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UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING AND THE ENVIRONMENT

PRESSURISED CARBON DIOXIDE AS A MEANS OF SANITISING SEWAGE
SLUDGE AND IMPROVING BIOGAS PRODUCTION

By

Maryam Mushtaq

A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

FACULTY OF ENGINEERING AND THE ENVIRONMENT

Doctor of Philosophy

PRESSURISED CARBON DIOXIDE AS A MEANS OF SANITISING SEWAGE
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The research reports on the potential for CO₂ pressurisation as a means of enhancing biogas production in the anaerobic digestion of co-settled sewage sludge, a technique reported in the commercial literature as showing great benefits. The possibility of using this technique to reduce the number of faecal indicator bacteria is also explored, as an alternative means of complying with the UK water industry's Safe Sludge Matrix.

Initial research using pure cultures of both *E. coli* (a strain isolated from sewage sludge) and *Salmonella enterica* showed that the results from different methods for isolating, recovering, resuscitating and enumerating *E. coli* were comparable. Further testing using heat stressed and unstressed *E. coli* showed the advantages of resuscitation and the MPN technique with 2-stage incubation was therefore used in the experiments to maximise the recovery of damaged but viable cells.

The results for pure cultures showed conclusively that under the conditions of time and pressure used CO₂ pressurisation and rapid depressurisation could cause irreversible cell damage and loss of viability in both test strains. The effect was reproducible and a time-pressure relationship was established for the apparatus used. It was shown, however, that the sanitising effect was influenced by culture volume, probably as this affected mass transfer of CO₂ and hence its penetration into the cells. This finding may limit the practical application of the approach and further work is needed to establish design parameters and develop reactor systems to overcome this issue. Even under the favourable conditions used, exposure times required for 6 log reduction were too long for commercial application. Optimisation of the pressurisation vessel design may improve this and should be a focus of any future investigations.

Batch and semi-continuous anaerobic digestion tests with co-settled sewage sludge were carried out to ascertain the effect of pressurised CO₂ treatment on biogas production. These showed conclusively that the treatment did not improve either biogas productivity or specific methane yield. Experimental work also showed that even treatment conditions which gave an 8 log reduction in *E. coli* in pure culture were ineffective in reducing the number of indigenous *E. coli* in the sludge, or of *S. enterica* when added to a sewage sludge matrix. These findings led to further investigation of the effects of the size of pressure vessel and sample used. The results showed that this was an important factor, but could not fully explain the lack of performance in comparison with pure culture. It was concluded that the sludge matrix itself must play a role in protecting the microbial consortia from the effects of pressurised CO₂. The exact reasons for this were not discovered but may be due to the effect of dissolved compounds present in the sludge and/or the structure and nature of the sludge flocs themselves.

Key words: Sewage sludge, Faecal Indicator Bacteria, CO₂ pressurisation, Anaerobic Digestion, Sanitisation, Safe Sludge Matrix.

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Author's Declaration

I, MARYAM MUSHTAQ declare that this thesis entitled PRESSURISED CARBON DIOXIDE AS A MEANS OF SANITISING SEWAGE SLUDGE AND IMPROVING BIOGAS PRODUCTION and the work presented in this thesis are both my own, and have been generated by me as a result of my own original research. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been presented and published as:

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List of acronyms and abbreviations

| | |
|--------------------------------|-------------------------------------|
| AD | Anaerobic Digestion |
| Ar | Argon |
| BGBB | Brilliant Green Bile Broth |
| BMP | Biochemical Methane Potential |
| BOD | Biochemical Oxygen Demand |
| BPW | Buffered Peptone Water |
| BRCA | British Retail Consortium |
| Ca(OH) ₂ | Calcium Hydroxide |
| CFU ml ⁻¹ | Colony Forming Units per millilitre |
| CH ₄ | Methane |
| COD | Chemical Oxygen Demand |
| CO ₂ | Carbon dioxide |
| CLSM | Confocal Laser Scanning Microscopy |
| CSS | Co-Settled Sewage Sludge |
| CSTR | Continuous Stirred Tank Reactor |
| DI | De Ionised |
| DS | Dissolved Solids |
| FC | Faecal Coliforms |
| FCM | Flow Cytometry |
| FIB | Faecal Indicator Bacteria |
| FSM | Fluoro Spectrometry |
| GB | Ground Beef |
| GHz | Giga Hertz |
| g Kg ⁻¹ | gram per Kilogram |
| GM | Growth Medium |
| HCl | Hydrochloric acid |
| HPH | High Pressure Homogenisation |
| Hrs | Hours |
| HRT | Hydraulic Retention Time |
| H ₂ | Hydrogen |
| H ₂ CO ₃ | Carbonic acid |

| | |
|-------------------------|---|
| H_2O_2 | Hydrogen peroxide |
| H_2SO_4 | Sulphuric Acid |
| IA | Intermediate Alkalinity |
| Kg hr^{-1} | Kilogram per hour |
| KGy | KiloGray |
| KHz | Kilo Hertz |
| KJ | Kilo Joule |
| KOH | Potassium hydroxide |
| kPa | kilo Pascal |
| K Rad | Kilo Rad |
| KV | Kilo Volt |
| L | Litre |
| LTB+BCP | Lauryl Tryptose Broth with Bromocresol Purple |
| MAD | Mesophilic Anaerobic Digestion |
| MCA | Mac Conkey agar |
| MHz | MegaHertz |
| MLGA | Membrane Lactose Glucoronide Agar |
| MPN | Most Probable Number |
| MRD | Maximum Recovery Diluent |
| MW | Microwave |
| NA | Nutrient Agar |
| NaCl | Sodium Chloride |
| NaOH | Sodium Hydroxide |
| NB | Nutrient Broth |
| NCIMB | National Collection of Industrial, Marine and Food Bacteria |
| NH_4Cl | Ammonium Chloride |
| N_2 | Nitrogen |
| N_2O | Nitrous oxide |
| OC | Overnight Culture |
| OD | Optical Density |
| OLR | Organic Loading Rate |
| O_2 | Oxygen |
| O_3 | Ozone |

| | |
|--------------------|--|
| PA | Partial Alkalinity |
| PB | Phosphate Buffer |
| PBS | Physiological Buffered Saline |
| P CO ₂ | Pressurised CO ₂ |
| PLA | Poly Lactic Acid |
| PLGA | Poly Lactic co Glycolic Acid |
| PS | Physiological Saline |
| PSI | Pounds Per Square Inch |
| PVC | Poly Vinyl Chloride |
| RPM | Revolutions Per Minute |
| RS | Ringers solution |
| SDS PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SC | Super Critical |
| SC CO ₂ | Super Critical Carbon dioxide |
| SCOD | Soluble Chemical Oxygen Demand |
| SEM | Scanning Electron Microscope |
| SEMs | Scanning Electron Micrographs |
| SRT | Solid Retention Time |
| SS | Sewage Sludge |
| Ss | Suspended solids |
| SSM | Safe Sludge Matrix |
| SS RVEB | Single Strength Rappaport Vassiliadis Enrichment Broth |
| STP | Standard Temperature and Pressure |
| TA | Total Alkalinity |
| TAN | Total Ammonia Nitrogen |
| TCOD | Total Chemical Oxygen Demand |
| TEM | Transmission Electron Microscope |
| TEMs | Transmission Electron Micrographs |
| TS | Total Solids |
| TSS | Total Suspended Solids |
| TW | Tryptone Water |
| uPVC | unplasticated Poly Vinyl Chloride |
| UV | UltraViolet |
| VFA | Volatile Fatty Acid |

| | |
|------|----------------------------------|
| VS | Volatile Solids |
| VSS | Volatile Suspended Solids |
| WAS | Waste Activated Sludge |
| WWTW | Wastewater Treatment Works |
| XLD | Xylose Lysine Desoxycholate Agar |

1 Introduction

Sewage Sludge (SS) is an inevitable product of waste water treatment: it is rich in organic matter and pathogens, has an obnoxious odour (Vesilind and Spinoso, 2001) and is amenable to biological decomposition (Luduice, 2001). Stabilisation plays a key role in solving these problems (Luduice, 2001). Anaerobic Digestion (AD) has a strong potential for stabilisation, sanitisation and minimisation of SS putrescibility. The outputs of AD are biogas (a mixture of carbon dioxide and methane) and an organic residue called digestate which can be recycled to agricultural land (Dohanyos and Zabranska, 2001) to return valuable carbon and nutrients to the soil. However, the bio-security threats associated with the pathogens in digestate used for arable land application cannot be ignored (Sahlström, 2003). Serious outbreaks of food-borne disease have been linked to the agricultural use of untreated animal manures and wastewater (Smith and Perdek, 2004), and increased public concerns have resulted in the implementation of strict regulations for the treatment of SS intended for land application (Tyson, 2002). Agricultural utilisation of SS in the UK is regulated by Safe Sludge Matrix (SSM) which determines criteria for the appropriate treatment of SS for pathogen removal in order to make it safe for land application (ADAS, 2001). Several pre-treatment techniques such as chemical (López Torres et al., 2008), mechanical (Nah et al., 2000), thermochemical (Sawayama et al., 1995), irradiation (Lafitte-Trouqué and Forster, 2002), ozonation (Weemaes et al., 2000), and ultrasound (Grönroos et al., 2005) have been used for sludge sanitisation and improvement of biogas production in AD.

