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Natural freezing as a wastewater treatment method: *E. coli* inactivation capacity

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ABSTRACT

Inactivation capacity of *E. coli* (strain ATCC 15597) in water by natural freezing was examined via two freezing methods: spray freezing and freezing in a freezer. The effect of freezing temperature (−5, −15 and −35 °C), storage time, freeze–thaw cycles on the survival of the test organism were investigated. In addition, the number of cells injured by the freezing process was also examined by using different growth media. The bacteria frozen at the warmer temperature (−5 °C) was most sensitive to storage and freeze–thaw cycles as compared to those frozen at −15 and −35 °C. In general, greater inactivation efficiencies were achieved under longer storage time and warmer freezing temperature conditions. Freezing and thawing caused cell injury. More cells were injured when frozen at −15 °C. The percentage of cells injured decreased as freeze–thaw cycles increased. The spray-freezing process was found more effective in killing the cells. On average, the log reduction rate for the spray ice with two-day storage time was about 4 log units higher than those without any storage after freezing. The results indicated that the natural freezing processes are not only cost-effective techniques for chemical and physical contaminant removal from wastewater or enhancing sludge dewaterability in cold regions but also effective in reducing *E. coli* concentration.

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1. Introduction

Studies on the potential pathogen inactivation capacity of freezing and thawing and the possible benefits in water or waste treatment are very limited. Sanin et al. (1994) investigated the pathogen reduction capabilities of freeze–thaw sludge conditioning and found that freeze–thaw resulted in a significant reduction in most pathogens of concern to public health, although it did not disinfect the sludge as effectively as pasteurization. Log reduction ranging from 0 for *Ascaris* ova to >8.0 for *Cryptosporidium parvum* oocysts were observed. Sanin and his coworkers were convinced that freeze–thaw as an economically feasible technique for sludge conditioning in regions where natural freezing was available was also effective for reducing pathogens in municipal wastewater sludge. Encouraged by results of Sanin et al. (1994), Chu and his co-workers carried out an experimental investigation using freezing and thawing to reduce microbial concentration in wastewater activated sludge. They also observed that freezing and thawing not only enhanced sludge dewaterability but also significantly reduced microbial concentration of the sludge under most freezing conditions. In a wastewater-freezing study, Parker et al. (2000) observed two to three log reduction for gram-negative coliforms while gram-positive and some gram-negative bacteria survived the multiple freeze–thaw cycles when wastewater was turned into snow by spraying.

It is known that when bacteria are frozen then thawed, survival is dependent on many parameters such as cooling

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and warming rates, holding temperature and duration after freezing. Knowledge of how these factors affect the survival of pathogenic microorganisms in water or wastewater is important with respect to proper design and applications of freezing-treatment processes. Previous research indicated that partial spray-freezing process, a modified natural-freezing process, was effective in removing various organic and inorganic contaminants from industrial wastewater (Gao et al., 2003, 2004a, b). However, it is not known whether the partial spray-freezing process will be effective in inactivating pathogenic microorganisms. Instigated by promising results from previous researchers' work, this study was carried out to investigate the effect of freezing temperature, storage time, freezing and thawing cycles on the survival of *E. coli* bacteria. Freezing and subsequent thawing may only cause injury of *E. coli* cells and the injured cells may be viable after freeze–thaw stress. Therefore, in this study, the percentage of cells injured by freezing and thawing processes was also investigated.

2. Materials and methods

2.1. Microbiological analysis

2.1.1. Experimental bacteria and growth conditions

E. coli ATCC strain 15597 (American Type Culture Collection, Rockville, MD) was used as the test organism. *E. coli* from a slant culture was inoculated into sterile tryptic soy broth (TSB) and incubated for 24 h at 35 °C with gentle air agitation. After growth, cultures were harvested and washed twice and resuspended in sterilized 0.05 M phosphate buffer solution. The *E. coli* stock solution was then maintained at 5 °C for use.

