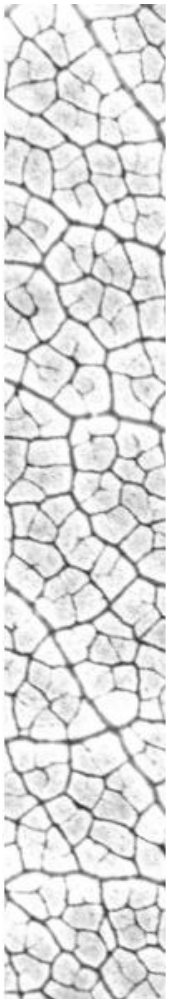

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Non-thermal carbon dioxide-mediated inactivation process of *Escherichia coli* using bespoke system

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Abstract
The reduction of the number of contaminated microbial cells in the biological and food solutions has desirably led to the development of many inactivation technologies aimed at the same goal--eradication of the microbial cells without harming the biological solution or generating toxic chemical agents. *E.coli* is known as food borne pathogen and cause serious problems in food industry and is used as a model organism in this study. The current CO_2 mediated approach was used as a cheap and emerging approach to inactivate bacterial cells, *E. coli*. CO_2 mediated approach is a non-thermal method, which has advantages over traditional methods such as autoclaving and γ-radiation. The results show that injecting CO_2 into the system reduced the bacterial population by ~2.5-Log after 90 min and to 3.6 Log reduction after lactic acid addition. Bacterial cells were suffered from morphological changes and shape changes were obvious. Efficient energy consumption, avoidance of hazardous substances usage and can be applied in situ, are among its advantages, making it a promising and emerging inactivation technology.

Keywords: bacterial inactivation, food spoilage, *Escherichia coli*, lactic acid additive

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INTRODUCTION

Sterilisation of contaminated food and biological solutions is a world-wide concern (Nwadibe et al., 2020). The presence and subsequent growth of microorganism in food raw materials and products result in spoilage of these products as well as reduction of the efficiency of downstream processes and its shelf life (Sudiarso and Prihandarini, 2020; Singh et al., 2020). Traditionally, pasteurisation, steam and autoclaving sterilisation methods are the widely used thermal inactivation techniques to sterilise these products. However, one of the disadvantages of these techniques is their unsuitability for heat-labile products such as those products containing sugars, proteins, vitamins and lipids (Nair, 1995) in addition to the high operating.

On the contrary, the use of alternative non-thermal technologies becomes one of the most promising alternatives to offset some of these disadvantages. Nevertheless, changing some of the mechanical characteristics of biomaterials as well as the carcinogenicity of some of these non-thermal methods such as ethylene oxide remain important obstacles in the using of ethylene oxide in the sterilisation process (Dillow et al., 1999; Hong and Pyun,1999 and Premnath et al., 1996).

Interestingly, inactivation with carbon dioxide is a non-thermal method to inhibit the metabolic pathways of many organisms such as bacteria and fungi through the destabilization of the biological systems within microbial cells (Mulakhudair et al., 2017 and Garcia-Gonzalez et al., 2007). Indeed, this option has drawn much attention because of its wide range applications.

The reaction of carbon dioxide with water occurs physically, with its solubility directly and indirectly dependent on pressure and temperature respectively (Spilimbergo and Bertucco, 2003). 0.02% of carbon dioxide would only be dissociated into carbonic acid, which decreases pH of treated solutions to around 4, due to the release of the two hydrogen ions through the following chemical reactions (Al-Mashhadani et al., 2012) **Table 1**.

The number of survived cells (as a ratio) is calculated by the following equation:

$$Log\ \frac{N}{N0} = k * t * 2.303\ (Eq.5)$$

Where N is the colony forming unit bacteria (CFU/ml)
N0 is the initial number colony forming unit bacteria (CFU/ml).
k is the sterilisation rate constant min-1(D-value)
t is the time (min).