The detrimental effect of carbon dioxide (CO_2) on bacterial growth has been recognized for a long time (Valley and Rettger, 1927). As a result of extensive research over the last two decades, Pressurised CO_2 (P CO_2) has emerged as a promising technique for non-thermal food sterilisation (Garcia-Gonzalez et al., 2007). Apart from a few exceptions, however, its application is confined to the food industry. This research aimed to explore the possible application of P CO_2 for pre-treatment of Co-settled Sewage Sludge (CSS) prior to AD to inactivate pathogenic microorganisms, in order to improve the bio-security of digestate and make it safe for land application.

Introduction

1.1 Research Background

The UK government has developed a strategy to minimise the landfill disposal of biodegradable waste, to retrieve energy from it and to support the renewable energy technologies one of the most important of which is AD (DEFRA, 2007). Therefore, initial funding of £ 10 million was allocated in April 2009 budget to enhance and encourage new applications for AD technology which offers benefits such as; waste stabilisation, renewable energy production and the potential use of digestate as a soil conditioner (DEFRA, 2008). SS is a huge bio-resource whose production has been consistently increasing in the UK and worldwide. Average annual sewage sludge production in the UK during 2006-07 was 1.73 million tonnes (dry weight) (Water UK, 2008) out of which 82.6 % was recycled to agricultural land and/or used for land reclamation (Water UK, 2007). AD is a highly recommended technology for sludge stabilisation which has a strong potential to generate renewable energy in the form of biogas as well as reduce the amount of volatile solids in sludge (Water UK, 2008). However, SS contains a high load of pathogenic microorganisms (Strauch, 1991) which survive during AD (Sahlström, 2006) and pose serious risks to human and animal health when the digestate is applied to arable land (Sahlström, 2003).

Increased public awareness due to the outbreak of food-borne diseases has intensified the demand of food safety. This resulted in an agreement between the British Retail Consortium (BRC) and UK Water in February 1998 known as 'The Safe Sludge Matrix' (SSM) which determines the criteria for land application of SS. Agricultural utilisation of untreated SS has been banned since 31st December 1999. In addition to this, proper sanitisation of SS for pathogen removal prior to arable land application is mandatory (ADAS, 2001).

Recently, a novel process called 'Cell Ruptor' (initially 'Bug Buster') developed by Eco-Solids International has been reported to enhance biogas production by around 30-40 % by using pressurised biogas (35-45% CO₂) to pre-treat secondary sludge bio solids before anaerobic digestion. The principle of pressurised CO₂ treatment as applied elsewhere is that CO₂ dissolves in the aqueous phase of a liquid to form carbonic acid (H₂CO₃) which diffuses into any microbial cells present. This causes a drop in intracellular pH (Daniels et al., 1985) which affects the enzymatic activities (Jones and Greenfield, 1982) and triggers

a series of complex interrelated processes leading to the loss of cell viability (Garcia-Gonzalez et al., 2007). During subsequent decompression, CO₂ dissolved in the cytoplasm expands causing the microbial cells to rupture and release their contents (Nakamura et al., 1994). Therefore, the cell contents of sludge microorganisms become readily available to anaerobic bacteria thus helping to overcome the rate-limiting step of AD i.e. hydrolysis (Spooner et al., 2007). This process could boost the biogas production and an additional benefit would be the production of much safer digestate since the majority of pathogenic bacteria are also expected to be inactivated in the pre-treatment with compressed biogas. However, this could only be verified by a detailed and well planned experimental investigation which is where this programme of work started.

1.2 Pathogens in Primary Sewage Sludge

Primary Sewage Sludge (PSS) mainly consists of settled solids from the waste water as a result of primary treatment and is potentially hazardous due to the presence of pathogens (Vesilind and Spinosa, 2001) which can be broadly classified as helminths, protozoa, fungi, viruses and bacteria. The major source of pathogenic bacteria in PSS is human and animal faeces in addition to soil, air and water (Da Silva et al., 2001). Table 1.1 gives a summary of pathogenic bacteria present in PSS and the diseases caused by them.

Table 1.1 Important pathogenic bacteria present in sewage sludge and the diseases associated with them.

(Arthurson, 2008)

| Pathogen | Disease(s) and/or symptoms |
|--|--|
| <i>Salmonella</i> spp | Salmonellosis, Typhoid |
| <i>Shigella</i> spp | Bacillary dysentery |
| <i>Escherichia.coli</i> (Enteropathogenic strains) | Gastroenteritis |
| <i>Pseudomonas aeruginosa</i> | Otitis externa, Skin infections (Opportunistic pathogen) |
| <i>Yersinia enterocolitica</i> | Acute gastroenteritis |
| <i>Clostridium perfringens</i> | Gastroenteritis (Food poisoning) |
| <i>Clostridium botulinum</i> | Botulism |
| <i>Bacillus anthracis</i> | Anthrax |
| <i>Listeria monocytogenes</i> | Listeriosis |
| <i>Vibrio cholera</i> | Cholera |
| <i>Mycobacterium</i> spp | Leprosy, Tuberculosis |
| <i>Leptospira</i> spp | Leptospirosis |

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| Pathogen | Disease(s) and/or symptoms |
|--------------------------|---|
| <i>Campylobacter</i> spp | Gastroenteritis |
| <i>Staphylococcus</i> | Impetigo, Wound infections, Food poisoning |
| <i>Streptococcus</i> | Sore throat, Necrotizing fasciitis, Scarlet fever |

In order to minimise the threats to human and animal health, it is important to reduce the pathogenic content of sludge and this becomes even more crucial if the sludge has to be finally recycled to agricultural land. Many developed countries have strict regulations for the removal of pathogens from sewage sludge to such a level which can allow it to be safely used in farming (Pinto, 2001).

Table 1.2 gives shows the limits for pathogenic organism removal from sewage sludge in various countries.

Table 1.2 Limits for pathogenic organism removal from sewage sludge in various countries

(Pinto, 2001, ADAS, 2001)

| Organism | USA (40 CFR 503) | | | European community | |
|-------------------------------------|--|--|---|--|--|
| | South | France | UK | (New proposal) | |
| Faecal coliforms | <1000 MPN g ⁻¹ of TS | <1000 MPN 10g ⁻¹ of TS | — | 2-6 Log ₁₀ reduction of <i>E. coli</i> | Reduction of 6 log units |
| Salmonella | <3 MPN 4g ⁻¹ of TS | 0 MPN 10g ⁻¹ 1 of TS | <8 MPN 10g ⁻¹ 1 of TS | Absent from 2g dry solids | 0 MPN 50g ⁻¹ of wet mass |
| Enterovirus | < 1 MPN 4 g ⁻¹ of TS | — | <3 MPN 10g ⁻¹ 1 of TS | — | — |
| Viable helminth eggs | < 4 viable eggs 4 g ⁻¹ of TS | 0 viable eggs 10 g ⁻¹ of TS | < 3 viable eggs 10g ⁻¹ of TS | — | — |

1.3 Anaerobic Digestion

Anaerobic digestion (AD) is one of the oldest and most widely used processes for stabilisation of municipal and industrial waste water sludge (Tchobanoglous et al., 2004). The main aim of AD is the biological decomposition of a significant portion of volatile

solids in the sludge (Gerardi, 2003) alongside the reduction of offensive odours and pathogens, both of which are very important to render the digestate suitable for land application (Vesilind and Spinosa, 2001). Table 1.3 presents a comparison of the composition of raw and anaerobically digested sludge.

Table 1.3 Comparison between raw and anaerobically digested sludge

(Luduice, 2001)

| Raw sludge | Digested sludge |
|---|---|
| Unstable organic matter | Stabilised organic matter |
| High biodegradable fraction of organic matter | Low fraction of biodegradable organic matter |
| High potential for generation of odours | Low potential for generation of odours |
| High concentration of pathogens | Concentration of pathogens lower than in raw sludge |

Biogas is a valuable product of AD which can be utilised as an environmentally friendly fuel in various ways such as; heat production, electricity generation and as a vehicle fuel (Appels et al., 2008). In spite of all these beneficial aspects, AD is not very efficient in terms of pathogen inactivation especially Faecal Coliforms (FC). Only 0.5 to 4 log reduction is of FC is achieved during Mesophilic Anaerobic Digestion (MAD) . Experimental evidence has shown that MAD can inactivate about 1-2 logs of *E. coli* and nearly 2 logs of *Salmonella* which can only meet the criteria of conventionally treated sludge (ADAS, 2001). Therefore, effective pre or post-treatment techniques are a pre-requisite for proper sanitation of SS in AD so that the digestate meets the set standards for pathogen removal and is safe for use as a soil conditioner.