2.1.2. Enumeration method

E. coli bacteria were enumerated by using Millipore HAGW047 membrane filters and the membrane filtration technique described in the *Standard Methods for the Evaluation of Water and Wastewater* (American Public Health Association (APHA), 1998). Two types of growth media were used: the standard m-FC agar (APHA, 1998) and a modified m-FC agar which was a nonselective agar prepared with the same components as m-FC agar, but eliminating 0.01% rosolic acid (Presswood and Strong, 1978).

2.1.3. Determination of the injured cells

In order to determine the population of injured cells, the method used by Presswood and Strong (1978) was followed. The dead cells did not form colonies either on a non selective medium (e.g. modified m-FC medium) or a selective medium (e.g. m-FC medium). These cells were destroyed by freezing and thawing processes and therefore had no ability to grow on these two types of media. The population of dead cells was determined by the difference between the number of colonies formed on a m-FC medium before and after freezing. A cell is termed injured if it could form colonies on a non selective medium, but not on a selective medium. The selective medium contains substances to which the test organism is usually resistant. Therefore, the difference in counts between the two growth media was used to estimate the portion of injured cells. The non injured population is made up of cells that are able to form colonies on a selective medium after freezing.

2.2. Freezing experiments

Two sets of freezing tests were performed: (1) batch-freezing test and (2) spray-freezing test. Inactivation was measured by comparing *E. coli* concentration (N) at a given experimental condition (i.e. freezing temperature, duration of storage time or freeze–thaw cycles) with the initial *E. coli* concentration (N_o). The log reduction or inactivation of *E. coli* bacteria at each experimental condition was calculated as $\log_{10}(N/N_o)$.

Batch-freezing test: In the batch-freezing test, 50 mL of *E. coli* solution with a concentration of 10^6 to 10^7 colony forming units per milliliter (CFU/mL) was placed in 100 mL beakers and frozen at −5, −15 or −35 °C in a freezer. The freezing of the liquid solution proceeded in multiple directions as the beakers were not insulated. The batch-freezing test was carried out to investigate the effect of the freezing temperature, storage time, and freeze–thaw cycles on the survival of *E. coli* bacteria. Thawing of the frozen *E. coli* solution was carried out at 5 °C.

Spray-freezing test: The same type of *E. coli* solution (10^6 to 10^7 CFU/mL) as used in the batch-freezing test was frozen by spraying. The *E. coli* solution was atomized through a nozzle and sprayed into a freezer. The freezer was located in a cool room where the temperature fluctuated between 1 and 5 °C. The freezer was kept at −15 °C during the spraying operation. The cover of the freezer was modified to fit the spraying system. During spraying the *E. coli* solution became small drops, the bacteria in the solution thus experienced higher cooling rates as compared to those in the batch-freezing test. The spraying system consisted of a 0.65 mm diameter nozzle (Evenmist™, Field Controls, NC) which can spray at a 60° angle, a diaphragm pump (Model: 8000-813-238, Garden Grove, CA) operated at about 689 kPa (100 psi) with adjustable flowrates (flow rates of 20 and 24 mL/min were used). The sprayed solution was collected in a plastic tray (460 mm × 360 mm × 130 mm) and placed inside the freezer. During the experiments, the temperature inside the freezer was monitored using temperature sensors and the data were collected via a data logger (Data Dolphin, Model-400).

Before each spraying operation, the spraying system was rinsed using 100 mL/L bleach solution for 20 min, and then followed by sterile water for 15 min. Finally, the system was washed with the sample solution for another 10 min.

The sprayed solution was only partially frozen and both ice and unfrozen liquid samples were collected. After spraying, half of the ice samples were stored at −15 °C for 2d and the other half was melted immediately. The storage temperature of the spray ice was the same as the freezing temperature. The spray ice was placed in insulated cylindrical Plexiglas storage columns ($d = 120$ mm, $H = 150$ mm) with a netted bottom. The melting test was carried out in the cool room. A 150 W infrared lamp was used to speed up the melting process. Meltwater was collected from the ice columns throughout the melting. After collection, the meltwater samples were analyzed for *E. coli* concentration.

E. coli inactivation efficacy of the spray freezing was determined. The effect of storage time on the survival of

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