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Table 1. Chemical reactions of dissociation of carbon dioxide in water

Reaction	Equation number
$CO_{2(g)} + H_2O \leftrightarrow CO_{2(aq)}$	1
$CO_{2(aq)} \leftrightarrow H_2CO_3$	2
$H_2CO_3 \leftrightarrow HCO_3^- + H^+$	3
$HCO_3^- \leftrightarrow CO_3^{-2} + H^+$	4

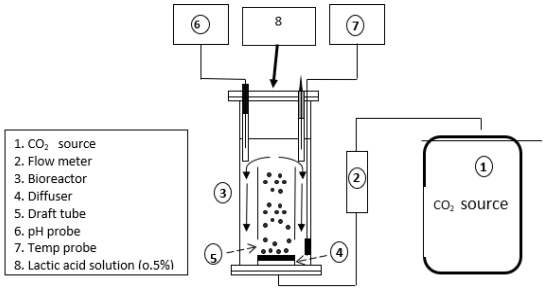

Fig. 1. The experimental set-up. Pure CO2 gas (100%) is fed into microporous plate

The mass transfer is preferentially enhanced with bubbles due to difference in concentration between the two phases and ratio of surface to volume.

Escherichia coli has the isoelectric point occurred at pH 3, below which, the net surface charge is positive and above pH 3, the charge is negative (Mulakhudair et al., 2016 and Nikolajeva et al., 2012). Indeed, the initial adhesion of bacterial cells to gas-liquid interfaces is promoted by surfaces hydrophobicity (Rochex et al., 2004). Similarly, the attachment of the positively charged gas-liquid interfaces and the negatively charged microbial cells increases with increasing ionic strength, indicating that the electrostatic attraction between microbial cells and bubbles interfaces strengthens the attachment. The time required for this process to take place is known as the induction time and this time should be less than the time required for the microbial cells to glide around and off the back the bubble (Schäfer et al., 1998).

Thereafter, the diffused CO_2 tends to be concentrated in the layer of phospholipid within the cytoplasmic membrane, and this would increase the liquidity of the membrane and exhibiting an anaesthesic effect on bacterial cells (Walls et al., 2014).

The present study aims to investigate using CO_2 mediated approach to reduce the survivor ratio of *E.coli* population, a common pathogen in food products and lactic acid is widely used in the food-related products and would improve the efficiency of the process, and to achieve more log reduction [- Log].

MATERIAL AND METHODS

The cultivation of *E. coli* on nutrient agar (Sigma-Aldrich, UK) was carried out at 37℃ for 24 hours, while the isotonic saline solution was provided and cooled for 24 h at 6℃. 900 ml of 18-24 hrs grown bacterial culture was completed to one litre by mixed with saline solution to prepare the diluted treatment solution, which was grown for 18-24 hrs, 6℃ was set as the final temperature.

In the present study, 3 L bioreactor was bubbled with pure carbon dioxide through a microporous plate as shown in **Fig.1**. All the experiments were carried out for 90 min, while the sampling process was done every 15 min. During this process, pH and temperature were recorded. Dilution of the samples were prepared with sterile saline and cultivated aseptically on agar plates (Nutrient agar) and incubated at 37℃ for 24 hrs. ColonyCount, 2018 (Promega corporation) was used to count the grown colonies. The culture with 30 – 300 colonies were counted, and CFU/ml were calculated.

Determination of CO_2 concentration

The SevenCompact™ pH-meter was used to measure the pH values and accordingly to determine the concentration of CO_2 in the solution using the following equation:

$$[CO_{2(aq)}] = \frac{(10^{-pH})^2\,(10^{-pH} - 10^{-14+pH})}{K_{a1}K_h\,[10^{-pH}] + 2K_{a1}K_{a2}K_h} \quad (Eq.6)$$

Where $K_h = [H_2CO_3]/[CO_{2(aq)}]$,

$K_{a1} = [HO^{-1}{}_3][H^+]/[H_2CO_3]$,

$K_{a2} = [CO_3{}^{-2}][H^+]/[HCO^-{}_3]$.

Morphological examination of the bacterial cells

All the microbial samples were stored using the saline solution (Oulé et al., 2006). Further, the samples were mounted on 12.5mm diameter carbon-sticky stubs coated in with TESCAN VEGA3 sputter, 25 nm of gold. Finally, they were examined with a TESCAN instrument (GmbH, Germany) with 20kV voltage acceleration.

RESULTS AND DISCUSSION

Reducing of survivor ratio of *E.coli* with lactic acid addition *E.coli* cells were treated using carbon dioxide enriched bubbles at 100 ml/min flowrate, 1 atm pressure and for 90 min (**Fig. 2**). The efficiency of the inactivation was measured using L-value and D-value, which estimated according to (Oulé et al.,2006). L-value is thetime to remain constant before starting the inactivation process, While the D-value is the time required for 1 log reduction in the microbial number. Using the carbon dioxide-enriched bubbles has led to 3.6-log reduction in cells' population, with 600 min of D-Value and 8 min of L-value. CO_2 effects and stress resulted from Oxidative are the vital players in this process (Kim and Park, 2014). The oxidative stress is resulted in disrupting the membrane-bound proteins with changing the membrane properties and lipid peroxidation.

