Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2022.28415

258 (2022) 190–196
May

Estimation of efficiency of water disinfection and preservation with low-pressure CO_2 using *Escherichia coli*

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Received 27 May 2021; Accepted 5 March 2022

ABSTRACT

The efficiency of *Escherichia coli* disinfection using low-pressure CO_2 (0.05–0.20° MPa) in the temperature range of 14°C–42°C has been studied. It has been shown that inactivation of *E. coli* in distilled water reaches 4.0–5.5-log orders of magnitude (the initial bacterial load is 1.3×10^4–9.0×10^5 CFU/cm^3) after 5° d from the moment of treatment and holding at a given pressure at all studied pressure values and temperatures. The highest rates of disinfection were observed at maximum investigated temperature and pressure. Inactivation of *E. coli* in control experiments (without CO_2) was only ~ 1.5-log orders of magnitude under similar conditions. The study of the process of inactivation of *E. coli* in distilled water containing nutrient broth showed a high preservative ability of CO_2 at a saturation pressure of 0.1 MPa. Despite the presence of nutrients, the growth of *E. coli* in a solution treated with CO_2 was not observed in the entire studied temperature range for 6 d, while the inactivation of microorganisms for the specified period was 4.2, 0.5, 6.0 and 6.0-log orders of magnitude at temperatures of 14°C, 22°C, 37°C and 42°C, respectively.

Keywords: Water disinfection; Water preservation; Low-pressure carbon dioxide; *Escherichia coli*

1. Introduction

The requirements for the quality of drinking water, which have increased in recent decades, necessitate the search for alternative, environmentally friendly and effective technologies for its preparation, ensuring the production of water that is safe for consumption by the humans. This, primarily, concerns the disinfection process, since traditional processes (chlorination, ozonation), providing a high inactivation of microorganisms in water, can be accompanied by the formation of especially toxic by-products [1–3] that constitute a serious threat to human health. Moreover, the latest research has established the formation of carcinogenic bromate ions, as well as bromine-containing organic substances during the treatment of bromide-containing waters with ferrate (Fe(VI)), which was still considered as a "green" oxidant [4].

In this regard, studies on the possibility of using an environmentally friendly "green" reagent for water disinfection, that is, carbon dioxide (CO_2), which is a vital component of the atmosphere, the end product of complete oxidation of organic carbon and a key substrate of the photosynthesis process are of particular interest [5,6]. Carbon dioxide treatment in sub- and supercritical conditions (SC-CO_2 treatment; Tcr. = 31.1°C, Pcr. = 7.38 MPa) is currently considered as the most promising method of inactivation of microorganisms in various food products [7–10] and biomedical materials [11,12] as an alternative to the thermal sterilization method, since the latter can cause undesirable changes in the properties of the treated objects.

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The advantages of using carbon dioxide also include the low cost of the reagent, ease of removal from the facility after use, and the status of a substance generally recognized as safe (GRAS), approved by Food and Drug Administration (FDA) [9].

Currently, there is no complete clarity in the mechanism of inactivation of microorganisms with CO_2 under sub- and supercritical conditions [5,7]. However, the available results of scientific research indicate an increase in permeability of cell membrane during such exposure, which may be due to both a decrease in the external cell pH and the direct effect of CO_2 molecules on its lipid layer [7,9,13]. Modification of plasma membrane promotes penetration of CO_2 and HCO_3^- into the cytoplasm, which causes a decrease in intracellular pH (pH_{in}) and inhibition of cellular metabolism [7,14,15], as well as disturbance of intracellular electrolyte balance. An increase in permeability of the cell membrane also leads to the loss of vital components by membrane itself and the cell [7,16].

The need to use high pressures for the implementation of SC-CO_2 treatment on the one hand, and the high efficiency of inactivation of microorganisms in this process on the other hand, initiate studies to investigate the possibility of disinfecting various microbiological objects using CO_2 in less severe conditions ($P < 4.5$ МПа) [14,15,17,18].

