A new approach for water purification from microbial pollution

E. Moosavi, V. Martirosyan, S. Ayrapetyan

Abstract—The fact that low concentrations of CO_2 have an activation effect on functional activity of microbes allows us to suggest that CO_2 could elevate the toxic effect of H_2O_2 on cells. To check this hypothesis the dependency of the toxic effect of H_2O_2 on wild type of *Escherichia coli K-12* on soluble concentration of CO_2 in culture media was studied. The obtained data show that culture media enriched with CO_2 leads to the increase of toxic effect of H_2O_2 on microbes at both cases when pH is constant and when it changes.

Keywords— water purification, antibacterial properties, CO_2, H_2O_2, Escherichia *coli K-12*

I. Introduction

As the hazardous effects of environmental pollution on organisms are realized mostly through the water medium, the problem of water purification is considered by the UNESCO and World Health Organization (WHO) as one of the global problems of the Modern Environmental Science [7, 24]. The World Health Organization estimates that about 4.000 children die from water-born diseases every day, while United Nation has shown that more than one billion people are still without access to clean drinking water, and 2.6 billion people have no access to proper sanitation [7]. The investigation of these problems calls forth a great number of researches to be conducted to identify robust new methods of purifying water at lower prices and with less energy [2, 9]. Our research is also directed to find alternative water purification method and namely methods and compositions for disinfecting water in artificial water systems (swimming pools etc.) using CO_2/H_2O_2 combination system in different environmental factors.

Currently H_2O_2 and CO_2 are being used as tools for water purification but separately. If the molecular mechanism of "killing" effect of H_2O_2 on bacteria is well documented [5], the mechanism of "killing" effect of CO_2 on bacteria still remains discussable [6]. Although it is already shown that $SCCO_2$ and H_2O_2 combination make inactivate spores to high degree [10], the authors of the mentioned article do not know the mechanism by which this inactivation takes place and also this combination has not been used as a water purification tool.

In our previous works we showed that the treatment of aqua nutrient medium by extremely low frequency electromagnetic field (ELF EMF) and infrasound (IS) could significantly modulate the growth and the development of microbes in water [21, 25]. Such treatment of water leads to the changes of CO_2 solubility in water and at the presence of oxygen it promotes the formation of reactive oxygen species (ROS) such as: singlet oxygen ($^1O_2$), superoxide radical (O_2^-), peroxide anion (O_2^{-2}), hydroxyl radical (OH·) and hydrogen peroxide (H_2O_2), through which the biological effects of this treatment are realized [16, 22]. On the other hand in our previous work, performed on cells of eukaryotes, it was shown that membrane proteins, determining cell membrane functional activity, are functionally in active and inactive (reserve) states. The ratio of these active and inactive molecules could be changed by the modulation of cell hydration [18, 20]. It was also shown, that metabolic poisons cause cell hydration [19] bringing to the increase of the number of functionally active receptors in the membrane [18]. On the one hand CO_2, being a strong metabolic poison, which leads to the increase of cell hydration, could serve as a potential factor able to elevate membrane sensitivity to H_2O_2 and on the other hand comparatively low concentrations of CO_2 have an activation effect on functional activity of microbes [6]. Thus, it was suggested that CO_2 could elevate the toxic effect of H_2O_2 on cell.

Therefore, working hypothesis for the present work was suggested: the activation of microbe metabolism induced by comparatively low concentrations of CO_2 could be used for the elevation of the toxic effect of H_2O_2 on microbes. This fact and data obtained from our published patent [17] let us suggest that a certain combination of both of "killer" molecules would give us opportunity to develop a novel and safe method for water purification. To check this hypothesis the following experiments were performed: *the study of different-exposure-time-dependent effects of CO_2 on growth of E. coli K-12*; *the study of dose-dependent effects of H_2O_2 on growth of E. coli K-12*; *the study of the antibacterial properties of CO_2/H_2O_2 combination on growth of E. coli K-12*.