On the other hand, the CO_2 mechanisms of action were described in depth previously (Cabisco et al., 2000

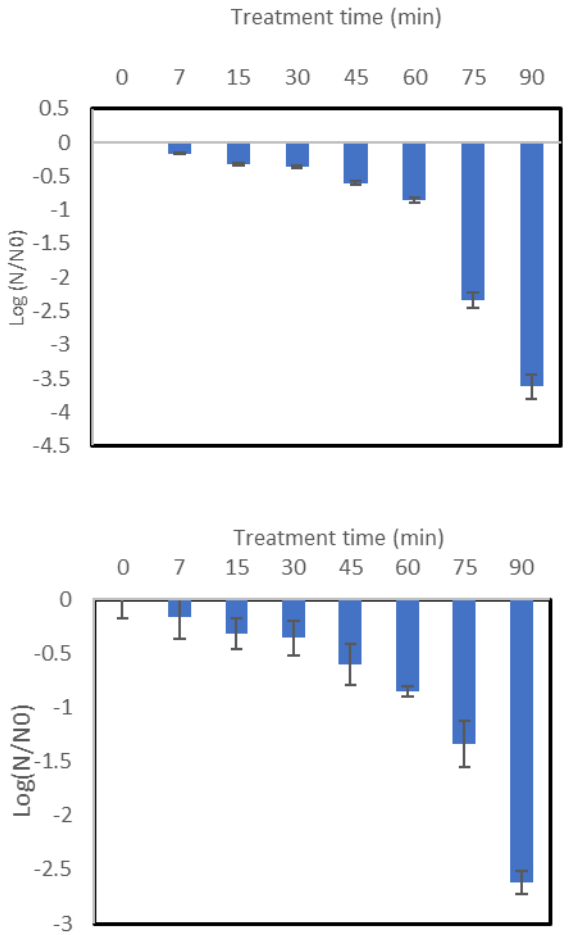

Fig. 2. Population number of *E.coli* after treatment with. (A) Carbon dioxde. (B) carbon dioxide and lactic acid 0.5% (v/v)

and Blass, 1999). With these mechanisms, high reduction of $_{E.coli}$ population can be reached if carbon dioxide enriched bubbles were used.

The temperature plays crucial role in controlling the solubility of carbon dioxide in solutions. Increasing the temperature reduces the solubility of that gas in water solutions, and vice versa (Carroll et al., 1991). According to **Fig. 3**, a gradual increase in temperature during the sparging course was shown (Temperature dropped from 12 ℃ to 6 ℃).

Some of organic substances may play role in enhancing the inactivation process if they present in the inactivation solution. These organic solvents are detrimental because its effect in increasing the permeability of the membrane, increasing the fluidity of the cell membrane, and it is preferentially concentrated in the cytoplasmic membranes (Conway, 1992 and Uday and Pramod, 1986). Additionally, lactic acid, is a membrane-active solvent and it was previously suggested to change the cytoplasmic membrane composition through changing the ratio of saturated and unsaturated fatty acids (Heipieper et al., 1991 and Heipieper et al., 1994). Lactic acid, as a weak organic

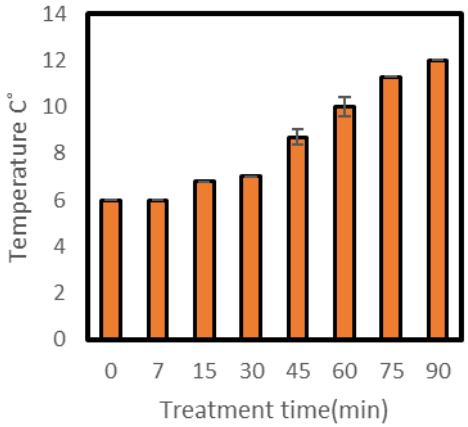

Fig. 3. Lineation of temperature during the pretreatment process

acid, has lipophilic activity and would penetrate the microbial cells through passive diffusion, whereby it dissociates directly in the cytosol. As a result, the internal pH of the bacterial cells would significantly decrease and the metabolic activity would be strongly reduced and the cell would eventually die. Another consequence of this is the change in the electrochemical gradient across the cytoplasmic membrane, an important demand for secondary transportation between cells and its environment. (Ingram, 1976).