1.4 Pressurised carbon dioxide

Carbon dioxide was discovered around 1630 by the Flemish scientist van Helmont who named it 'gas sylvestre'. It was known to be able to extinguish a flame and cause asphyxiation in humans and animals. This property was probably one of the reasons which prompted the scientists to investigate the influence of CO₂ on microbial growth (Valley and Rettger, 1927). CO₂ plays a duplicate role in the physiology of microorganisms whereby it can both trigger or retard their growth (Liu et al., 2005). This is linked to the ability of CO₂ to act as a substrate in carboxylation reactions while also being a product of decarboxylation (Jones and Greenfield, 1982). Initially, CO₂ was utilised in the food industry to extend the shelf life of food products. It was considered that the bacteriostatic

Introduction

action of CO_2 was the result of extension of the lag phase and generation time of spoilage microorganisms (Daniels et al., 1985). Later on, it was found that the antimicrobial action of CO_2 was enhanced at elevated pressure (Kamihira et al., 1987, Haas et al., 1989).

Since then extensive research has been carried out, as a result of which P CO_2 is now recognised as a sterilisation technique with strong potential to replace the traditional thermal sterilisation (Spilimbergo and Bertucco, 2003, Garcia-Gonzalez et al., 2007). Some of the attractive features which make CO_2 an ideal sterilisation agent are, it is non-flammable, non-toxic, inexpensive and physiologically safe (Zhang et al., 2006). It has a low critical point (31.1°C and 7380 kPa) after which it enters in the supercritical state whereby it possesses gas like diffusivity and liquid like dissolution power thus making it an ideal solvent for effective sterilisation (Garcia-Gonzalez et al., 2007).

In spite of the well-known fact that P CO_2 can inactivate microorganisms, the exact mechanism of inactivation is still unclear. Initially, it was considered that CO_2 replaced oxygen (O_2) and retarded microbial growth by making O_2 un-available for cellular metabolism. This theory was later rejected. Extracellular and intracellular pH reduction was identified as a key factor contributing to the antimicrobial property of CO_2 (Daniels et al., 1985). Enzymatic activities were affected by the intracellular pH drop as a result of dissolution of CO_2 in the aqueous phase of cytoplasm. Dissolved CO_2 was found to alter the nature of proteins and hence their function by reacting with their Amino ($-\text{NH}_2$) group. Whereas carbonic acid (H_2CO_3) a product of the dissolution of CO_2 in water, interfered with the positively charged groups of proteins through a dipole- protein interaction (Jones and Greenfield, 1982). Biological membranes were found to be the major target of P CO_2 (Sears and Eisenberg, 1961, Dixon and Kell, 1989). Plasma membrane lipids were replaced by the molecules of CO_2 thus causing the order loss of lipid chain and increasing the membrane fluidity, a phenomenon termed as the ‘anaesthesia affect’ as a consequence of which, the membrane loses its ability to uptake various ions (Jones and Greenfield, 1982, Isenschmid et al., 1995). It can be concluded that microbial inactivation by P CO_2 is a consequence of closely interrelated processes which could be summarised in

Figure 1.1.

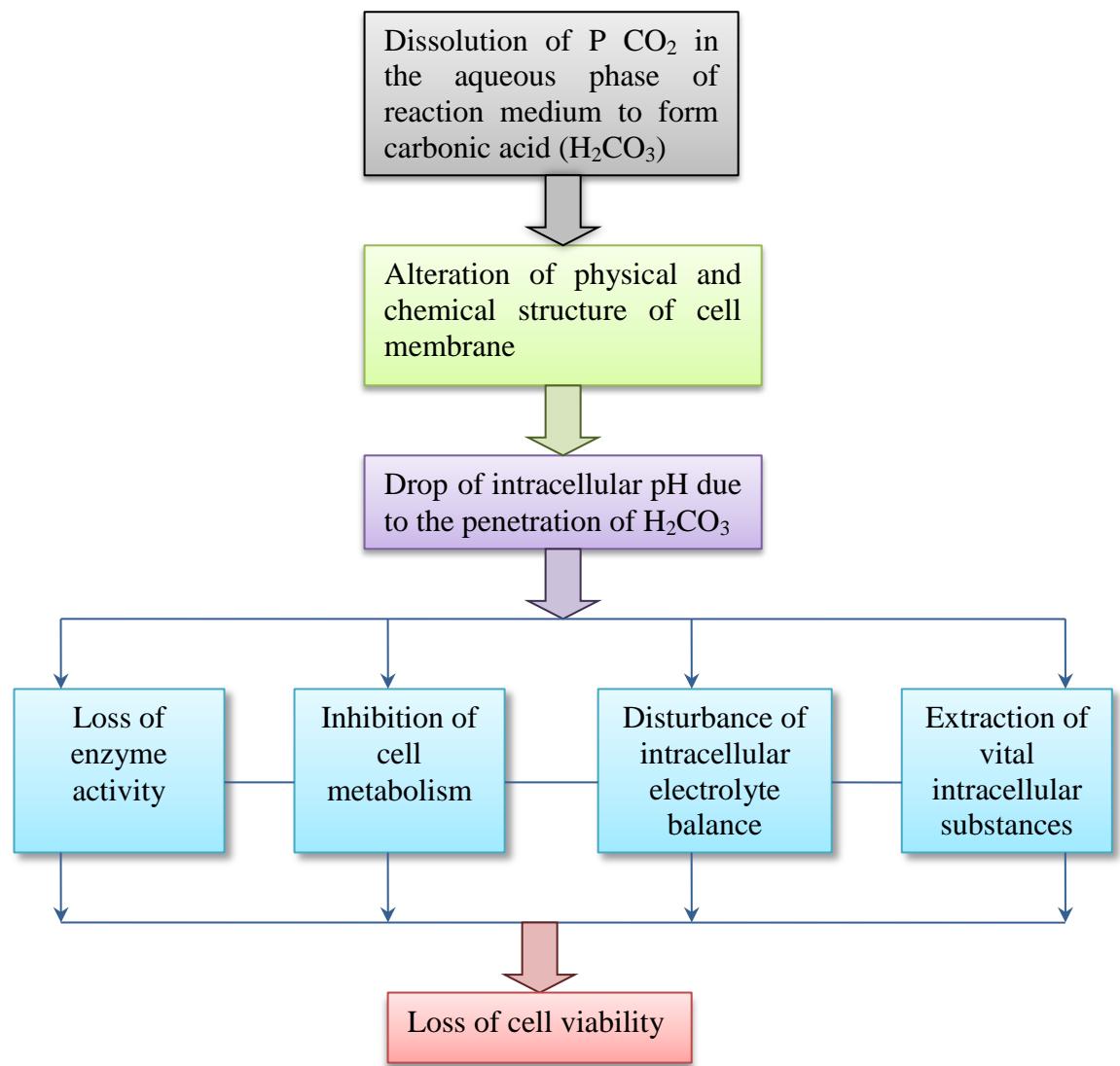


Figure 1.1 Various steps involved in the inactivation of microorganisms by P CO₂

1.5 Aim

The aims of this research were

- To assess the possibility of applying P CO₂ for improving the bio security of PSS.
- To gain insight into the sterilising mechanism of P CO₂.
- To determine the impact of P CO₂ pre-treatment on the biodegradability and sanitisation of PSS in MAD.
- To explore the possible upgrading and application of this technique in a commercial AD plant.

1.6 Objectives

To achieve these aims, the following objectives were put forward:

- To identify and isolate *Escherichia coli* from CSS and maintain in pure culture.
- To determine the optimised conditions for inactivation of Faecal Indicator Bacteria (FIB) suspended in liquid medium with regard to process parameters such as; pressure, treatment time and agitation.
- To investigate whether the FIB are able to recover and regain viability following P CO₂ treatment by employing resuscitation techniques.
- To understand the mechanism of bactericidal action of P CO₂. This will be achieved by performing various techniques such as:
 1. **Scanning and Transmission Electron microscopic studies:** These will be conducted to elucidate the changes in cell morphology and internal structure as a consequence of P CO₂ treatment.
 2. **Spectroscopic studies:** These will be carried out on the cell supernatant before and after P CO₂ treatment to determine the absorbance of Ultraviolet (UV) light as a clue to the extraction of proteins and nucleic acids from microbial cells.
 3. **Enzymatic activities of treated and untreated cells:** These will be compared using enzyme detection kit (APIZYM) which will give an insight whether P CO₂ alters the enzymatic activities of microorganisms.
- To ascertain whether the optimized process parameters determined for pure cultures are equally efficient for the inactivation of FIB in CSS.
- To carry out the Biochemical Methane Potential (BMP) test of raw and P CO₂ pre-treated CSS in order to elucidate the effect of P CO₂ pre-treatment on the biogas kinetics.
- To perform semi-continuous MAD of raw and P CO₂ pre-treated CSS in Continuous Stirred Tank Reactors (CSTR) and monitor the performance of reactors with respect to these parameters; specific methane and biogas production, biogas composition, alkalinity, ammonia, Volatile Solids (VS) and *E. coli* destruction.