II. Problem Formulation

Due to increases of human population and rapid industrialization nowadays water pollution is becoming a serious problem. So, one of the global challenges of the 21st century is to find the best method for cleanup of aquatic ecosystems from microbes, which will be cheap, safe for human health and environmentally friendly. Our research

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relates to a new approach of cleaning water from microbial pollution, which will give an opportunity to solve some of the above mentioned problems. To achieve our goals we have used following methods and materials.

A. Experimental setup and apparatus

The processing apparatus used in this work contained 50 liter CO_2 vacuum tank (Fig. 1 a), the airline tubing with regulator valve (Fig. 1 b) and the CellStar CO_2 incubator (Fig. 1 c) (USA, SWJ500TV BA.), where the samples were located for CO_2 exposure. All the samples, which were incubated in CellStar incubator, were treated by CO_2 for different exposure time period. The appropriated sham tube (control tube) was also placed in the same CO_2 incubator. Compressed carbon dioxide was supplied from a gas cylinder, which was linked to the pressure vessel by a valve (Fig. 1 d). A precision manometer (Fig. 1 e) was installed in the vessel to measure the pressure. The pressure in the vacuum tank could be adjusted between 5 kPa to atmospheric pressure, and the pressure at the end of the experiment could be controlled.

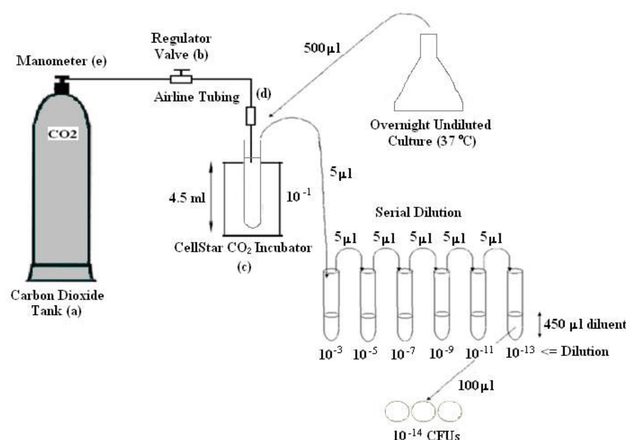

Fig. 1 A schematic blow-up of carbon dioxide into the bacterial culture and schematic representation of the serial dilution and culture.

B. Design and process of experiments

As for the experimental design, three factors were studied, which are the following: CO_2 concentration (mol l^{-1}) or exposure time (min) of CO_2, pH and H_2O_2 concentration (mmol l^{-1}). The main responses of the above mentioned factors bring to the inactivation of bacteria and the acidity of the medium. The experimental tubes containing bacterial culture were placed in the CellStar incubator at the beginning of each experiment, and the temperature was kept constant in this incubator. Than carbon dioxide (Air Liquide, 99.7% pure) was injected into the incubator at the selected pressure (atmospheric pressure). Microbial cells were treated with CO_2 for various exposure times. The apparatus allows us to inject carbon dioxide very quickly into the bacterial culture (within 10 s). The tubes in the incubator were closed and removed, and the survived cells were immediately counted (CFUs count). The inside surfaces of the incubator were wiped clean with cotton moistened with a 70% alcohol solution before each test.

C. Bacterial strains and culture media used

We used the wild type of *Escherichia coli K-12*, which was obtained from the Armenia Collection of Microorganisms (Institute of Microbiology of the Center of Microbiology and Microbial Depository of Armenian NAS). It is a good model of organism for this work, because *E. coli* are widely distributed in waters including natural waters, particularly in tropical regions of the globe and it is closely related to other pathogenic bacteria in the enteric family (*Shigella, Salmonella*, etc.). During the experiment as a culture media we have used nutrient enriched broth (NEB) and nutrient enriched agar (NEA), containing: NEB + 1.5% agar (Difco), final pH 7.1 ± 0.2 at 37ºC.