However, Oulé et al. [17] reported that 90 min CO_2 treatment of an *E. coli* suspension in a nutrient broth with initial load of 1.0 × 10^6 colony forming units (CFU)/cm^3 at a pressure of 2.5 MPa and a temperature of 40°C does not lead to a bactericidal effect, that is, the number of microorganisms after treatment is equal to the initial quantity.

Kobayashi et al. [14], Kobayashi and Odake [15,18] proposed a device for saturation of an aqueous solution with CO_2 microbubbles (MB-CO_2 treatment), which increases the efficiency of gas dissolution in water, and provides effective inactivation of *Escherichia coli* and *Saccharomyces pastorianus* at a pressure of 2.0 MPa and a temperature above 40°C. For example, under the indicated conditions and the initial load of *E. coli* in physiological solution of 1.0 × 10^6 CFU/cm^3, inactivation reaches 6-log after 30 min of treatment [14]. When the pressure decreases to 0.5 MPa, the 2-log reduction in *E. coli* population occurs for the same period. At the same time, with a decrease in temperature to 25°C, treatment with CO_2 at a pressure of 2.0 MPa does not cause inactivation of *E. coli* with treatment duration of 1 h.

Klangpetch et al. [19] reported that inactivation of *E. coli* after low-pressure (1.0 MPa) CO_2 treatment (~ 15 min) and following heating at 55°C (1 min), reaches 3.5-log orders and is not related to physical damage in the *E. coli* cells, but causes physiological damage of the cells, including a decrease in amount of intracellular ATP. Following the depletion of intracellular ATP, the failure of the cells to discard protons caused decrease in pH_{in} (from 6.7 to 5.5). Kobayashi and Odake [15] reported that inactivation of *S. pastorianus* by two-stage MB-CO_2 treatment (1.0–2.0 MPa) might be induced by both damage to the cellular membrane (at 45°C and 50°C) and lowering of the pH_{in} (at 40°C).

The purpose of this work was to study the possibility of applying CO_2 for water disinfection at low operating pressures (0.05–0.2 MPa), which could significantly simplify the equipment used for implementation of the process and reduce the cost of its realization, while achieving high environmental friendliness of the process and receiving safe drinking water.

2. Materials and methods

The efficiency of the process of water disinfection and preservation by CO_2 was assessed on the cells of the sanitary indicative test microorganism *E. coli*. The culture of *E. coli* was obtained from the collection of the State Research Institute of Standardization and Control of Medical Biological Preparations (Moscow).

For preparation of the bacterial suspension, the bacteria culture *E. coli* was grown in nutrient broth (NB) and cultivated for 18–24 h at 37°C to the stationary growth phase. Then daily-aged culture was centrifuged at 7,000 × g for 15 min, washed three times with sterile 0.9% sodium chloride (physiological saline) solution and re-suspended to a concentration of 10^8 CFU/cm^3 determined by optical density (a KFK-2 photocolorimeter, λ = 540 nm). Prior to experiments, the initial suspension was diluted to a corresponding concentration with distilled water.

The block diagram of the installation for water treatment with carbon dioxide is shown in Fig. 1.

When studying the disinfecting effect of CO_2, a model solution (0.4 dm^3) contaminated with microorganisms (prepared with distilled water) was introduced into a mixing vessel (0.5 dm^3) with lower tube. After that, the vessel was hermetically connected to a cylinder containing carbon dioxide, and the latter was fed through an aerator submerged to the bottom of the vessel with valve 5 being initially open (for ~ 0.5 min), and then – closed (for ~ 1 min) until the required pressure was reached (0.05–0.20 MPa). The pressure in the mixing vessel was controlled by a pressure gauge. After the treatment mixing vessel was held at a given pressure at all studied pressure values and temperatures.