2 Review of Literature

2.1 Anaerobic digestion

Anaerobic digestion (AD) is a natural process of putrefaction in which biodegradable organic matter is decomposed to simple compounds in the absence of oxygen by the combined action of several groups of microorganisms. This process occurs naturally in warm and wet environments such as swamps, river sediments, marshes and the digestive tract of ruminants (Evans, 2001). AD is a complex process in which the various groups of microorganisms constitute a balanced ecosystem and each group plays a specific role (Chernicaro, 2005). Generally, AD can be considered as a two phase process. Complex organic compounds (Carbohydrates, Proteins and Lipids) are converted in to simpler substances such as Volatile Fatty Acids (VFA) by the action of anaerobic bacteria in the first phase. In the second phase, VFAs and hydrogen are converted in to CH_4 and CO_2 by the action of methanogens, a group of strict anaerobes (Chernicaro, 2005). The two phases of AD can be divided in to four interrelated steps; hydrolysis, acidogenesis, acetogenesis and methanogenesis.

Figure 2.1 shows a schematic diagram of the four steps of AD, their interrelationship and the microorganisms involved in each step.

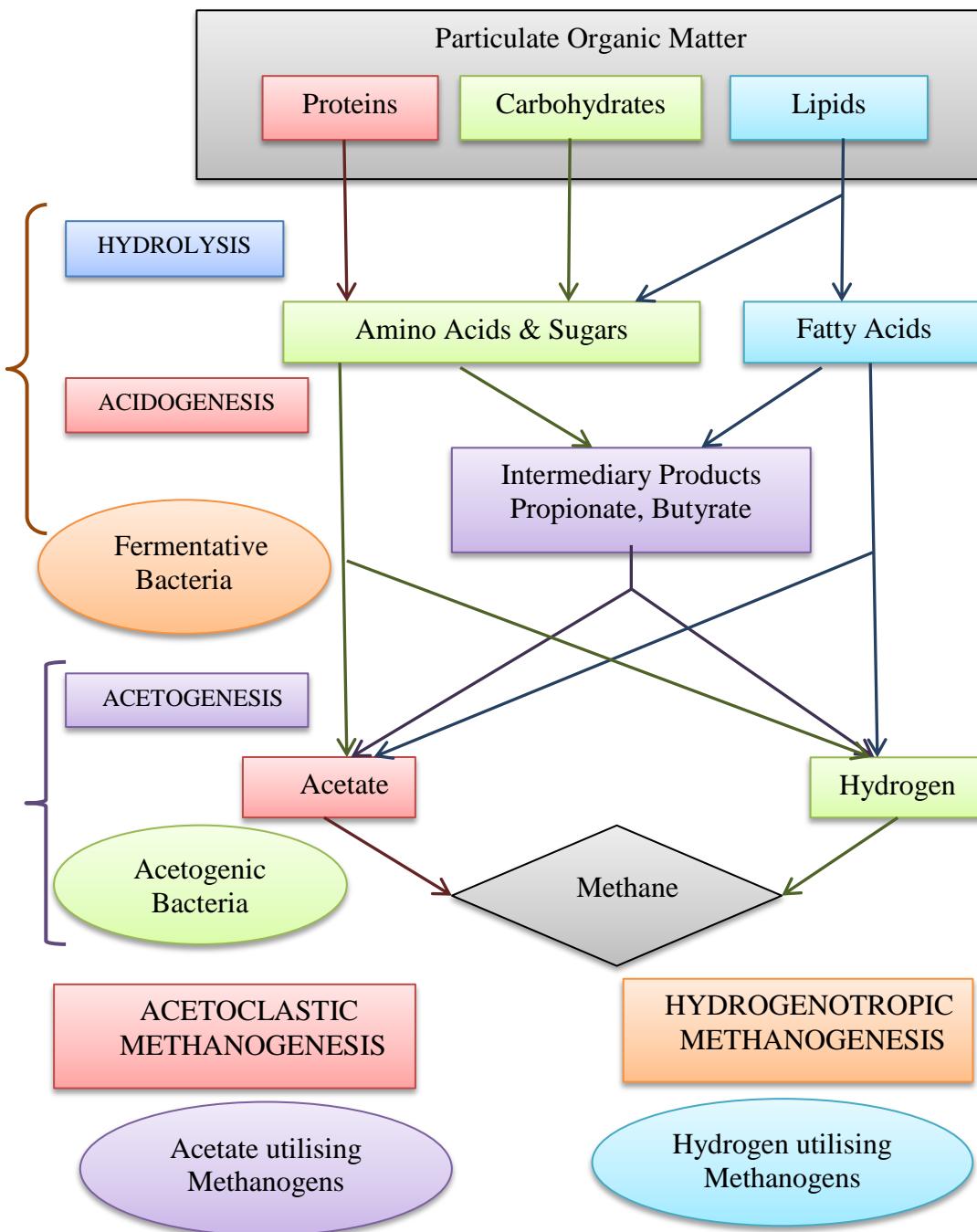


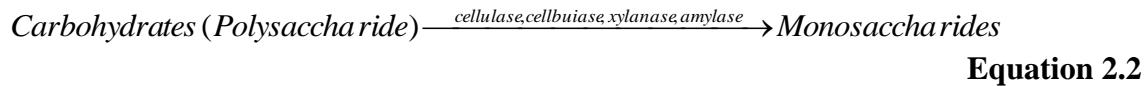
Figure 2.1 Various steps of AD, their interrelationship and the microorganisms involved in them.

(Gujer and Zehnder, 1983)

2.1.1 Hydrolysis

The first step of AD is hydrolysis during which complex biopolymers such as carbohydrates, proteins and fats are decomposed by the extracellular enzymes of hydrolytic bacteria into simple sugars (monosaccharides), fatty acids and glycerol and

amino acids respectively (Al Seadi et al., 2008, Evans, 2001), as shown in the following equations. Hydrolysis is often considered as the rate limiting step of AD.



2.1.2 Acidogenesis

Products of hydrolysis i.e. simple organic molecules easily penetrate through the cell membrane of acidogenic bacteria where they are converted into methanogenic substrates: VFAs (Acetic, lactic and propionic acid), alcohols, Ammonia (NH_3), Hydrogen sulphide (H_2S), CO_2 and H_2 (Chernicaro, 2005). The exact proportion of different products depends partly on the environmental conditions, and largely on the bacterial species present in the anaerobic digester (Evans, 2001).

2.1.3 Acetogenesis

The role of acetogenic bacteria is to convert the products of acidogenesis which cannot be utilised directly by methanogenic bacteria such as alcohols and VFAs other than acetic acid into methanogenic substrates i.e. H_2 , acetate and CO_2 (Al Seadi et al., 2008). During this process the pH might drop due to the production of H_2 . However, this H_2 is consumed by methanogenic bacteria to produce methane and VFAs (propionic and butyric acids) by the reaction of H_2 , CO_2 and acetic acid. The VFAs are further disintegrated by acetogenic bacteria to produce acetic acid and H_2 (Chernicaro, 2005).

2.1.4 Methanogenesis

Methanogenesis is the final stage of AD which involves the production of CH_4 and CO_2 from the products of previous stages. In AD systems treating wastewater and similar mixed substrates at typical organic loadings, nearly 70% of CH_4 is generated from acetate whereas the remaining 30% originates from the reaction of H_2 and CO_2 (Al Seadi et al., 2008). Two groups of methanogenic bacteria are responsible for the production of methane using different substrates. Acetoclastic methanogens produce methane from

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acetate, whereas hydrogenotrophic methanogens are responsible for methane production from H_2 and CO_2 (Chernicaro, 2005). Methanogenesis is the most critical and slowest step of AD and is highly sensitive to the changes in operational conditions. Temperature, pH, feedstock composition and loading rate are factors which can affect methanogenesis. Pronounced variations in temperature and loading rate or entry of large amount of O_2 in the digester can severely inhibit the process of methane production (Al Seadi et al., 2008).

2.2 Role of Anaerobic Digestion in waste water treatment

The efficiency of AD for stabilisation of raw sludge was well known to sanitary engineers nearly 150 years ago. AD was used initially in 1860 for the stabilisation of domestic waste water using an air-tight septic tank called 'Moura's automatic scavenger' (McCarty, 2001). The technique of AD progressed steadily and resulted in the development of highly efficient anaerobic digesters in the late 20th century for the treatment of sewage and industrial wastewater (Gijzen, 2002).