D. Enumeration of living microorganisms

The viability of *E. coli K-12* was determined by counting the number of colony forming units (CFUs) for per milliliter. Measuring techniques involve indirect viable cell counts, also called plate counts, which is performed by plating out (spreading) a sample of a culture on a nutrient agar surface by triplicate plating. Each treated sample (5 µl) was diluted with 450 µl of an aqueous NEB solution before plating. The contents of the tubes were then shaken well by magnetic stirring for two min. By shaking the tubes the microorganisms dispersed in the solution. The dispersion obtained was then diluted repeatedly, and 100 µl of the appropriate dilution was plated on triplicate petri dishes containing a suitable growth medium. After plated on a suitable medium and after incubation in thermostat (37°C) each viable unit grew and formed colony and the number of CFUs was related to the viable number of bacteria in the sample. Microbial cells in the control samples were counted by the same procedures described for treated samples. The acidity of each treated sample was expressed by measuring the pH of the NEB media.

E. The method of CO_2 concentration measurement

There are several ways of determining the level of CO_2 in liquid media. Common analyze which was done for the measurement of the concentration of CO_2 comprised a reaction of a sample during which a certain amount of alkaline fluid was added, e.g. NaOH [23], to neutralise the carbon dioxide. Through titration the remaining amount of OH^- we can determine the amount of dissolved CO_2. So the content of CO_2 in bacterial culture media was determined by acid-base titration method (quantitative chemical analysis). The time for one titration was about 10 min. The end-point was determined by potentiometrical use of pH meter.

F. Statistical analysis

Statistical analysis was conducted using JMP software (Version 5.1.2, SAS Institute Inc., Cary, NC, USA, 2004). All

experiments were repeated at least three times. The differences among the means of treatments were tested by using *t* test.

III. Problem Solution

In present work we have investigated the effect of CO_2 and subsequent H_2O_2 treatment on the inactivation processes of the bacterial culture of *E. coli K-12*. The increase of CO_2 concentration in liquid and the subsequent addition of H_2O_2 made the inactivation processes of bacterial culture more effective. To check out the above mentioned hypothesis the following studies were done.

A. The study of different-exposure-time-dependent effects of CO_2 on growth of E. coli K-12

Carbon dioxide has a dual physiological role in microorganisms since it can both stimulate and inhibit cell development [3]. At comparatively low concentrations of CO_2 the latter has strong stimulatory effect on the growth and development of microbes [6] while in higher concentrations and in the case of longtime exposure it has inhibitory effect on the growth and development of microbes [14]. Various hypotheses have been proposed to explain the microbicidal activity of carbon dioxide. CO_2 dissolves in water to form carbonic acid. Thus, dissolved CO_2 acts by lowering the pH of the medium, and the resulting acidity leads to a disturbance of some biological systems within cells. It was therefore suggested that microbial inhibition was due to an alteration in the properties of cell (membrane, cytoplasm, enzymes, etc.) [12]. However, a reduction in the pH of the medium is not sufficient to account for the antimicrobial action of CO_2, since it shows a specific inhibitory effect, which is greater than that of the other acids used to lower medium acidity (hydrochloric acid, phosphoric acid, etc.). These acids do not penetrate the microbial cells as easily as carbon dioxide [8]. Therefore, it was suggested that the comparatively low concentration of CO_2-induced activation of the metabolism of microbes could serve as a convenient method for the increase of the sensitivity of microbes to the toxic effect of low concentration of H_2O_2.

To find out direct and indirect effects of CO_2 on growth of *E. coli K-12* we have studied the effects of CO_2 in constant and changing pH conditions. Since CO_2 dissolves in aqueous solutions, to form an acid we need to keep constant the level of pH in order to avoid the effects of latter on bacterial killing processes. We have kept the level of pH constant by adding NaOH (0.1 mol l^{-1}) during CO_2 treatment. It should be mentioned that we have kept the value of pH constant (pH=6), because in case of this value the growth of *E. coli* bacterial culture is maximal [11]. So to find out the direct effect of CO_2 we have studied the influence of time-dependent exposure of CO_2 on the growth of *E. coli K-12* at constant pH (pH=6) (CO_2=0.55 mol l^{-1}) (Fig. 2). The same figure also illustrates the indirect effect of CO_2 as this kind of treatment was done in different pH values (Fig. 2).

