2 level from the lowest IPTG concentration to the highest one in pETmIL-4 harboring cells (Fig. 6B). However the wild type cells didn't display considerable variations in H_2O_2 concentrations (Fig. 6A). Besides, although wild type cells demonstrated almost the same growth pattern in various IPTG concentrations (Fig. 7A), a t reduction was observed in the growth rate of pETmIL-4 harboring cells in the presence of various concentrations of IPTG (Fig. 7B).

DISCUSSION

E. coli is a versatile bacterium for production of recombinant proteins. Introduction of foreign DNA and expression of recombinant protein often lead to metabolic load. Metabolic load lowers the cell's growth rate (1−9). ROS such as H_2O_2 can be generated by both normal aerobic metabolism and exogenous factors and could result in oxidative stress (15−17). Catalase detoxifies harmful effects of H_2O_2 by converting it to H_2O (18,19).
Reviewing literature didn't show any report about the effect of recombinant protein expression on ROS production. Having noticed the lack of connection in literature between ROS generation and recombinant protein production in microorganisms, the effect of cytoplasmic recombinant protein expression on ROS

production was investigated. The present study relies on the hypothesis that recombinant protein expression might induce ROS generation that eventually harms the recombinant cells.

It is clear that transformation of empty pET21a(+) vector into the cells means introduction of foreign DNA along with expression of β-lactamase as recombinant ampicillin resistant protein. Introduction of pEThIL-2 and pETmIL-4 vectors followed by hIL-2 and mIL-4 expression will have the same consequences as the introduction of empty vector pET21a(+), except that the extent of recombinant protein expression in the prior cases will be remarkably more than its amount in the latter case. Assuming that the only difference between recombinant protein expressing cells and pET21a(+) transformed cells is in hIL-2 and mIL-4 production, the difference in H_2O_2 concentration and catalaze activity in hIL-2 and mIL-4 producing cells with empty vector transformed cells may be attributed to the absolute effect of recombinant protein expression on H_2O_2 concentration and catalase activity. This is while, comparison of these values in empty vector transformed cells with wild type cells, mainly reflects the influence of foreign DNA introduction.

Growth rate analysis (Fig. 3) showed that wild type cells entered exponential phase before recombinant cells. Empty vector transformed cells grew slower than wild type cells and faster than hIL-2 and mIL-4 expressing cells. These results are in accordance with the alteration in H_2O_2 concentration and catalase activity in these cell types. Wild type cells had the lowest amount of H_2O_2 compared to recombinant cells with a significant difference (Fig. 4). Among the recombinant cells, H_2O_2 concentration in hIL-2 and mIL-4 producing cells was significantly higher than its concentration in empty pET21a(+) transformed cells. These results clearly confirmed the increasing effect of foreign DNA introduction and recombinant protein expression on H_2O_2 concentration. Catalase activity assay (Fig. 5) demonstrated a significant reduction in this enzyme's activity in the empty vector transformed cells compared to wild type cells. Catalase activity was further reduced significantly in hIL-2 and mIL-4 expressing cells compared to empty vector transformed cells demonstrating reduction in enzyme activity upon foreign DNA introduction and recombinant protein expression. On the other hand, the effect of various concentrations of IPTG on H_2O_2 level (Fig. 6B) demonstrated that increase in IPTG concentration in pETIL-4 harboring cells results in higher increase in H_2O_2 concentration in mIL-4 expressing.

Accordingly, these results led us to conclude that: (I) recombinant proteins (hIL-2 and mIL-4) expression and introduction of foreign DNA induce a condition in the cells that result in a significant increase in H_2O_2 concentration and decrease in catalase activity from the early stages, (II) recombinant protein expression has more effect on H_2O_2 elevation and catalase activity reduction than introduction of empty vector, (III) there is a direct relation between the reduction of catalase activity and H_2O_2 accumulation that might be one of the reasons for H_2O_2 accumulation , (IV) cell growth depression directly correlates with recombinant proteins production, H_2O_2 concentration elevation and reduction in catalase activity.

This observation is quite important and interesting issue that could be addressed as H_2O_2 upshift stress caused by recombinant protein expression during which catalase activity is also reduced. Further reports regarding different aspects of ROS generation in recombinant cells are in progress from our laboratory.

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