Major operational steps in a wastewater treatment plant can be listed as (Vesilind and Spinosa, 2001);

- Preliminary treatment
- Primary treatment
- Secondary treatment
- Tertiary treatment
- Disinfection
- Sludge treatment

Sludge constitutes the solid by-products of the wastewater treatment process which after proper biological treatment is transformed in to 'biosolids', a term used to indicate the beneficial aspects of utilisation of stabilised SS in agriculture and land reclamation (Vesilind and Spinosa, 2001). Figure 2.2 represents the various steps involved in the treatment of sludge, with the objective of each step and the techniques that could be utilised to achieve these objectives (Sperling and Chernicharo, 2005a).

| 1. Thickening | 2. Stabilisation | 3. Conditioning | 4. Dewatering | 5. Disinfection | 6. Final disposal |
|--|--|--|---|--|---|
| Objective Volume reduction | Objective Mass reduction | Objective Preparation for Dewatering | Objective Volume reduction | Objective Pathogen removal | Objective Destination of by-products |
| Techniques <ul style="list-style-type: none">• Gravity thickening• Floatation• Centrifuge• Belt filter press | Techniques <ul style="list-style-type: none">• Anaerobic digestion• Aerobic digestion• Thermal treatment• Chemical stabilisation | Techniques <ul style="list-style-type: none">• Chemical conditioning• Thermal conditioning | Techniques <ul style="list-style-type: none">• Drying beds• Sludge lagoons• Filter press• Centrifuge• Belt filter press• Vacuum filter• Thermal drying | Techniques <ul style="list-style-type: none">• Lime addition• Thermal treatment• Composting• Wet air oxidation• Gamma radiation | Techniques <ul style="list-style-type: none">• Agricultural recycling• Land farming• Composting• Incineration• Sanitary landfill |

Figure 2.2 Various stages of sludge management, their objectives and the main processes used
(Sperling and Chernicharo, 2005a)

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AD is currently one of the most widely used processes for sludge stabilisation in municipal wastewater treatment plants where it degrades about 80% of the organic matter (Gerardi, 2003). In addition to this, in most cases AD of municipal wastewater sludge can generate sufficient biogas to meet the energy requirements of the AD plant (Tchobanoglou et al., 2004). Moreover, the biosolids produced by AD can be used as an organic fertiliser; however, proper disinfection is a pre-requisite for unrestricted use (Sperling and Chernicaro, 2005a). Given below is a summary of the beneficial aspects and drawbacks of anaerobic systems (Table 2.1).

Table 2.1 Advantages and disadvantages of the anaerobic processes

(Chernicaro, 2005)

| Advantages | Disadvantages |
|--|---|
| <ul style="list-style-type: none">• Low production of solids; about 3 to 5 times lower than in aerobic processes• Low energy consumption; usually associated with an influent pumping station, leading to very low operational costs• Low land requirements• Low consumption costs• Production of Methane, a highly calorific fuel gas• Possibility of preservation of the biomass, with no reactor feeding, for several months• Tolerance to high organic loads• Application in small and large scale• Low nutrient consumption | <ul style="list-style-type: none">• Anaerobic microorganisms are susceptible to inhibition by a large number of compounds• Process start-up can be slow in the absence of adapted seed sludge• Some form of post-treatment is usually necessary• The biochemistry and microbiology of anaerobic digestion are complex and still require further studies• Possible generation of bad odours, although they are controllable• Possible generation of effluents with unpleasant aspect• Unsatisfactory removal of Nitrogen, Phosphorus and pathogens |

2.3 Pros and cons of land application of sewage sludge

Land application of SS can be beneficial in many respects as it could: supply macronutrients (Nitrogen, Phosphorus) and micronutrients (Iron, Copper, Zinc, Manganese) to the crops, improve the physical properties of the soil, increase soil organic matter, enhance the water and mineral holding capacity of soil, reduce surface run-off and water erosion (Kelley et al., 1984, UNEP, 2005). Beneficial aspects of the land application of SS for soil and the environment can be summarised as in Table 2.2.

Table 2.2 Beneficial impacts of land application of sewage sludge on soil and the environment

(Andreoli et al., 2007)

| Biosolid | Action in soil | Consequences in the soil | Effects on environment |
|----------------|---|--|--|
| Organic matter | <ul style="list-style-type: none"> • Aggregation of soil particles • Cation exchange capacity improvement • Improvement of soil structure • Plant nourishment | <ul style="list-style-type: none"> • Increases water infiltration • Increases resistance against rainfall impact • Reduces nutrient leaching losses • Improves soil fertility • Fosters plant growth • Increases microbial biomass • Accelerates plant growth | <ul style="list-style-type: none"> • Reduces surface run-off • Reduces surface water pollution • Reduces nutrients leaching and groundwater contamination • Increases soil covering • Improves soil aggregation |
| Nutrients | | | |

The presence of large quantities of Nitrogen and Phosphorous in SS make it a useful organic fertiliser (Andreoli et al., 2007). In addition to this, the potential beneficial impacts on the physical, chemical and biological characteristics of soil from the application of SS support its use in agriculture and land reclamation (Kelley et al., 1984). Land recycling of SS may therefore be seen not only as an eco-friendly option but also as economically favourable, as it has the potential to partially replace chemical fertilisers (UNEP, 2005).

Despite these beneficial aspects, there are some serious issues linked to the land application of SS (Kelley et al., 1984), including the presence of toxic metals such as Mercury, Lead, Copper, Cadmium, and Arsenic; persistent organic pollutants; and pathogenic organisms such as viruses, protozoa, helminths and bacteria (Arthurson, 2008). In order to assure safe, environmentally friendly and economical land recycling of SS, these issues need to be properly considered (Kelley et al., 1984).

Dissemination of pathogens due to the land application of untreated SS has long been recognised. However, serious disease outbreaks due to the use of untreated SS or animal manure (Table 2.3) have intensified the need of proper sanitisation in order to achieve the maximum advantages of land recycling of SS while safeguarding human, animal and plant health (Gerba and Smith, 2005).

Table 2.3 Examples of manure related outbreaks
(Gerba and Smith, 2005)

| Location | Year | Pathogen | Impact | Suspected source |
|----------------|------|----------------------------|----------------|------------------|
| Walkerton, ON, | 2000 | <i>E. coli</i> O157:H7 and | 6 deaths, 2300 | Runoff from farm |

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| Location | Year | Pathogen | Impact | Suspected source |
|---------------------------------|------|--|--|--|
| Canada | | <i>Campylobacter</i> spp | cases | fields entering town's water supply |
| Washington County, New York, US | 1999 | <i>E. coli</i> O157:H7 and <i>Campylobacter</i> spp. | 2 deaths, 116 cases | Runoff at fairgrounds |
| Sakai City, Japan | 1995 | <i>E. coli</i> O157:H7 | 12 680 cases, 425 hospitalized, 3 deaths | Animal manure used in fields growing alfalfa sprouts |
| Bradford, UK | 1994 | <i>Cryptosporidium parvum</i> | 125 cases | Storm runoff from farm fields |
| Milwaukee, Wisconsin, US | 1993 | <i>Cryptosporidium parvum</i> | 400 000 cases, 87 deaths | Animal manure and/or human excrement |
| Cabool, Missouri, US | 1990 | <i>E. coli</i> O157:H7 | 243 cases, 4 deaths | Water line breaks in farm Community |
| Swindon and Oxfordshire, UK | 1989 | <i>Cryptosporidium parvum</i> | 516 excess cases | Runoff from farm fields |
| Carrollton, Georgia | 1989 | <i>Cryptosporidium parvum</i> | 13 000 cases | Manure runoff |

It is evident from the above table that the causative agents of a substantial number of these disease outbreaks are bacteria. Raw SS has a considerable proportion of pathogenic bacteria (see Table 1.1), the main source of which is human faeces (Arthurson, 2008). The majority of these bacteria are zoonotic i.e. they can infect both humans and animals (Sahlström et al., 2004); can resist commonly used stabilisation practises and adapt to environmental changes (Kearney et al., 1994); and therefore survive for long time intervals in the soil (Da Silva et al., 2001). All these characteristics make proper monitoring of bacterial pathogens in SS a high priority for safe land recycling.

2.4 Bacterial pathogens and faecal indicator bacteria

The group of bacterial pathogens in SS has been extensively studied due to its epidemiological significance and well developed methods existing for analysis, quantification, and standardisation (Sahlström et al., 2004).

Isolation, identification and quantification of pathogenic microorganisms from SS is relatively complicated, however, due to the fact that only a small fraction of population suffers from waterborne diseases at a specific time, a limited number of pathogens are found in the faeces of these inhabitants and these are diluted in the sewerage system. This

results in a very low final concentration of pathogens per unit volume in the SS. This issue is resolved by the use of 'indicator organisms' for detecting the presence of pathogens. The concentration of these indicator organisms in SS is relatively high, they are basically non-pathogenic and their detection is fairly easy and economical (Sperling and Chernicharo, 2005b).