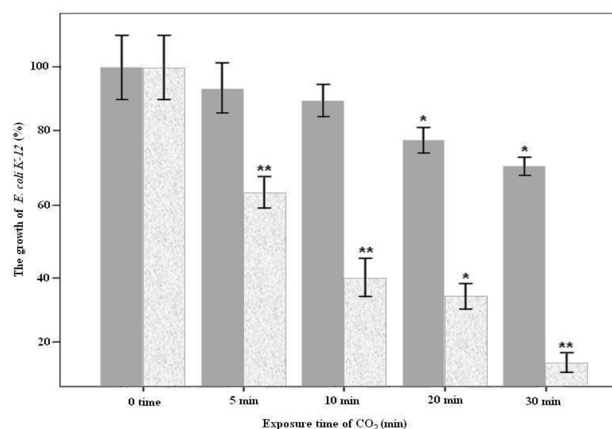

Fig. 2 The ratio of killed *E. coli K-12* (%) depending on exposure time of CO_2 (0, 5, 10, 20 and 30 min) compared with control sample (0 time), columns illustrate the direct ( ) and indirect ( ) effects of CO_2 on the growth of bacterial culture at constant (pH=6; CO_2=0.55 mol l^{-1}) and at different pH conditions, correspondingly: 0 time (pH=7.29; CO_2=0.11 mol l^{-1}), 5 min (pH=6.34; CO_2=0.51 mol l^{-1}), 10 min (pH=5.66; CO_2=0.81 mol l^{-1}), 20 min (pH=5.65; CO_2=0.84 mol l^{-1}), 30 min (pH=5.61, CO_2=0.85 mol l^{-1}). The p value of the treatments were 0.01(**), 0.05(*) and the number of experiment was 3.

We have checked the rate of inactivation of the *E. coli K-12* as a function depending on exposure time. The ratio of surviving cells after treatment with CO_2 was compared with the ratio of cells in the control culture (0 time) and the rate of inactivation was expressed in percent (Fig. 2). After CO_2 exposure samples were incubated for 2 h at 20°C, then plating was done and the samples were incubated at 37°C thermostat for 22 h. The number of living cells was determined by measuring the number of colonies grown on each agar-medium. Zero time of treatment represents the control samples or zero time of the treatment means that the sample was not exposed to CO_2. The number of cells in the suspension was counted in nutrient agar plates with the surface plating method (CFUs for per ml of sample 10^{10}). The inactivation of microbial cells was enhanced by the increase of time of exposure.

As it can be seen the indirect effect of CO_2 at different pH conditions on microbes is higher than the direct effect of CO_2 when pH is constant (pH=6, CO_2=0.55 mol l^{-1}) (Fig. 2), but the direct effect of CO_2 on microbes has also significant decreasing effect as compared with control group (0 time). These data correspond with the literature data that the toxic effect of CO_2 on microbes could not be explained only by acidification medium, probably because of its higher membrane permeability has direct poisoning effect on cell metabolism. Thus, the obtained data show that CO_2-induced depression of microbe growth was more pronounced than in case of equivalent decrease of pH by adding HCl acid. This conclusion completely corresponds with the previous literature data on this subject [4, 8]. The different drop in pH, compared with the control sample (pH=7.29), was observed in all of the experiments (Fig. 2). Since CO_2 dissolves in aqueous solution

to form an acid pH was lowered after each treatment, but with further increases in experimental condition values, the measured pHs was not changed significantly, which means that the end-point of acidity was not a function of the exposure time. So we can see that the initial inhibition is increased with the elevation of exposure time of CO_2 (Fig. 2).

B. The study of dose-dependent effects of H_2O_2 on the growth of E. coli K-12

It is known that the response of *E. coli* to H_2O_2 highly depends on its concentration. Low or high concentrations of the oxidant bring to the production of different species leading to cell death via two different mechanisms: at low concentrations microbes are forming filaments, while at higher concentrations microbes are shrinking [1, 5, 13]. In the present work we have checked the possibility to elevate the toxic effect of comparatively low doses of H_2O_2 on microbes by preliminary increase of the metabolic activity of microbes by pre-incubation of the latter in the aqua medium enriched with CO_2. Before all these procedures we studied the dose-dependent effects of H_2O_2 on the growth of *E. coli K-12* (Fig. 3).

