Effectiveness of pressurised carbon dioxide for inactivation of *Escherichia coli* isolated from sewage sludge

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**ABSTRACT**

This research explored the possible application of pressurised carbon dioxide (P CO₂), a promising non-thermal sterilisation technique, for the treatment of sewage sludge (SS) before anaerobic digestion to inactivate pathogenic microorganisms. *Escherichia coli* was selected as the test organism and was isolated from SS and maintained in pure culture. The growth curve of the isolated strain was determined by measuring the optical density (OD) in liquid culture medium and relating this information to the spread plate count so that a culture of known cell density could be grown for optimisation experiments. Inactivation of *E. coli* was enhanced by increase in pressure (1,500, 2,000 and 2,800 kPa) and treatment time (from 0.75 to 24 h). A short exposure time at high pressure was sufficient to provide a degree of inactivation which could also be achieved by longer exposure at lower pressure. Complete inactivation (8 log₁₀ reduction) was possible at all three pressures. Scanning (SEM) and transmission (TEM) electron microscopy studies of *E. coli* treated with P CO₂ revealed that the cell walls were ruptured, and the cytoplasm was unevenly distributed and had lost its density, indicating the possible leakage of intracellular substances.

**Key words** | anaerobic digestion, pathogens, pressurised carbon dioxide, sewage sludge

**INTRODUCTION**

Sewage sludge (SS) from primary and secondary wastewater treatment is a major bio-resource whose production has been consistently increasing in Europe and elsewhere. Annual SS production in the UK is about 1.7 million tonnes dry weight, over 70% of which is recycled to agricultural land after treatment (Luduice 2001; Water UK 2010).

Anaerobic digestion (AD) is a preferred technology for SS stabilisation (Water UK 2008) and offers additional benefits such as the production of biogas for renewable energy, and of digestate which can be used as a soil conditioner (Dohanyos & Zabranska 2001). However, SS contains a high load of pathogenic microorganisms (Strauch 1991) which can survive the digestion process (Sahlström 2006) and pose risks to human and animal health when the digestate is applied to arable land (Sahlström 2003). Agricultural utilisation of SS in the UK is regulated by the Safe Sludge Matrix (SSM), a voluntary agreement between the British Retail Consortium and Water UK (ADAS 2001). The SSM requires SS intended for land recycling to be properly treated in order to ensure a 4 or 6 log₁₀ removal of *Escherichia coli*. Several pre-treatment techniques have been developed for sludge sanitisation and improvement of biogas production but those aimed at cell disruption, such as mechanical, thermo-chemical and ultrasound methods, all have a high energy demand and low-cost alternatives are thus of interest.

Pressurised carbon dioxide (P CO₂) has emerged over the past two decades as a promising technique for non-thermal food sterilisation (Garcia-Gonzalez et al. 2007). The majority of studies have employed CO₂ in the supercritical state (31.1 °C and 7,380 kPa) (Kamihira et al. 1987; Ishikawa et al. 1995; Kim et al. 2007a, b) whereby it possesses gas-like diffusivity and liquid-like dissolution power, thus making it an ideal solvent for effective sterilisation. Capital and operating costs are high, however, due to the type of vessel required to withstand the elevated pressure (Garcia-Gonzalez et al. 2007), making this approach unsuitable for a large-volume, low-value product such as SS. Earlier research has shown that the mechanism of action of both subcritical and supercritical CO₂ is the same except that...
intracellular penetration is faster in supercritical conditions. This situation results in more rapid inactivation compared to the longer treatment duration required to achieve the same degree of inactivation in subcritical conditions (Lin et al. 1992). Recently, an attempt has been made to use P CO₂ in subcritical conditions for enhancement of biogas production by treatment of SS prior to AD. Spooner et al. (2007) described a novel process named ‘Cellruptor’ (initially ‘Bug Buster’), and presented preliminary results from a unit installed at Old Whittington Wastewater Treatment Works (Yorkshire Water, UK): this finding was reported to increase biogas production by 30–40% by pumping compressed biogas at 400–600 kPa through SS prior to AD. This phenomenon was explained by the rupture of microbial cells upon decompression causing release of intracellular components, which became readily available to anaerobic bacteria, resulting in an increase in biogas production (Spoonser et al. 2007). The current work aimed to investigate whether such a process could also offer enhanced digestate sanitation due to inactivation of pathogenic microorganisms by the pretreatment of SS with subcritical P CO₂, thus potentially offering further cost savings in sludge treatment.