An ideal indicator organism should meet the following criteria:

- It must be present wherever there is faecal contamination.
- The number of indicator organism should be greater than or equal to that of pathogens.
- It should have a relatively longer survival time in the environment than the pathogenic organism for which it is being used as an indicator.
- The isolation, identification and quantification of the indicator organism should be easier compared to that of pathogenic organisms.
- The indicator organism must be a member of the intestinal micro-flora of warm-blooded animals (Tchobanoglous et al., 2004, Sharma, 2005).

The most commonly used indicator organisms are total and faecal coliforms, *E. coli*, *Klebsiella*, Faecal Streptococci, Enterococci, *Clostridium perfringens* and *Pseudomonas aeruginosa* (Tchobanoglous et al., 2004).

2.5 Regulations to treat sewage sludge for safe land application

In order to address the threats to human, animal and plant health linked to the land application of untreated SS, strict regulations have been imposed on the proper treatment of SS prior to agricultural use in most of the developed countries. The regulations for controlling pathogen dissemination are based on the concept of multiple barriers such as reduction of pathogens and vector attraction, restrictions on the crops grown on land amended with SS and specific intervals between sludge application and harvesting or grazing (Godfree and Farrell, 2005).

The driving factor for regulating the agricultural use of SS in UK was increased public concern linked to the farm animal diseases such as Bovine Spongiform Encephalopathy and *Salmonella* outbreaks due to malpractices in the poultry industry. In 1997, the British Retail consortium (BRC) took the issue of food safety to the UK water industry. Their main demand was to review the use of raw SS on arable land. In February 1998, the BRC and UK Water agreed on an agenda known as the Safe Sludge Matrix (SSM). In 1999, UK

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Water voluntarily decided to implement the SSM in a series of phases long before it was given the status of law in January 2007. Application of raw SS on the land used to grow food crops was banned from 31st December 1999 (Tyson, 2002).

Different types of sludge and their quality criteria defined in SSM are as follows (ADAS, 2001):

a. Untreated sludge

Sludge which is screened for removing litter but not stabilised.

b. Conventionally treated sludge

Sludge which is stabilised employing any available treatment technique in order to remove 99% pathogens. This type of sludge must ensure the removal of $2 \log_{10}$ of *E. coli*.

c. Enhanced treated sludge

Sludge stabilised by using a technique capable of eradicating almost every pathogen in the SS ($6 \log_{10}$ removal of *E. coli*). This type of sludge must be free from *Salmonella*.

Table 2.4 shows the microbiological standards for different types of sludge as described in the SSM.

Table 2.4 UK standards for treated and enhanced treated sludge

(Godfree and Farrell, 2005)

| Sludge category | Reduction in numbers of <i>E. coli</i> across sludge treatment process | Maximum number of <i>E. coli</i> in final product | Maximum number of <i>Salmonellae</i> in final product |
|------------------|--|---|---|
| Treated | $2 \log_{10}$ | 10^5 g^{-1} dry solids | No standard |
| Enhanced treated | $6 \log_{10}$ | 10^3 g^{-1} dry solids | Absent from 2 g dry solids |

In the USA, agricultural use of SS is regulated by ‘Standards for the use and disposal of sewage sludge’ which is title 40 of the Code of Federal Regulations commonly known as the ‘40 CFR part 503’. These were published in the Federal Register on 19th February 1993 and became effective on 22nd March 1993. Three classes of biosolids were distinguished in subpart D of part 503. Two classes (A and B) include the standards for indicator bacteria and or pathogens and the third class (Exceptional Quality biosolids)

includes standards for heavy metals in addition to those for pathogens (US EPS, 1993, US EPA, 1994).

Different classes of sludge and the quality criteria as described in the 40 CFR part 503 are:

a. Class B biosolids

Sludge with faecal coliform concentration less than $2 \times 10^6 \text{ g}^{-1}$ of dry weight.

b. Class A biosolids

Biosolids which meet the following quality criteria in 4 g of dry weight are designated as class A biosolids.

| | |
|---------------------|-----------|
| <i>Salmonella</i> | <1000 MPN |
| Faecal Coliforms | <1000 MPN |
| Enteric Viruses | <1 |
| Viable Helminth ova | <1 |

c. Exceptional quality biosolids

The biosolids included in this class conform to the heavy metal limits of part 503 alongside maintaining the microbiological standards for class A biosolids.

The microbiological standards for class A and class B biosolids are summarised in Table 2.5.

Table 2.5 End product microbiological standards for Class A and B biosolids (US EPS, 1993)

| Standard (4g ⁻¹ dry wt) | Class A | Class B |
|---|---------|------------|
| Faecal coliform, g ⁻¹ dry solids | <1000 | <2 000 000 |
| <i>Salmonellae</i> , 4 g ⁻¹ dry solids | <3 | |
| Enteroviruses, pfu 4 g ⁻¹ dry solids | <1 | |
| Parasite ova, 4 g ⁻¹ dry solids | <1 | |

2.6 Pre-treatments for improving the digestibility of sludge during anaerobic digestion

AD is one of the most extensively used methods for sludge treatment (about 70% of sludge is treated by AD) since it offers the benefits of reducing volatile solids in sludge,

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generating energy-rich biogas and producing a nutrient-rich organic residue (digestate). Hence, AD is a vital process in a WWTP (Mata-Alvarez et al., 2000). However, poor biodegradation efficiency (20-50%) and long retention time (20-50 days) are the major bottlenecks in this process, which are linked to the first step of AD i.e. hydrolysis (Neis, 2000). Various techniques have been applied for the pre-treatment of sludge with the aim of improving biodegradability, shortening retention time (Tyagi and Lo, 2011) and enhancing biogas yield. Some other aspects taken into account in these techniques are COD solubilisation, dewaterability, laboratory/pilot to full-scale application, capital, operational and maintenance cost and pathogen reduction.

Although the pathogen load of SS is substantially reduced during AD (about 0.5 - 4 logs) (Pinto, 2001) however, it does not comply with the microbiological standards for rendering the SS safe for land recycling (ADAS, 2001, US EPS, 1993).

Table 2.6 summarises the extent of pathogen inactivation achieved during different sludge stabilisation techniques.

Table 2.6 Pathogen reduction during different sludge treatment processes (Godfree and Farrell, 2005)

| Log reduction | | | | |
|--------------------|---------------------|-------------------|-----------------|------------|
| Treatment | | Coliform Bacteria | Enteric viruses | Parasites |
| Mesophilic | anaerobic digestion | 0.5 to 4 | 0.5 to 2 | 0 |
| Aerobic digestion | | 0.5 to 4 | 0.5 to 2 | 0 |
| Composting | | 2 to > 4 | 2 to > 4 | 2 to > 4 |
| Air drying | | 0.5 to 4 | 0.5 to > 4 | 0.5 to > 4 |
| Lime stabilisation | | 2 to > 4 | > 4 | 0 |

The microbial cells present in sludge are highly resistant to degradation since they are composed of glucan strands cross-linked by peptide chains (Weemaes and Verstraete, 1998b). Various pre-treatment techniques have been employed for disintegrating microbial cells in sludge (Appels et al., 2008) and improving biogas production. Pathogen reduction is not the priority of most of these techniques, but since majority of them target the disintegration of bacterial cell walls, therefore they contribute indirectly towards the

improvement of sludge sanitation. Following section summarises the achievements, advantages and disadvantages of sludge pre-treatment techniques.

2.6.1 Chemical pre-treatment techniques

Strong acid or alkali in combination with high temperature was originally used for the chemical pre-treatment of sludge. Recently, however, ozonation and peroxidation have also emerged as effective methods of chemical pre-treatment (Tyagi and Lo, 2011).

2.6.1.1 Acidic and alkaline pre-treatment

Principle

This approach involves the solubilisation of sludge at ambient temperature by the addition of an acid or alkali (Appels et al., 2008) which results in the decomposition of carbohydrates, fats and protein molecules into smaller and relatively more soluble units such as polysaccharides, aliphatic acids and amino acids respectively (Chiu et al., 1997, Mukherjee and Levine, 1992). Addition of alkali raises the pH of the medium which causes the loss of turgor pressure of cells resulting in their disintegration. Moreover, it causes the solubilisation of membrane lipids through the process of saponification, leading to the release of intracellular substances. Not only does it make the sludge more easily degradable in AD but it also renders it sanitised (Neyens et al., 2003a). The most extensively used chemical for thermochemical hydrolysis is sodium hydroxide (NaOH) (Chishti et al., 1992, Karlsson and Smith, 1991, Mavinic et al., 1995, Tanaka et al., 1997); however, in some studies other chemicals have been used including sulphuric acid (H_2SO_4), hydrochloric acid (HCl) (Smith and Göransson, 1992, Woodard et al.) and calcium hydroxide $Ca(OH)_2$ (Karlsson and Smith, 1991, Mavinic et al., 1995). An overview of studies conducted on the effect of acid/ alkaline pre-treatments on the solubilisation is presented in Table 2.7.