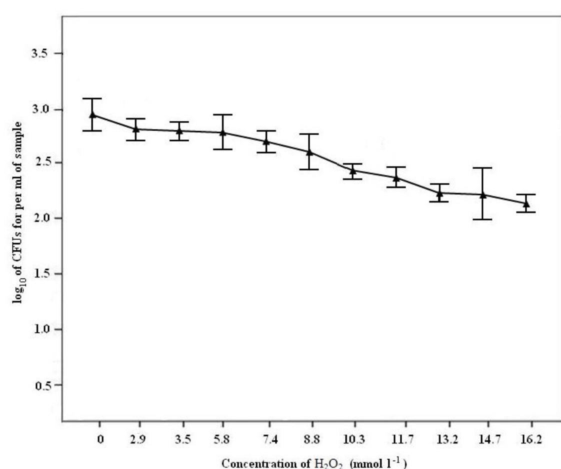

Fig. 3 The dose-dependent effects of H_2O_2 on the growth of *E. coli K-12*. The values are the average of three experiments with the standard deviation.

For hydrogen peroxide challenge, a fresh hydrogen peroxide solution was prepared immediately before starting the experiments and added to the bacterial culture bringing to the corresponding concentration of hydrogen peroxide in aqua media. Samples were incubated for 2 h at 20°C. The number of cells in the suspension with H_2O_2 concentration of several dilutions from 0 to 16.2 mmol l^{-1} (or 0.055-0.01%) was counted in nutrient agar plates with the surface plating method, plates were inoculated at 37°C for 22 h and colonies were counted to determine the number of survived cells after H_2O_2 exposure: then the average and standard deviation of each point were calculated. After obtaining the average value of each of CFUs (three per datum point) it was transformed into the logarithm with base 10 ($\log_{10}$ CFUs ml^{-1}). We can see that the higher the concentration of H_2O_2 is the higher the inhibition effect of growth of the bacterial culture is, so we need to find alternative method that could induce sensitivity of microbes to comparatively low concentrations of H_2O_2.

C. The study of the antibacterial properties of CO_2/H_2O_2 combination on the growth of E. coli K-12

The examination of the above mentioned hypothesis shows that the toxic effect of low concentrations of H_2O_2 on *E. coli K-12* was increased in cell bathing medium enriched with CO_2 for 30 min (maximum) (Fig. 4, (▲)), than in case of non CO_2 enriched bathing medium (Fig. 4, (■)). So fig. 4 shows that the antibacterial effect of H_2O_2 could be elevated with CO_2 enrichment.

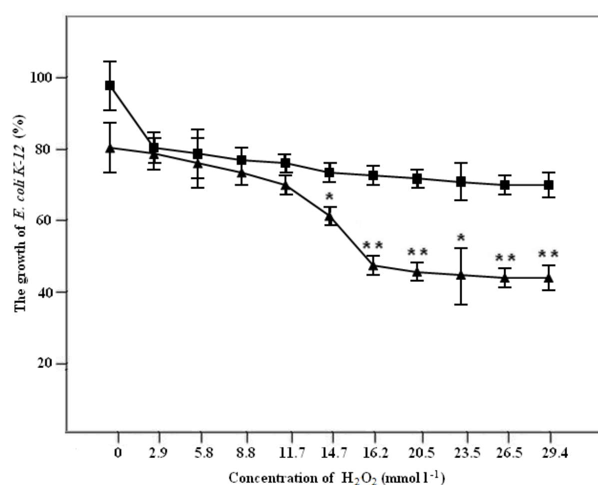

Fig. 4 The effect of different concentrations of H_2O_2 (mmol l^{-1}) on the growth of *E. coli K-12* (the percent of killed *E. coli K-12*) in 30 min CO_2 enriched bathing medium (▲) and in case of non CO_2 enriched bathing medium (■). p=0.01 (**), p=0.05 (*), n=3.