The addition of lactic acid reduced the pH of the pretreatment solution to 3. Further, carbon dioxide is existed in an aqueous phase within acid conditions, where the carbonate species present at very limited concentrations (Ingram, 1977). It was hypothesied that carbon dioxide is directly working on the cell membrane, and CO_2 low pH would consider the most important factor that explains the elevated penetration rate of CO_2 into bacterial cells (Garcia-Gonzalez et al., 2007).

Additionally, remaining of the used acid in the treated samples has a minimum inhibitory effect (Heipiepe et al., 1996).

CO_2 concentration during CO_2-enriched bubbles sparging plus lactic acid

Equations 6 was used to determine CO_2 concentrations at different pHs as shown as in **Fig. 4**. It can be seen that in conjunction, increasing carbon dioxide increases the inactivation process. The increased CO_2 level is concentrated in the phospholipid layers of the plasmic membrane, and thus leads to increase in the penetrated CO2 (Trček et al., 2015). It can be also noticed that the survivor ratio of *E.coli* population can be declined if dissolved CO_2 in culture media increases, as a result of the positive relationship between CO_2 concentration and inactivation ability of CO_2 enriched bubbles. the lactic acid addition to the solution decreases pH to around 3 and this would be an obstacle for the dissociation of CO_2.

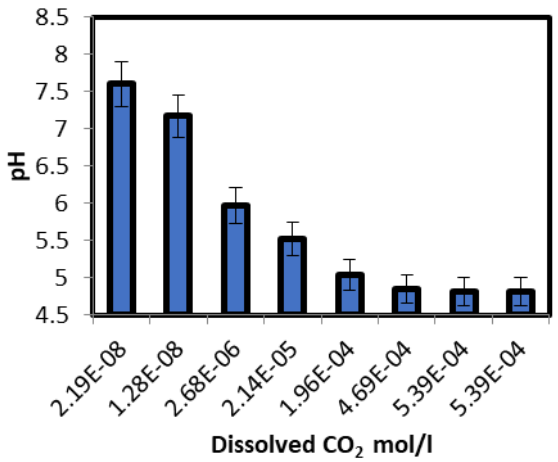

Fig. 4. Concentration of CO2 during the pretreatment process. The points are representative of triplicate results

Henry constant of CO_2 is relatively low, thus; it has good solubility in water in comparison with other gases such as oxygen and nitrogen. The chemical reaction of CO_2 with water to produce carbonic acid and its ions, can play an important role in determining the value of CO_2 solubility. However, concentration of CO_2 in the water is still a function of pH, since the concentration of CO_2 in water decreases when pH decreases (VanBogelen et al.,1987). The main reason of this decreasing is that these chemical reactions (Tab.1) thermodynamically have become unspontaneous reactions due to increase in the concentration of hydrogen's ions in the water according to Le Chatelier's principle (Al-Mashhadani et al., 2012) due to the lactic acid addition. Thus, the CO_2 at acidic pH (3 to 4) is still as an aqueous gas or undissolved gas and in general ; microorganisms are likely to interact with bubble gas-liquid interfaces, whereby the CO_2 shows its activities directly.

Morphological changes on *E.coli* cells

The changes in morphological of bacterial cells and eventually in their number was observed examine the manifestations of the inactivation process. It was observed that the number of bacterial cells was substantially reduced in comparison with control experiments. This reduction was combined with

Table 2. Chemical reactions with their corresponding reaction rate constants used to integrate the CO2 concentration equations in the current study

