Inactivation of *Escherichia coli* by several types of gas bubbles

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**Abstract.** The ability of gas bubbles to inactivate *Escherichia coli* suspended in distilled water at varied temperature and several types of gas, namely carbon dioxide, nitrogen and argon, was investigated. The results of the experiment showed that carbon dioxide gas bubbles gave the better inactivation of *Escherichia coli* than other gasses and the number of surviving *Escherichia coli* was reduced by approximately 0.76-log from the treatment of carbon dioxide gas bubbles at 4-10°C and gas flowrate of 2.5 ml/min for 40 min. The pH of solution after treatment with carbon dioxide gas bubbles for 40 min was 3.8. Furthermore, the number of surviving *Escherichia coli* was reduced by approximately 1.62-log when the solution temperature was increased to 50°C with the same condition of the treatment of carbon dioxide gas bubbles at 4-10°C. It was found that the addition of carbon dioxide gas bubbles together with increasing the solution temperature can significantly reduce the number of *Escherichia coli*. The most suitable of inactivation kinetics reaction was first order and the rate constant, \( k \), at 4-10°C, 30°C and 50°C was 0.0220, 0.0013 and 0.0475 min\(^{-1}\), respectively. The decimal reduction time \( D \) at 4-10°C, 30°C and 50°C was 45.45, 769.23 and 21.05 min, respectively.

1. **Introduction**

Bacteria are the most abundant forms of life on earth and are a natural component of lakes, rivers, and streams. Most of these bacteria are harmless to humans but a relatively small number of species causes disease. However, certain bacteria inhabited in body of organisms such as the intestinal tract of warm-blooded animals and a human stomach have the potential to cause sickness and disease in humans [1].

*Escherichia coli* is the most common gram-negative pathogen, causing urinary tract infections, septicemia and new-borns meningitis [2]. It was first isolated from the stools of children by Theodor Escherich in 1885 [3]. *Escherichia coli* is classified in the family *Enterobacteriaceae* and is a rod-shaped bacterium, commonly found in terminal small intestine and large intestine of human and many warm-blooded animals [4-6]. The optimum temperature for *Escherichia coli* growth is 37°C but growth can occur between 4-45°C as well. The optimal pH for growth is in a range of pH 6-8. It can survive as low as pH 4 and as high as pH 9 [3,6,7].

Most strains of *Escherichia coli* are harmless. However, *Escherichia coli* O157:H7 can cause several diseases [8, 9]. The infective dose of *Escherichia coli* O157:H7 as low as 10 cells can be caused disease that lower than infective dose of most foodborne pathogens is greater than 10,000 cells [8,9]. Waterborne transmission of *Escherichia coli* O157:H7 is an emerging concern to human health. Water is essential to life. Improving quality of safe drinking water has a profound effect on health [10]. *Escherichia coli* has usually been used as test microorganisms because it was a hygiene indicator bacterium [11]. There are several inactivation methods of *Escherichia coli* in water, such as increasing
temperature, pressure, treating with chemicals and dissolved gases, and exposure time. [12-15]. Kobayashi F. et al., 2016 [14] reported that the number of surviving *Escherichia coli* cells in physiological saline was decreased 1-log following micro-bubble carbon dioxide treatment at 35°C and 1.0 MPa for 10 min and the number of surviving *Escherichia coli* cells was decreased with increasing pressure or temperature. Adrian et al., 2019 [16] found that dissolved carbon dioxide solution contained with 0.17 M NaCl could decreased 0.6-log of surviving *Escherichia coli* cells at pH of 4.1, temperature of 38°C and atmospheric pressure. In addition, argon and nitrogen gas were also reported an inhibitory effect on inactivation of bacteria in modified atmosphere packaging for maintaining the quality of meat and fruit [17-19]. Hence, this research aimed to study the effects of gases, temperature and contact time for *Escherichia coli* inactivation by using several types of gas bubbles and the inactivation kinetics of *Escherichia coli* were also investigated.