At the same time it has also been shown that through the antibacterial effect of 5min-CO_2-exposure (minimum) twice could be elevated the effect of 5.2 mmol l^{-1} H_2O_2 (Fig. 5).

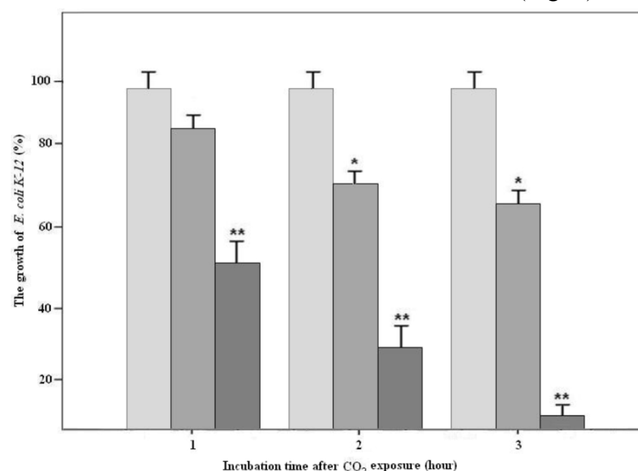

Fig. 5 The ratio of killed *E. coli K-12* (%) as a function of time-dependent inhibition of the growth of bacterial culture

after 5 min of CO_2 exposure for two times: incubation at 20°C: 5.2 mmol l^{-1} H_2O_2 containing (**1**), CO_2 enriched (one time) medium with 5.2 mmol l^{-1} H_2O_2 (**2**) and CO_2 enriched (two times) medium with 5.2 mmol l^{-1} H_2O_2 (**3**): control or 0 time ( ), after 1 h incubation ( ) and after 2 h incubation ( ). 5 min CO_2 exposure for two times was done before and after 1 h incubation at 20°C and then samples were incubated in thermostat for 2 h with 5.2 mmol l^{-1} H_2O_2, after that we have done plating and then samples were incubated at 37°C for 22 h. p = 0.01 (**), p = 0.05 (*), n = 3.

So the toxic effect of H_2O_2 is realized by its oxidative properties, consequently, it depends on the initial metabolic activity of microbes [1]. It is suggested that the factor having stimulatory effect on cell metabolism could elevate the toxic effect of H_2O_2 on microbes. On the basis of the fact that CO_2 at comparatively low concentrations [6] has strong stimulatory effect on cell metabolism it could be suggested that cells pretreated by CO_2 could have high sensitivity to toxic effect of H_2O_2 (Fig. 4, 5). It should be mentioned that stimulation of growth occurs because some anabolic reactions involve CO_2 fixation and in the absence of an external source of the gas, CO_2 concentration in cell can be rate limiting for these reactions, with resultant decreased growth rates [15]. The basis of CO_2 inhibition has not been clearly established.

IV. Conclusion

The obtained data in the present work clearly show that CO_2 increases the toxic effect of H_2O_2 on microbes and it could be used as a promising tool for water purification in places having higher environmental pollution with CO_2. Although, the obtained data in the present work did not allow us to make final conclusion on the nature of the mechanism through which the CO_2 poisoning metabolism could increase cell membrane sensitivity to H_2O_2, but corresponds to the literature data on CO_2-induced swelling of microbes [4], and our previous data obtained on eukaryotes, on cell swelling-induced increase of a number of chemoreceptors in the membrane [18], allow us to explain CO_2-induced elevation of the toxic effect of H_2O_2 on microbes by the increase of the number of membrane receptors for H_2O_2 in the result of cell swelling. But for final conclusion we need more detailed investigation. So this research is a new approach for decreasing microbial pollution in water, as well as for better understanding of the fundamental mechanisms controlling the microbial growth inhibition depending from environmental factors. We believe that this work will represent the first systematic investigation of the effect of CO_2/H_2O_2 combination at comparatively low concentrations on the reduction of microbial pollution of water.

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