When studying the preserving effect of CO_2 on *E. coli* cells, prior to carbon dioxide treatment, nutrient broth (LLC "Farmaktiv" Ukraine) was introduced into distilled water contaminated with microorganisms, supporting the growth of microorganisms, which simulates secondary water pollution [20]. Nutrient broth was added to the culture solution at a NB/solution volume ratio of 1:50. On the one hand, such an amount of NB is necessary and sufficient to maintain the vital activity of microorganisms in comparison with the control of culture without NB (it is known that the number of viable cells of *E. coli* culture in water during storage in the absence of nutrients decreases [21]). On the other hand, it does not cause rapid growth of microorganisms, as this takes place in case when the culture is directly introduced into the NB (data not shown).

At certain time intervals, an aliquot of the treated water was taken through the sampler (lower tube of the container) for microbiological analysis and pH measure. In the latter case, an I-130 M pH meter was used for control. If in the process of sampling the pressure in the system deviated from the preset one, it was corrected by supplying an additional portion of CO_2.

The number of surviving *E. coli* cells was determined by plate count method using Endo agar medium (LLC "Farmaktiv" Ukraine) [22,23]. The plates were incubated at

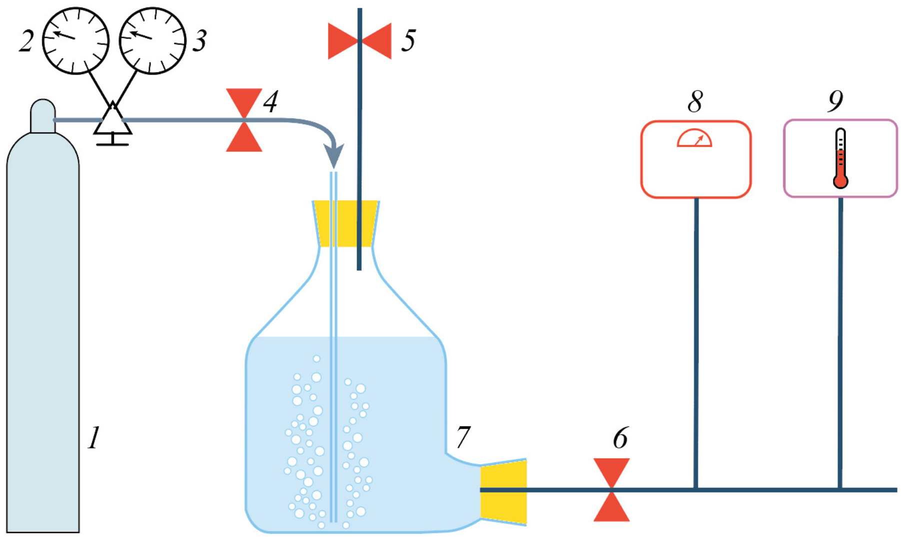

Fig. 1. Block diagram of the installation for water treatment with CO_2. (1) CO_2 cylinder; (2) cylinder pressure gauge; (3) delivery pressure gauge; (4–6) valves; (7) mixing vessel; (8) pH meter; (9) temperature sensor.

37°C for 18–24 h, and the colonies were then counted. The inactivation ratio was expressed as $\log(N_t/N_0)$, where N_t is the cell count after treatment and N_0 is the initial cell count.

The possibility of bacteria regrowth in water after its treatment with CO_2 was investigated by the method [24]. This method allows identifying microorganisms in water in viable but non-culturable (VBNC) state. The essence of this method is to introduce a certain volume of analyzed water into a synthetic microbiological medium M9 [25] and cultivate it in a thermostat at 37°C for 24 h. After that samples are plated onto a Petri dish with Endo agar. Further incubation and counting of reactivated cells are carried out similarly to the procedure described.

Glass mixing vessels used in experiment were thoroughly washed with water and autoclaved at 1 atm for 30 min.

To study the effect of temperature on water disinfection and preservation using CO_2, water samples were thermostated at 42°C, 37°C, and 22°C in the TS-80M thermostat. Some experiments were carried out without thermostating at temperature 14°C. All experiments were done in triplicate. The data presented are the means with standard errors of the results of triplicate experiments.