Table 2.7 Overview of acidic/alkaline pre-treatment studies

| Reference | Chemical used/ Conditions | Prominent findings |
|------------------------|------------------------------|---|
| Wood and Wukasch, 1994 | H_2SO_4 | <ul style="list-style-type: none"> • 50-60% solubilisation |
| Knezevic et al., 1995 | NaOH | <ul style="list-style-type: none"> • No apparent improvement in VS reduction • Improvement in biogas production with the increase in dose of NaOH |
| Lin et al., 1997 | NaOH | <ul style="list-style-type: none"> • 52% increase in COD removal |

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| Reference | Chemical used/ Conditions | Prominent findings |
|-----------------------------|---|---|
| Tanaka et al., 1997 | NaOH, 130°C | <ul style="list-style-type: none"> 163% increase in biogas production 20% increase in biogas yield 50% rise in CH₄ production |
| Penaud et al., 1999 | KOH | <ul style="list-style-type: none"> KOH was more effective than NaOH |
| Heo et al., 2003 | NaOH | <ul style="list-style-type: none"> 55°C was the best temperature as compared to 25 and 35°C 38.3% COD solubilisation 88% increase in CH₄ production |
| Kim et al., 2003 | NaOH, KOH, Mg(OH) ₂ Ca(OH) ₂ | <ul style="list-style-type: none"> 30% increase in VS reduction |
| Neyens et al., 2003 a | Ca(OH) ₂ | <ul style="list-style-type: none"> Removal of all pathogens Increase in sludge dewaterability |
| Carballa M and Omil F, 2004 | CaO | <ul style="list-style-type: none"> 85% removal of faecal coliforms and faecal streptococci at pH 12 No significant improvement in AD |
| Chen et al., 2007a, 2007b | NaOH, HCl | <ul style="list-style-type: none"> Alkaline treatment (pH 9-11) performed better than the acidic treatment (pH 4-5) Total VFA concentration was improved at pH 8-11 |
| Li et al., 2009 | NaOH | <ul style="list-style-type: none"> 50% VSS solubilisation |
| Lin et al., 2009 | NaOH | <ul style="list-style-type: none"> 83% COD solubilisation 83% increase in CH₄ production |

Advantages

- Simple.
- Energy efficient.
- Effective for pathogen inactivation (Tyagi and Lo, 2011).

Disadvantages

- Extreme pH values (acidic or alkaline) cause a loss of methanogenic activity and the sludge has to be re-neutralised.
- Generation of foul odour.
- Corrosion and fouling of equipment (Appels et al., 2008).

2.6.1.2 Ozonation

Principle

Ozone is a strong oxidant capable of oxidising a wide range of organic and inorganic substances. In addition to causing cell lysis, which kills the sludge microorganisms and

oxidises intracellular substances (Saktaywin et al., 2005, Cui and Jahng, 2006), ozone reacts with the cell wall components and cell membrane of microorganisms transforming them into simple substances and causing the release of cytoplasm in the form of soluble COD (SCOD) (Yasui and Shibata, 1995, Weemaes et al., 2000, Goel et al., 2003a). Ozonation decomposes the sludge in three sequential steps; floc disintegration, membrane solubilisation and oxidation of intracellular substances (Ahn et al., 2002, Lee et al., 2005, Chu et al., 2009). Table 2.8 summarises research conducted to study the effect of ozonation on sludge decomposition and AD.

Table 2.8 Overview of ozonation studies

| Reference | Conditions | Findings |
|-------------------------|--|--|
| Song et al., 1992 | Catalytic wet oxidation | <ul style="list-style-type: none"> 93.8% removal of SCOD |
| Weemaes et al., 2000 | 0.1 g O ₃ g ⁻¹ COD | <ul style="list-style-type: none"> 112% increase in CH₄ production 64% rise in COD destruction |
| Deleris et al., 2000 | 0.05 g O ₃ g ⁻¹ Volatile Suspended Solids (VSS) | <ul style="list-style-type: none"> 70% reduction in sludge volume |
| Yeom et al., 2002 | 0.05 g O ₃ g ⁻¹ Total Suspended Solids (TSS) 0.1 g O ₃ g ⁻¹ TSS | <ul style="list-style-type: none"> 19.6% increase in COD solubilisation 25.7% increase in COD solubilisation |
| Battimelli et al., 2003 | 0.16 g O ₃ g ⁻¹ TSS | <ul style="list-style-type: none"> 58% rise in COD removal |
| Goel et al., 2003b | 0.045 g O ₃ g ⁻¹ Total Volatile Solids (TVS) | <ul style="list-style-type: none"> 81% VSS removal 1.3 times increase in biogas production |
| Goel et al., 2003 c | 0.015 g O ₃ g ⁻¹ Total Solids (TS) 0.05 g O ₃ g ⁻¹ TS | <ul style="list-style-type: none"> 19% sludge solubilisation 37% sludge solubilisation 90% VS removal |
| Sievers et al., 2004 | 0.03 kg O ₃ g ⁻¹ TSS 0.06 kg O ₃ g ⁻¹ TSS | <ul style="list-style-type: none"> 11% increase in COD solubilisation 16% increase in COD solubilisation |
| Bougrier et al., 2006 | 0.10-0.16 g O ₃ g ⁻¹ TS | <ul style="list-style-type: none"> No significant rise in biogas production |
| Chu et al., 2008 | 0.06-0.16 g O ₃ g ⁻¹ TSS 0.02 g O ₃ g ⁻¹ TSS | <ul style="list-style-type: none"> 31% COD solubilisation 80% inactivation of microorganisms |
| Carballa et al., 2009 | 20 mg O ₃ g ⁻¹ TSS | <ul style="list-style-type: none"> 63% removal of <i>Salmonella</i> and faecal <i>Streptococci</i> |
| Chu et al., 2009 | 20 mg O ₃ g ⁻¹ TSS | <ul style="list-style-type: none"> 30% increase in sludge solubilisation |
| Yan et al., 2009 | 0.22-0.27 g O ₃ g ⁻¹ TSS 0.27 g O ₃ g ⁻¹ TSS | <ul style="list-style-type: none"> 60% TSS disintegration 40% sludge solubilisation 26% increase in sludge mineralisation |

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| Reference | Conditions | Findings |
|--------------------|--|---|
| Zhang et al., 2009 | 50 mg O ₃ g ⁻¹ Dissolved Solids (DS) | <ul style="list-style-type: none"> • 46.7% sludge disintegration |
| Erden et al., 2010 | 0.10g O ₃ kg ⁻¹ TS | <ul style="list-style-type: none"> • 30% VS reduction as compared to 22.8% in case of raw sludge |
| Kim et al., 2010 | 0.32 g O ₃ g ⁻¹ SS | <ul style="list-style-type: none"> • 3670 mg l⁻¹ rise in SCOD • 23% TSS solubilisation • 19% VSS solubilisation |

Advantages

- Ozonation is a practically tested and trusted technology now being successfully installed at full-scale.
- Improves sludge settle ability and dewaterability.
- Reduces foaming.
- Effective for solids degradation.
- Improves CH₄ production.

Disadvantages

- Expensive in terms of energy consumption.
- 20% rise in electricity consumption of a WWTP for every 30% reduction in sludge yield (Boehler et al., 2006).
- Ozonation results in the release of soluble bio-refractory compounds in sludge which can pose serious health threats to the animal and plant life of receiving water bodies (Liu, 2003).

2.6.1.3 Peroxidation

Principle

Peroxidation utilises the oxidation of Hydrogen peroxide (H₂O₂) by Iron (Fe²⁺) salts and disintegrates extracellular polymeric substances to rupture the cell walls and release the intracellular substances. It transforms refractory COD into soluble and readily available BOD and therefore increases biogas yield (Tyagi and Lo, 2011). Table 2.9 shows research conducted to study the influence of peroxidation on sludge pre-treatment and AD.