2. Materials and methods

2.1 Inoculum preparation and inoculation procedure
A pathogenic strain of *Escherichia coli* TISTR 780 (ATCC 8739) was used as the test strains. The cell culture was obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The activated cells were cultured individually in nutrient broth (Himedia laboratories pvt.ltd.) and then incubated at 37°C for 48 h before using as the working inoculum. The final bacterial concentrations in the inoculum samples were approximately controlled in the range of 8 log CFU/ml in the nutrient broth.

2.2 Washing conditions

2.2.1 Preparation of dissolved gas solutions. Each dissolved gas solution was prepared by filling 200 ml of distilled water in a 300 ml beaker placed in a water-ice mixture bath. N₂ (99.99 %, Bangkok Industrial Gas Co., Ltd., Thailand), Ar (99.995 %, Bangkok Industrial Gas Co., Ltd., Thailand), CO₂ (99.5%, Bangkok Industrial Gas Co., Ltd., Thailand), 50CO₂:50N₂ v/v or 50CO₂:50Ar v/v were dissolved into distilled water via a gas diffuser at flowrate of 2.5 ml/min and 20 min to final saturation. The flowrate of gases was controlled by a flowmeter with a needle valve and gas mixtures between CO₂ and N₂ or Ar were mixed in a tube before feeding it into the distilled water. Gas and gas mixtures were flushed into the distilled water until the dissolved oxygen was nearly zero measured by the YSI 52 DO/BOD meter. The temperature of the deoxygenated water was measured by a thermometer and was controlled in the range of 4-10°C, 30°C or 50°C.

2.2.2 Inactivation. After preparing the dissolved gas solution, 2 ml of *Escherichia coli* inoculum was dropped into the solution and the solution was then mixed by the flow of gas via a gas diffuser until the end inactivation period. The initial concentration of *Escherichia coli* in solution was approximately controlled in the range of 6 log CFU/ml. After the inactivation time at 0, 10, 20, 30 and 40 min, one milliliter of each sample was taken from each solution for measuring the number of *Escherichia coli*.

2.3. Microbial analysis and enumeration
One milliliter of diluted solution was made serial dilution (1:10), adding 1 ml of the solution to 9 ml of water, before measurement. Then, one milliliter of each diluted sample was dropped in a commercial count plate, a 3M *Escherichia coli* count plate and all plates were incubated at 35 (+1°C) for 24 ± 2 h (AOAC, 2002) [20]. The amount of *Escherichia coli* was counted after incubation and was calculated the number reverse before dilutions. The colony counts are expressed as log CFU/ml. All experiments were performed in duplicate, and the data presented are averaged from the results of duplicate experiments.
2.4 Inactivation kinetics

The kinetic of inactivation model was developed based on the basic kinetic equation for chemical reaction rate. The first-order kinetic model assumes an exponential reduction of the number of survivors over treatment time [20]. The model for the inactivation of microorganisms can be written as follows:

\[
\frac{d(N/N_0)}{dt} = -k \left( \frac{N}{N_0} \right)
\]

where \(N\) and \(N_0\) are the surviving number and the initial number of the microorganisms, respectively, \(t\) is time (min) and \(k\) is first-order of rate constant (min\(^{-1}\)). The integration from \(t = 0\) to \(t = t\) in equation (1) can be written as follows:

\[
\frac{N}{N_0} = e^{-kt}
\]

The values of \(k\) in the model were used to estimate the time needed to inactivation of microorganisms and the time at a given temperature to reduce the population by 1 log cycle (\(D\) value) will be equal to

\[
D = \frac{2.303}{k}
\]

and substitution of equation (3) into equation (2) yields the first-order survival model:

\[
\log \frac{N}{N_0} = -\frac{t}{D}
\]

where \(D\) is the decimal reduction time (min). When one log reduction is achieved, it means 90% of relevant microorganisms have been killed [21].