Table 1 provides information on the concentration of carbon dioxide in water and the pH of water at the saturation pressures used in this work.

3. Results and discussion

Fig. 2 shows the kinetics of *E. coli* inactivation using CO_2 at a pressure of 0.1 MPa and different temperatures. The initial culture load is 1.3×10^4–9.0×10^5 CFU/cm^3. As it can be seen in Fig. 2, despite the relatively low operating pressure of CO_2 in the system, during the experiment inactivation of *E. coli* is observed and 4.0–4.5-log reduction in *E. coli* population is archived after 5 d of storage at all studied temperature values. In the control experiment (without CO_2, pH = 5.6) inactivation is also observed due to the lack of nutrients, but in this case only ~ 1.5-log reduction of *E. coli* occurred in 5 d (Fig. 2).

Noteworthy is the change in the shape of the kinetic curve of *E. coli* inactivation using CO_2 with increasing temperature (Fig. 2). So, at a lower temperature (14°C and 22°C), the phase of slow death of *E. coli* is replaced by a phase of more rapid death, while at a temperature of 37°C, on the contrary, the phase of more rapid inactivation precedes the slow phase. Thus, with a decrease in the duration of contact of the *E. coli* suspension with CO_2, the effect of temperature on inactivation becomes more significant. These two types of kinetic curves of cell inactivation were also observed by Ortuño et al. [26] when studying the effect of SC-CO_2 on an *E. coli* culture at different stages

Table 1
Concentration of CO_2 (C_{CO_2}) in water and pH of water at different CO_2 pressures

P, MPa	C_{CO_2}, mg/dm^3 (20°C), literature data	pH (20°C), literature data [26]	pH (experimental data obtained in this work)	
			22°C	37°C
0.05	711 [27]	4.05	4.10	4.13
0.10	1,488 [27]	3.92	4.10	4.13
0.15		3.85	4.06	4.10
0.20	2,972 [27]	3.80	3.82	3.80

of its growth. However, it is natural that the duration of the above-mentioned phases in the kinetic curves in the cited work was measured not in days (as in our case), but in minutes. Authors explain the results obtained by the difference in the permeability of cell membranes at different stages of growth in relation to CO_2, which affects the rate of CO_2 penetration into cells and, consequently, the rate of their inactivation.

Change in the shape of the kinetic curve in Fig. 2 with an increase in temperature to 37°C, is obviously due to an increase in the diffusion rate of CO_2 and, as a consequence, the rate of its penetration through the plasma membrane. In addition, with an increase in temperature, the permeability of the cell membrane also increases and the activity of cell enzymes decreases, which facilitates the inactivation of cells by various inhibitors [27,28].

Kobayashi et al. [14] also ascribe the discovered increase in efficiency of inactivation of *E. coli* by MB-CO_2 (P = 2.0 MPa) at the temperature growing from 25°C to 40°C to increasing infusibility of CO_2 and fluidity of the cell membrane at higher temperatures. It is noted that despite the fact that dissolved CO_2 concentration in solution at 25°C was higher than at other temperatures (in general, solubility of gas in liquid decreases with increase in temperature) 6-log reduction on *E. coli* population was reached at 35°C and 40°C for 40 and 30 min respectively while at 25°C a reduction was not observed for 60 min. Kobayashi and Odake [15] reported about significant decrease of pH_{in} cells *S. pastorianus* (from 4.94 to 3.42) in the process MB-CO_2 treatment (2.0 MPa, 5 min) when the temperature rises from 35°C to 50°C which is also explained by the increase of CO_2 diffusivity or fluidity of the cell membrane.