**MATERIALS AND METHODS**

All chemicals used in this study were obtained from Fisher Scientific (Loughborough, UK). Co-settled Primary Sewage Sludge (PSS) was collected from Millbrook Wastewater Treatment Works (Southern Water), Southampton, UK. The E. coli used was isolated from this PSS. P CO₂ treatment was carried out in the pressurisation unit of a CAL2k bomb calorimeter (Digital Data Systems Ltd, South Africa) using compressed CO₂ (99% purity) obtained from BOC (UK).

*Escherichia coli* was enriched from freshly collected PSS according to the procedure in SCA (2003). *Escherichia coli* was identified by the change of lauryl tryptose broth with bromocresol purple from purple to yellow. MacConkey agar (MCA) in Petri plates was inoculated by sterile loop from the confirmation stage, which was carried out in tryptone water (TW) after incubation at 37 °C for 24 h. The isolated colonies showed a characteristic ‘rose red’ colour on MCA. Confirmation of *E. coli* was conducted by the indole test (SCA 2003). The isolated culture was sub-cultured and tested a further three times to ensure that the strain was a pure culture. Storage of the isolated strain was carried out by sub-culturing the colonies from MCA in TW and streaking on to nutrient agar (NA) slants which were incubated at 37 °C for 24 ± 2 h then stored at 4 °C. These slants were used to inoculate TW to grow a pure culture in each subsequent experiment. *Escherichia coli* was sub-cultured on fresh NA slants every fortnight to retain viability.

A culture of *E. coli* was prepared by inoculating 100 ml TW in an Erlenmeyer flask from NA slants and incubating in a shaking incubator at 37 ± 2 °C for ~12 h. An amount of 0.1 ml of this culture was aseptically transferred to 100 ml TW and placed on an orbital incubator at 37 ± 2 °C. At hourly intervals a sample was aseptically removed, a portion of which was placed into a cuvette for OD determination. One millilitre was transferred into 9 ml of Maximum Recovery Diluent (MRD). This aliquot was then serially diluted with 0.1 ml transferred and spread-plated on MCA petri plates. The inoculated MCA petri plates were incubated at 37 °C for 24 ± 2 h, after which the colonies of *E. coli* were counted. This procedure was continued hourly until the culture reached stationary phase. OD was measured using a Cecil 3000 spectrophotometer (Cecil Instruments, Cambridge, UK) at 400 nm against a blank of TW. The growth rate and doubling time were calculated as follows:

\[
\text{Growth rate} = \frac{\log_{10} \left( \frac{N}{N_0} \right) - \log_{10} (10)}{(T - T_0)} \tag{1}
\]

\[
\text{Doubling time} = \log_{10} \frac{N - N_0}{N_0} / \log_{10} \frac{N}{N_0} \tag{2}
\]

where \(N_0\) and \(N\) are the initial and final cell counts and, \(T_0\) and \(T\) the start and end times.

For each test run, a culture was grown in TW to a cell density of ~10⁸ CFU ml⁻¹, based on its OD. This culture was split into two portions: an untreated control and a 5 ml sample, which was then pressure treated with CO₂ at one of the selected pressures (1,500, 2,000, 2,800 kPa) and treatment times (24, 18, 12, 9, 6, 3, 1.5, 1 and 0.75 h) in the pressurisation unit (Figure 1). Immediately before treatment, 1 ml of the control sample was serially diluted in MRD, spread-plated on MCA and incubated at 37 °C for 24 ± 2 h. This control (control 1) gave a value for the initial cell count. Another control (control 2) was maintained at ambient pressure and temperature until the end of the pressurisation period then serial diluted and spread-plated alongside the treated sample to quantify any change in cell numbers for the untreated material that occurred during the treatment period.