3. Result

The results of colony counting of *Escherichia coli* showed only dark blue spots and some gas bubbles (Fig. 1) after incubating for 24 hours same as the results reported by Gangar, *et al.* 1999[22]. The effect of gas bubbles on the inactivation of *Escherichia coli* in distilled water is shown in Fig. 2. The number of surviving *Escherichia coli* was reduced by approximately 0.76-log from the treatment of carbon dioxide gas bubbles at 4-10°C and gas flowrate of 2.5 ml/min for 40 min, 0.46-log reductions, 0.11-log reductions by 50%CO\(_2\):50%N\(_2\) v/v and N\(_2\), respectively, while Ar and 50%CO\(_2\):50%Ar v/v increased the surviving *Escherichia coli*. The pH of solution after carbon dioxide gas bubble treatment at 40 min was 3.8 while the optimum pH for *Escherichia coli* growth is in the range of 6-8, however, growth can occur as low as pH 4 and as high as pH 9 as well [3,6,7]. It seems that the reduction of pH after treating with carbon dioxide gas bubbles could reduce the number of *Escherichia coli*. Adrian *et al.*, 2018 [23] reported that the pH of dissolved carbon dioxide solution with 0.17 M NaCl at room temperature was dropped from 5.9 to 4.1 and the reduction of *Escherichia coli* added to the solution was found only 0.05-log. Satoshi I. *et al.*, 2010 [24] explained that the critical pH of some bacteria including *Escherichia coli* was approximately 4.7. The synergistic effects of the pH below the critical point and dissolved carbon dioxide could have the *Escherichia coli* inactivation. Dissolved carbon dioxide in solutions easily diffused into the phospholipid bilayer of the membranes [16]. These results were supported by others [25, 26] who reported that dissolved carbon dioxide in solutions easily diffused into bacterial cell, resulting in a decrease in intracellular pH and cell death.

Fig. 3 shows the effect of temperature on inactivation of *Escherichia coli* in distilled water by carbon dioxide gas bubble. The number of surviving *Escherichia coli* was reduced by approximately 1.62-log from the treatment of carbon dioxide gas bubbles at 50°C and gas flowrate of 2.5 ml/min for 40 min, in comparison with the number reduction of *Escherichia coli* by 0.76-log and 0.05-log at 4-10°C and 30°C, respectively. This result was confirmed by the previous works [3,6,7] that *Escherichia coli* can survive between 4-45°C. The inactivation at 50°C could be explained by thermal inactivation mechanism...
resulting the reduction of surviving *Escherichia coli* more than at 30°C. In comparison with results of Stephane Guyot, et.al. 2014 [12] who reported that the addition of carbon dioxide gas and the increase of temperature can significantly reduce the number of *Escherichia coli*. On the other hand, the reduction of temperature in the inactivation process to 4-10°C gave better results than the inactivation at 30°C. This may explain that the *Escherichia coli* cells are halted with ‘cold shock response’, indicating that this strain has no capacity to efficiently adapt to low temperatures [27, 28].

![Escherichia coli Colony with gas bubbles](image)

**Figure 1.** Colony of *Escherichia coli* after incubating for 24 hours at inactivation by carbon dioxide gas bubbles at 30°C and 30 min.

![Log N/No vs Time](image)

**Figure 2.** Effect of gas bubbles on inactivation of *Escherichia coli* in distilled water by gas bubble of N₂(●), Ar(■), CO₂(▲), 50%N₂:50%CO₂/v/v (■) and 50%Ar:50%CO₂/v/v (●) at 4-10°C