It should be noted that despite the observed difference in the kinetics of *E. coli* inactivation at different temperatures (Fig. 2), pH values of solution treated with CO_2 under these conditions differed slightly (Table 1) and amounted to 4.10–4.13 (initial pH 5.6). Thus, our results are consistent with studies [14], according to which the pH of the solution

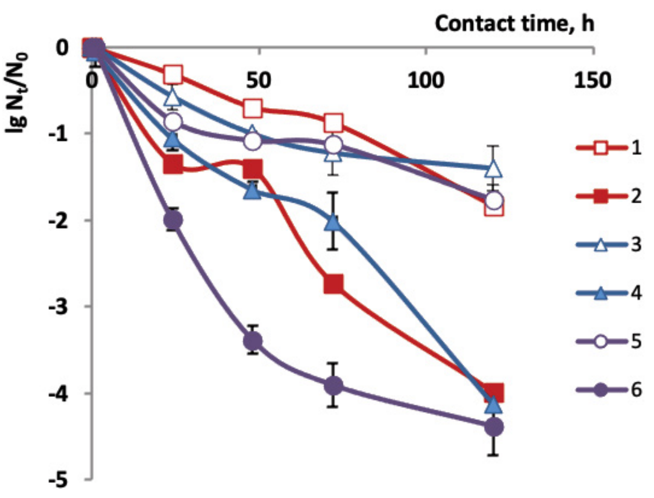

Fig. 2. Kinetics of *E. coli* inactivation in distilled water (1, 3, 5) and water saturated with CO_2 at a pressure of 0.1 MPa (2, 4, 6). Temperature: 14°C (1, 2); 22°C (3, 4) and 37°C (5, 6).

has a little effect on the inactivation of *E. coli* by MB-CO_2 treatment.

Investigation of the process of *E. coli* inactivation in distilled water containing nutrient broth showed a high preservative ability of CO_2 at a saturation pressure of 0.1 MPa and temperatures of 14°C–42°C (Fig. 3). The pH of *E. coli* suspensions after treatment at different temperature was 4.10–4.20.

Despite the presence of nutrients, the growth of *E. coli* in the solution treated with CO_2 was not observed in the entire studied temperature range for 6 d, while the inactivation of microorganisms for the specified period was 4.2, 0.5, 6.0 and 6.0-logs at temperatures of 14°C, 22°C, 37°C and 42°C, respectively. At the same time, in control experiments (without CO_2) at temperatures of 22°C, 37°C and 42°C, the growth of culture was observed, amounting in 6 d to 3.2, 3.0 and 0.5 orders of magnitude respectively.

As is known, after water treatment with disinfectants, microorganisms are able to enter into the viable, but non-culturable state [29,30], which can lead to their secondary growth [31]. In particular, Kobayashi and Odake [15] reported that two-stage MB-CO_2 treatment at 40°C might bring *S. pastorianus* cells to VBNC state. Zhao et al. [32] indicated that *E. coli* O157:H7 in 0.85% NaCl solution (pH 7.0) was able to enter the VBNC state by high pressure CO_2 treatment (5 MPa) and lower temperatures (25°C–37°C).

In our work, in water samples treated with carbon dioxide, no secondary growth of the *E. coli* culture was detected for one month (observed period) both in the presence of nutrients and in their absence. Besides, secondary culture growth was not observed in samples of inactivated water, which after depressurization of the mixing vessel contacted with atmospheric air for one month.

The effect of pressure (0.05–0.2 MPa) on the inactivation of *E. coli* in distilled water by CO_2 treatment at a temperature of 22°C and 37°C is shown in Fig. 4.

As it was expected, an increase in CO_2 pressure during processing leads to an increase in the degree of inactivation of microorganisms. Obviously, this is due to a significant increase of the dissolved CO_2 concentration in the solution under these conditions, which, in particular, is demonstrated by the data in Table 1, as well as the results obtained by Kobayashi et al. [14]. An increase in the concentration of dissolved CO_2 in the solution with an increase in pressure accelerates its diffusion through cell membrane, causing a significant decrease in intracellular pH_{in}, which was experimentally confirmed in the study of inactivation by low-pressure carbon dioxide of *S. pastorianus* [15], *Saccharomyces cerevisiae* and *Listeria innocua* [33,34].