At the end of the treatment period the pressure was rapidly released, the pressure vessel was opened and the pH was immediately measured in treated and untreated samples using a flat-tip solid state pH probe (PH K555-558 F Thermo Fisher Scientific UK) calibrated in buffer solutions at 4 and 7 (Fisher Scientific UK).
Degree of inactivation was determined by the enumeration of viable cells in both treated and untreated (control 2) samples and then employing the following equation:

\[
\text{Inactivation} = \log_{10} N_2 - \log_{10} N \tag{3}
\]

where \( N_2 \) is the cell count of control 2 and \( N \) is the cell count after treatment.

In order to investigate the mechanism of inactivation of bacterial cells by \( \text{P CO}_2 \), scanning (SEM), and transmission (TEM) electron microscopy studies were conducted with the untreated and treated (2,800 kPa, 9 h) \( \text{E. coli} \) cultures. Samples for microscopic study were centrifuged at 10,000 g, 4 °C for 30 min in a high-speed refrigerated centrifuge (Varifuge 20 RS, Henderson Biomedical Ltd, UK). For TEM, the supernatant was decanted and the cell pellets were re-suspended in 1 ml of primary fixative comprising 3% glutaraldehyde, 4% formaldehyde in 0.1 mol/l PIPES buffer (pH 7.2). For SEM, the samples were again centrifuged and half of the primary fixative was removed followed by re-suspension of the cell pellet in the remainder of the fixative. This small amount of sample was then left for 30 min on the surface of a 13 mm cover slip coated with 3-aminopropyl tri-ethoxy silane (APES) kept in a glass vial. The coated cover slip provided a static charge for holding the bacterial cells. The next steps were buffer rinsing, post-fixation in 1% buffered osmium tetroxide and dehydration in alcohol series. After this step, the cover slip with bacterial cells attached to it was dried in a critical point drier (CPD 030, Balzers, Germany) followed by mounting on aluminium stubs, which were coated with gold palladium in a sputter coater (SEM coating unit E 5100 Polaron Equipment Ltd, UK). The samples were then viewed on a scanning electron microscope (FEI Quanta 200, Netherlands).

For TEM, the samples were prepared according to Method 4, Page et al. (1994). Gold sections were cut on an Ultra Cut E microtome (Leica, Germany), stained with Reynolds lead stain and viewed on a transmission electron microscope (FEI Tecnai 12, Japan) equipped with a SIS mega view III digital camera.

RESULTS AND DISCUSSION

Growth curves for \( \text{E. coli} \) measured by OD and by \( \log_{10} \) CFU are shown in Figure 2(a) and (b). OD versus \( \log_{10} \) CFU for the exponential growth phase of approximately 4 h is shown in Figure 2(c).

In the test runs the culture was grown for 3–4 h to an OD of around 0.8 giving a cell density of \( \sim 10^8 \) CFU ml\(^{-1}\), starting from an inoculum of 0.1 ml into 100 ml of TW with an initial OD between 0.13 and 0.15. This result was found to be reproducible between different test runs and provided a method for ensuring uniform conditions at the start of each run. The growth rate of the \( \text{E. coli} \) was around 0.6 h\(^{-1}\) corresponding to a doubling time for the culture of 1.0–1.2 h.

\( \text{P CO}_2 \) treatment resulted in a fall in pH of the cell suspension from 7.0 to 5.0 at all pressures and treatment times used. This finding was expected, as the higher pressure increases the solubility of \( \text{CO}_2 \) in the aqueous phase of the
reaction medium, which then forms carbonic acid that lowers the pH (Garcia-Gonzalez et al., 2017). The similar final pH values suggested that successful dissolution had occurred in all cases.

Table 1 shows the degree of inactivation of *E. coli* expressed as log_{10} reduction in CFU. Complete inactivation was achieved at the longest treatment time at all of the selected pressures (Figure 3).

The degree of inactivation decreased with decreases in pressure and in treatment time. These results were in agreement with previous studies (Sirisee et al. 1998; Erkmen 2001; Mazzoni et al. 2001; Liao et al. 2007) which reported an increased inactivation rate at higher pressure and longer treatment time. In each pressure range the increase in log_{10} reduction with duration was approximately linear up to complete inactivation, but the rate of increase was much higher at 2,800 kPa. Although the CO₂ pressurisation process has been tested at a larger scale for improvements in biogas production, the work did not consider the potential for pathogen reduction; the current results suggest this finding could be an important factor, but higher pressures or exposure periods may be required to produce satisfactory inactivation. At the highest pressure of 2,800 kPa a 7.8 log_{10} reduction was achieved in 1.5 h, which would satisfy the requirements of the SSM and may also represent a feasible duration for practical application in wastewater treatment.