Kinetic models are an important tool for inactivation of microorganisms. The inactivation rate can be usually expressed using the values of $k$ and, a decimal reduction time, ($D$). Each microorganism strain is a specific $D$ value for each inactivation process. Fig. 3 showed the inactivation of *Escherichia coli* for this study. A linear regression analysis from Equation (4) showed in straight lines and the results of kinetic parameters were shown in Table 1. The rate constant $k$ at 50°C was more than at 4-10°C and at 30°C. The decimal reduction time $D$ implied the retention time required to achieve the inactivation of 90% of the microorganisms, which showed that *Escherichia coli* was inactivated at 50°C more than at 4-10°C and at 30°C, which was consistent with the results above.
4. Conclusion

Gas bubbles were effective in the reduction of *Escherichia coli* in distilled water and its effectiveness varied concomitantly with temperature, time and types of gas in the solution. In addition, the number of surviving *Escherichia coli* was reduced by approximately 1.62-log from the treatment of carbon dioxide gas bubbles at 50°C and gas flowrate of 2.5 ml/min for 40 min. The rate constants $k$ for the inactivation model at 4-10°C, 30°C and 50°C were 0.022, 0.0013 and 0.0475 min$^{-1}$, and 45.45, 769.23 and 21.05 min for the decimal reduction time $D$, respectively. These results suggest that carbon dioxide gas bubble treatment may become a future inactivation method and it can be used in washing process for cleaning of fruit, vegetables, fish and poultry in the future.

Table 1. kinetic parameters after solving (eq. 2, eq. 3 and eq.4) to describe inactivation of *Escherichia coli* in distilled water by carbon dioxide gas bubbles.

| Temperature | $k$ (min$^{-1}$) | $D$ (min) | $R^2$ |
|-------------|-----------------|-----------|-------|
| 4-10°C      | 0.0220          | 45.45     | 0.9503|
| 30°C        | 0.0013          | 769.23    | 0.6406|
| 50°C        | 0.0475          | 21.05     | 0.9617|

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References

[1] Russell J B, Diez-Gonzalez F and Jarvis G N 2000 Symposium: farm health and safety Invited Review: Effects of Diet Shifts on *Escherichia coli* in Cattle *J. Dairy Sci.* **83**(4) 863-73
[2] Sarff L D, McCracken G H, Schiffer M S, Glode M P, Robbins J B, Orskov I and Orskov F 1975 Epidemiology of *Escherichia coli* K1 in healthy and diseased newborns *The Lancet* 1099-101
[3] Escherich T 1885 Die darmbakterien des neugeboren und sauglings *Fortschritte der Medizin* **3**(2) 515-22
[4] Mackie R I, Sghir A and Gaskins H R 1999 Developmental microbial ecology of the neonatal gastrointestinal tract *Am J Clin Nutr.* **69**(5) 1035-45
[5] Gilligan P H 2013 Identification of pathogens by classical clinical tests *The Prokaryotes* (Berlin Heidelberg: Springer) chapter 4 pp 57-89
[6] Serres M H and Riley M 2006 *Volume 1: Symbiotic associations, biotechnology, applied
microbiology, genomics and metabolism in Escherichia coli The Prokaryotes (Berlin Heidelberg: Springer) chapter 1 pp 261-74

[7] Doyle M P and Schoeni J L 1984 Survival and growth characteristics of Escherichia coli associated with hemorrhagic colitis Appl Environ Microbiol. 8(4) 855-56

[8] Armstrong G L, Hollingsworth J and Morris Jr J G 1996 Emerging foodborne pathogens: Escherichia coli O157:H7 as a model of entry of a new pathogen into the food supply of the developed world Epidemiologic Reviews 18 29-51

[9] Shallow S, Daily P and Rothrock G 1997 Foodborne diseases active surveillance network 1996 Morbidity and Mortality Weekly Report 46(12) 258-261

[10] WHO (World Health Organization) 2008 Guidelines for Drinking-water Quality Incorporating 1st and 2nd Addenda Vol 1 Recommendations 3rd ed WHO Geneva Switzerland

[11] Damar S and Balaban M O 2006 Review of dense phase CO2 technology: microbial and enzyme inactivation, and effects on food quality J. Food Sci. 71 R1-R11