At the same time, as our studies have shown, the pH of *E. coli* suspensions after treatment at different CO_2 pressure differed insignificantly (Table 1). This is consistent with the data reported in the literature [14], and indicates, as it has been already mentioned above, the insignificant effect of external cellular pH on the inactivation of *E. coli* in the process under study.

Fig. 4 also demonstrates the above-described phenomenon of change in the course of kinetic curves of *E. coli* inactivation using CO_2 with increasing temperature, which is explained by the acceleration of the process of CO_2 diffusion through the cell membrane.

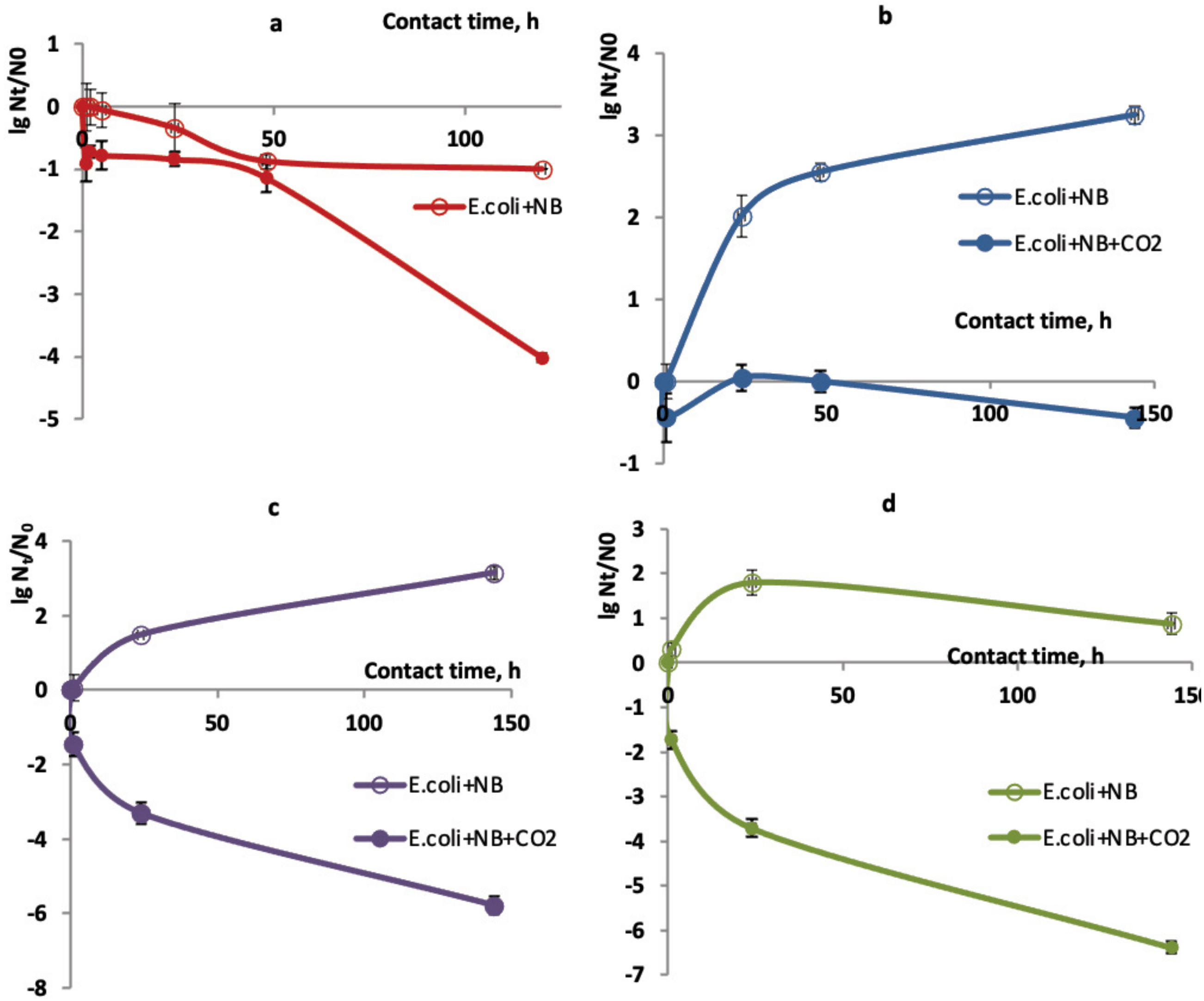

Fig. 3. Kinetics of *E. coli* inactivation and conservation using CO_2 (P = 0.1 MPa) in distilled water in the presence of NB at different temperatures. Temperature: (a) 14°C, (b) 22°C, (c) 37°C, and (d) 42°C.

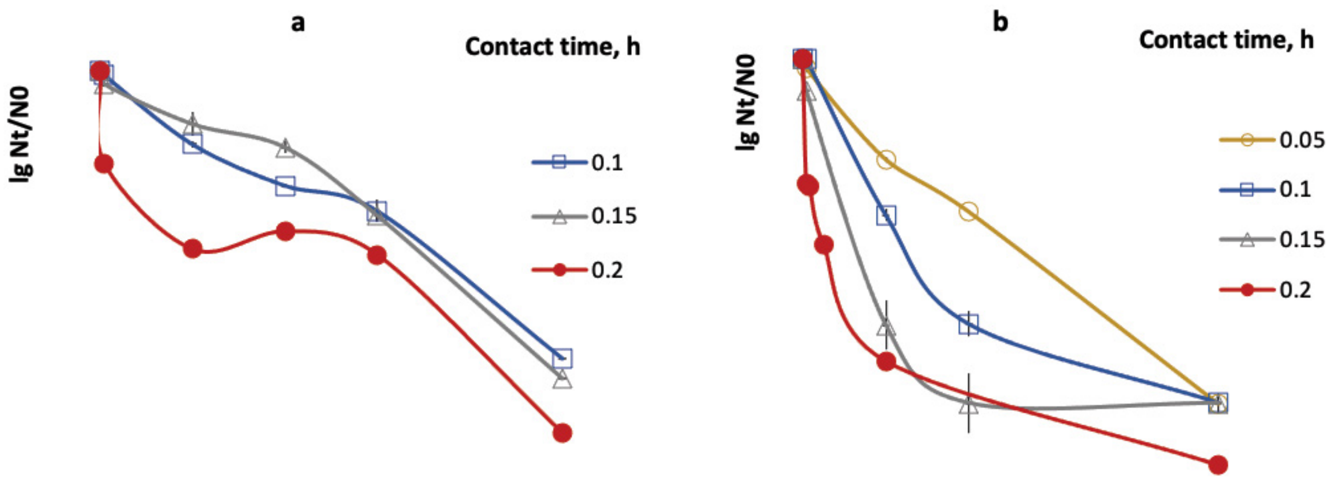

Fig. 4. Kinetics of *E. coli* inactivation in distilled water by CO_2 at various pressures (numbers indicated near curves) and temperatures. Temperature: (a) 22°C and (b) 37°C

4. Conclusions

The principal possibility of disinfection of *E. coli* by CO_2 at a pressure of 0.1–0.2 MPa is shown. An increase in temperature to 37°C–42°C significantly intensifies the process and reduces the time required to achieve a given degree of inactivation. At the indicated temperature even in the presence of nutrients, the degree of disinfection of *E. coli* reaches 6-log orders of magnitude at pressure 0.1 MPa in 6 d.

Despite the need for a long-term contact of CO_2 with the disinfected object in order to ensure the effective inactivation, low-pressure CO_2 treatment can be considered as an alternative to traditional methods of disinfection, especially considering the environmental friendliness of the process and the possibility of obtaining drinking water, that does not contain toxic by-products, safe for human consumption. The additional benefit is also the low costs of the process and the simplicity of the equipment used for its implementation. The results obtained in this work can be used for the development of new strategies for guaranteeing high quality and safety of drinking water.

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