Scanning electron micrographs of *E. coli* treated with P CO₂ (2,800 kPa for 9 h) showed clear signs of rupture, holes

<table>
<thead>
<tr>
<th>Pressure (kPa)</th>
<th>1,500</th>
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<th>2,800</th>
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<tr>
<td>Hours 24</td>
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<td>8.3</td>
<td>8.2</td>
</tr>
<tr>
<td>18</td>
<td>4.6</td>
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<td>6</td>
<td>2.5</td>
<td>2.9</td>
<td>8.1</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>2.2</td>
<td>7.9</td>
</tr>
<tr>
<td>1.5</td>
<td>0.3</td>
<td>1.5</td>
<td>7.8</td>
</tr>
<tr>
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<td>0.2</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td>0.75</td>
<td>0.2</td>
<td>0.4</td>
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</tr>
<tr>
<td>Slope</td>
<td>0.32</td>
<td>0.60</td>
<td>10.18</td>
</tr>
<tr>
<td>R²</td>
<td>0.960</td>
<td>0.949</td>
<td>0.994</td>
</tr>
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*Slopes (log₁₀ reduction in CFU per hour of treatment.***
and damage to the cells (Figure 4(a)) as compared with the smooth appearance of untreated cells (Figure 4(b)). This finding was considered likely to be a result of mechanical damage during the rapid release of applied pressure. Cell debris was clearly visible close to the treated cells, but this debris was not seen on the micrographs of untreated cells.

Transmission electron micrographs showed that the cytoplasm of treated cells (Figure 4(d)) (2,800 kPa, 9 h) was unevenly distributed and had empty spaces, again indicating the possible loss of cell components, whereas that of untreated cells was evenly distributed (Figure 4(c)).

Previous work has suggested that the mechanism of inactivation due to P CO₂ could be due to carbon dioxide diffusing across the cell membrane and reaching equilibrium within the cytoplasm; then on release of pressure coming rapidly out of solution and causing expansion and rupture of the cell. It has also been proposed that acidification caused by the carbon dioxide contributes to microbial inactivation. In this study it is clear that cell rupture is occurring at the highest pressures used. Cell structures after treatment at lower pressure were not examined, and it therefore cannot be ruled out that pH change also plays a role. Intracellular pH was not measured, but a reduction in the cell suspension by two units (from pH 7 to pH 5) was observed in all test runs: this finding indicates that pH change in the bulk suspension did not play a major role.

Although simple damage through pH reduction may not have occurred, it has also been suggested that the higher solvating power of CO₂ at elevated pressure could extract vital intracellular cell components (Kamihira et al. 1987; Lin et al. 1992, 1993), and thus contribute to disruption, as seen in Figure 4(c).
CONCLUSIONS

P CO₂ treatment of E. coli showed that inactivation could be achieved under subcritical conditions given a suitable exposure time. A reduction of 8 log₁₀ units was achieved at all three selected pressures at the longest treatment time (24 h). Reduction of 7.8 log₁₀ was achieved in 1.5 h at the highest pressure (2,800 kPa), which may represent a practically feasible duration from the perspective of wastewater treatment. SEM micrographs clearly showed ruptured cells which must have suffered physical impairment due to loss of cell contents. TEM micrographs also suggested that the treated cells had lost intracellular substances. These findings suggest the potential for employing P CO₂ pre-treatment to enhance the sanitation of digestate, and possibly also the treated cells had lost intracellular substances. These findings suggest the potential for employing P CO₂ pre-treatment to enhance the sanitation of digestate, and possibly also the biodegradability of SS in AD. Further studies on the AD of raw and P CO₂ pre-treated SS and comparison of E. coli concentrations in the final digestate product are required to verify the applicability of the technique when applied to a real sludge matrix.

REFERENCES


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