[12] Stéphane G, Laurence P, Alain H, Mélanie R, Julia H T, Paul M, Eric F and Patrick G 2014 Extremely rapid acclimation of Escherichia coli to high temperature over a few generations of a fed-batch culture during slow warming Microbiologypopen. 52-63

[13] McWilliam L E C and Stewart C S 2002 Susceptibility of Escherichia coli O157:H7 and non-O157 isolates to lactate Lett Appl Microbiol. 35 176-80

[14] Kobayashi F, Odake S, Miura S and Akuzawa R 2016 Pasteurization and changes of casein and free amino acid contents of bovine milk by low-pressure CO2 microbubbles LWT-Food Sci and Tech. 71 221-26

[15] Hongmei L, Zhong K, Liao X, and Hu X 2014 Inactivation of microorganisms naturally present in raw bovine milk by high-pressure carbon dioxide Inter J. Food Sci Tech. 49 696-702

[16] Adrian G S, Richard P and Barry N 2019 Virus and bacteria inactivation by CO2 bubbles in solution JPN Clean Water 5 1-9

[17] Spencer K C 1995 The use of argon and other noble gases for the MAP of foods International Conference on Modified Atmosphere Packaging and Related Technologies Campden & Chorleywood Research Association, Chipping Campden, UK. 6-7

[18] Chung H S and Moon K D 2009 Browning characteristics of fresh-cut ‘Tsugaru’ apples as affected by pre-slicing storage atmospheres Food Chem. 114 1433-37

[19] Ulrike H, Sonja R, Meik A K and Judith K 2013 Comparison of argon-based and nitrogen-based modified atmosphere packaging on bacterial growth and product quality of chicken breast fillets Poultry Science 92 1348-56

[20] AOAC 2002 Association of Official Analytical Chemist, Official Method 998.08 Escherichia coli Count in Poultry, Meat and Seafood J. AOAC Inter. 82 73

[21] Hayriye B, Jairus R D D, Ryan J T, D Scott L and P Michael D 2016 Thermal Inactivation Kinetics of Sporolactobacillus nakayamae Spores, a Spoilage Bacterium Isolated from a Model Mashed Potato–Scallion Mixture J. Food Prot. 79(9) 1482-89

[22] Gangar V, Curiale M S, Lindberg K and Lenarz S G 1999 Dry rehydratable film method for enumerating confirmed Escherichia coli in Poultry, Meat, and Seafood: Collaborative Study J. AOAC Inter. 82(1) 73-78

[23] Garrido A, Pashley R M and Ninham B W 2018 Water sterilisation using different hot gases in a bubble column reactor J. Enivir Chem Engi 6 2651-59

[24] Satoshi I, Katsuhisa K and Satoshi H 2010 Effects of pH on Bacterial Inactivation in Aqueous Solutions due to Low-Temperature Atmospheric Pressure Plasma Process and Polymer 7 33-42

[25] Garcia G L, Geeraerd A H, Spilimbergod S, Elsta K, Van L G, Debevere J, Van I J F and Devlieghere F 2007 High pressure carbon dioxide inactivation of microorganisms in foods: the past, the present and the future Inter J Food Microbiology 117 1-28

[26] Kobayashi F, Daisuke S, Tetsuya T and Hiromi I 2012 Inactivation of Lactobacillus fructivorans in physiological saline and unpasteurised sake using CO2 microbubbles at ambient temperature

6
and low pressure *Inter J Food Sci and Tech.* 47 1151-57

[27] Asif S, Shafiqul I, Md Fahad H and Talha B E 2019 Cold Shock and Thawing Effect on the Growth of *Escherichia coli* EC *Microbiology* 15(1) 36-43

[28] Beran R K and Simons R W 2001 Cold-temperature induction of *Escherichia coli* polynucleotide phosphorylase occurs by reversal of its autoregulation *Molecular Microbiology* 39(1) 112-125