Chemical reaction	Reaction rate constant	Unit	Reference(s)
$H_2O \overset{K^f}{\rightarrow} H^+ + OH^-$	$K^f = 5.5 * 10^{-6}$	$1/s$	32
$H^+ + OH^- \overset{K^r}{\rightarrow} H_2O$	$K^r = 3 * 10^3$ $K_{eq} = 1.8 * 10^{-16}$ $K_w = 1 * 10^{-14}$	$1/mole/sec$	33 34
$CO_2 + H_2O \overset{K^f}{\rightarrow} H_2CO_3$	$K^f = 0.043$	$1/mole/sec$	35
$H_2CO_3 \overset{K^r}{\rightarrow} CO_2 + H_2O$	$K^r = 14.98$	$1/s$	
	$K_{eq} = 2.87 * 10^{-3}$		
$H_2CO_3 \overset{K^r}{\rightarrow} HCO_3 + H$	$K^f = 10^{6.9}$		
$HCO_3 + H^+ \overset{K^r}{\rightarrow} H_2CO_3$	$K^r = 4.67 * 10^{10}$ $K_{eq} = 1.7 * 10^{-4}$	$1/mole/sec$	
$HCO_3 \overset{K^f}{\rightarrow} CO_3 + H^+$	$K_{eq} = 5.62 * 10^{-11}$		36

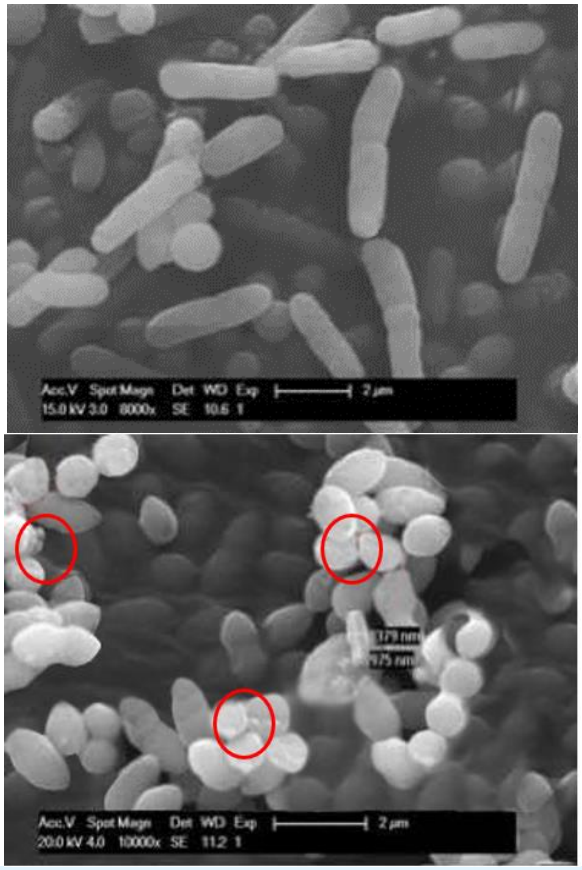

Fig. 5. Population number of *E.coli* after treatment with. (A) Carbon dioxde. (B) carbon dioxide and lactic acid 0.5% (v/v)

changes in cells' morphology such as shrinking and shortening of the cells. Further, images showed changes in the morphology of the bacteria cells such as they were shortened and shrinkaged.

This observation was previously marked by Fraser, 1951, when the author observed that bacterial cells under supercritical conditions were burst after releasing the pressured gas. Thereafter, this theory was confirmed and broadened to extract some cellular components such as enzymes (Hallsworth et al., 2003). From **Fig. 5**, the size of *E.coli* cells was reduced due to the treatment in combined with lactic acid addition at the beginning of the inactivation process. The reduction in bacterial number may be due to the lack of tolerance in *E.coli* to the passive diffusion of lactic acid within the plasmic membrane, which intensify the effects of dissolved CO_2. Decreasing the cell size consequently decreases the tolerance to lactic acid due to the decrease in relative area for passive diffusion (Hwang et al., 2009 and Isenschmid et al., 1995). Therefore, reducing cells size in the current experiment suggests that bacterium lacks the mechanism to tolerate the weak acid in combined with increase the membrane permeability via CO_2.

CONCLUSIONS

Changing from high energy consuming inactivation methods to non-thermal ones has been explored. *Escherichia coli* was used as a model in the current study. CO_2 approach is a relatively new technology, which uses the greenhouse gas, carbon dioxide, to inactivate microorganisms *in situ*. Lactic acid was chosen due to wide spread usage in the food products and the minimum detrimental effects that would cause in downstream processes such as foodstuff manufacturing. The treatment with CO_2 alone caused around 2.5 Log, wherease around 3.6 Log reduction was achieved after lactic acid addition. Addition of lactic acid intensified the activities of CO_2 and reduced the population of *E. coli*. Morphological changes one bacterial cells were observed, and they range from changing in shapes to changing in numbers with several lesions and severe injuries signs and cell death.

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