Table 2.9 Overview of peroxidation research

| Reference | Conditions | Findings |
|-----------------------|---|--|
| Neyens et al., 2003 b | 25 g H ₂ O ₂ kg ⁻¹ Dry Solids (DS) (1.67 g Fe ²⁺ ions kg ⁻¹ DS) pH 3, ambient temperature and pressure | <ul style="list-style-type: none"> • Considerable reduction of DS and organic DS • Improved dewaterability • 60% reduction of sludge volume |

| Reference | Conditions | Findings |
|------------------------------|---|---|
| Valo et al., 2004 | 150 milli molar (m mol) l^{-1} H_2O_2 5 m mol l^{-1} $FeSO_4$, 90°C, 60 min | <ul style="list-style-type: none"> • 20% rise in cake DS content • 16% increase in biogas production |
| Rivero et al., 2006 | 2 g H_2O_2 g^{-1} VSS, 90°C, 24 Hrs 2 g H_2O_2 g^{-1} VSS, 90°C, 60 Hrs | <ul style="list-style-type: none"> • 17.9% increase in COD removal • 22.4% increase in COD removal |
| Dewil et al., 2007 | 390 g H_2O_2 l^{-1} sludge, pH 3 | <ul style="list-style-type: none"> • Considerable rise of COD/BOD ratio • 75% increase in biogas production • No significant rise of methane percentage of biogas (65-70%) |
| Eskicioglu et al., 2008 b | Coupling of H_2O_2 oxidation with microwave 1g H_2O_2 g^{-1} TS, 60-120°C | <ul style="list-style-type: none"> • 11-34 % loss of TS and total COD |
| Pham et al., 2010 | 0.01 ml H_2O_2 g^{-1} SS, ambient temperature for 1 hour | <ul style="list-style-type: none"> • Decrease in viscosity and shear thinning behaviour of sludge • Enhancement of sludge dewaterability |

Advantages

- Effective sludge decomposition.
- High biogas yield.

Disadvantages

- Extremely corrosive due to very low pH.
- High operational and maintenance cost.

2.6.2 Thermal pre-treatment techniques

Thermal pre-treatment was demonstrated as an effective method of sludge disintegration as early as 1970. It involves the exposure of sludge to a temperature range of 60-180°C (Neyens and Baeyens, 2003b). The input of heat energy results in the disintegration of the gelatinous matrix of sludge (Smith and Göransson, 1992) as well as the breakdown of chemical bonds in the cell wall and cell membrane, resulting in the solubilisation of cell components (Appels et al., 2008). Thermal pre-treatment has been reported to increase methane production to a considerable extent (Appels et al., 2008). Generally, thermal pre-treatment has a positive impact on AD; however; the extent of solubilisation mainly depends on the type of sludge (Weemaes and Verstraete, 1998b). It can also be helpful in net energy generation from AD due to both enhanced biodegradability and the reduced requirement of digester heating (Haug et al., 1983). Conventional heating and microwave irradiation are the two basic forms of thermal pre-treatment which are described in detail in the following sections.

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2.6.2.1 Conventional heating

Carbohydrates and lipids of sludge are comparatively easily degradable as compared to the proteins which are protected by the cell wall. Destruction of the cell walls of microorganisms by exposure to a temperature range of 60-180°C can make the intracellular substances readily available for biodegradation by the methanogens (Neyens and Baeyens, 2003a). Table 2.10 summarises studies conducted to date on the influence of thermal pre-treatment on sludge biodegradability and performance of AD.

Table 2.10 Overview of thermal pre-treatment research

| Reference | Treatment Conditions | Findings |
|----------------------------|-------------------------|---|
| Brooks, 1970 | 170°C | <ul style="list-style-type: none">• 40-60% solubilisation of WAS• 20-35% solubilisation of mixture of WAS and primary sludge |
| Haug, 1977 | 175°C, 30 min | <ul style="list-style-type: none">• 60-70% increase in CH₄ production |
| Haug et al 1978 | | |
| Haug et al., 1983 | | |
| Hiraoka et al., 1985 | 60-100°C | <ul style="list-style-type: none">• Maximum increase in biogas yield from the sludge pre-treated at 60°C• 5-10% VS reduction at 100°C |
| Pinnekamp, 1989 | 220°C | <ul style="list-style-type: none">• 55% DS reduction of WAS• 34% DS reduction for sewage sludge |
| Li and Noike 1992 | 90°C | <ul style="list-style-type: none">• 25-45% increase in sludge solubilisation |
| | 170°C, 60 min | <ul style="list-style-type: none">• 30% increase in VSS degradation• 100% increase in CH₄ production• 5 days reduction in HRT of anaerobic digester |
| Tanaka et al., 1997 | 180°C, 60 min | <ul style="list-style-type: none">• 30% increase in VSS solubilisation• 90% increase in CH₄ yield |
| Zheng et al., 1998 | 220°C, 30 seconds | <ul style="list-style-type: none">• 55% reduction in VS• 80% increase in biogas production |
| Kepp et al., 2000 | CAMBI 165°C, 30 minutes | <ul style="list-style-type: none">• Significant increase in biogas production |
| Kepp U and Solheim, 2001 | | |
| Elliott and Mahmood, 2007 | | |
| Panter K and Kleiven, 2005 | | <ul style="list-style-type: none">• 27% increase in net electricity production• 60% VS destruction• Improvement of sludge dewaterability |
| Kim et al., 2003 | 121°C, 30 minutes | <ul style="list-style-type: none">• 30% rise in VS reduction• 34.3% increase in CH₄ production• 67.8% enhancement in SCOD removal |
| Dohanyos et al., 2004 | 170°C, 60 seconds | <ul style="list-style-type: none">• 49% rise in biogas yield |
| Valo et al., 2004 | 170°C, 15 minutes | <ul style="list-style-type: none">• 80% sludge solubilisation• 71% TCOD degradation• 59% rise in TS reduction• 92% increase in biogas production |
| Graja et al., 2005 | 175°C, 40 minutes | <ul style="list-style-type: none">• 65% TSS reduction |
| Bougrier et al., 2006 | 170°C, 30 minutes | <ul style="list-style-type: none">• 76% increase in CH₄ production at 24 Hrs Solid Retention Time (SRT)• 51% increase in CH₄ production at 20 days SRT |

| Reference | Treatment Conditions | Findings |
|---------------------------|-----------------------|---|
| Ferrer et al., 2006 | 70°C, 9-72 Hrs | <ul style="list-style-type: none"> Positive effect on biogas production in thermophilic AD High temperature (110-134°C) had no positive effect |
| Climent et al., 2007 | 70-134°C, 9 min-9 Hrs | <ul style="list-style-type: none"> 50% rise in biogas production from thermophilic AD of sludge pre-treated at 70°C for 9 Hrs Temperature higher than 70°C was ineffective |
| Perez-Elvira et al., 2008 | 170°C, 30 minutes | <ul style="list-style-type: none"> 50% increase in CH₄ production |
| Carballa et al., 2009 | 130°C, 1 Hour | <ul style="list-style-type: none"> Complete eradication of <i>Salmonella</i> and <i>Shigella</i> Spp Number of total coliforms, <i>E. coli</i> and faecal <i>Streptococci</i> was 4, 15 and 24 MPN g⁻¹ TS respectively and <i>Clostridium perfringens</i> was 1700 CFU g⁻¹ TS |
| Nges and Liu, 2009 | 50°C, 48 Hrs | <ul style="list-style-type: none"> 11% increase in CH₄ production |
| Wang et al., 2009 | 170°C, 30 minutes | <ul style="list-style-type: none"> 45-55% VS removal 62% reduction of total COD 92% removal of SCOD during AD at 10 days SRT |
| Ge et al., 2010 | 50-70°C for 2 days | <ul style="list-style-type: none"> 54% VS reduction 25% increase in CH₄ production |
| Wett et al., 2010 | 170-180°C, 60 minutes | <ul style="list-style-type: none"> 75% increase in biogas production 43% rise in COD solubilisation |

Advantages

- Relatively simple.
- Improvement of sludge dewaterability.
- Enhanced sludge sanitation (Tyagi and Lo, 2011).

Disadvantages

- Fouling of heat exchangers.
- Odour generation.
- High energy requirement.
- High operational and maintenance cost.

2.6.2.2 Microwave (MW) irradiation

Microwave radiation is a form of electromagnetic radiation in a frequency range of 300 Megahertz (MHz) to 300 Gigahertz (GHz) corresponding to a wavelength in the range of 1 meter to 1 millimetre (Banik et al., 2003).

Principle

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Microwaves generate rapid direct heat both internally and at the surface of the material being heated via the alignment of dipoles. Since sludge is a mixture of water, organic substances, minerals and microbial cells (Qiao et al., 2008), microwave (MW) irradiation performs its action in two ways: athermal and thermal effects. As the name indicates, the athermal effect is not associated with a rise in temperature (Hong et al., 2004). It involves the lining up of polarised parts of macromolecules alongside the poles of the electromagnetic field causing the breakage of hydrogen bonds. The thermal effect corresponds to the generation of heat as a consequence of absorption of MW radiation by polarised molecules (Yu et al., 2010). The combined action of athermal and thermal effects results in the disintegration of sludge flocs. This causes the release of extracellular and possibly intracellular substances (in the case of application of high intensity MW irradiation), such as proteins, polysaccharides and small amounts of nucleic acids (DNA and RNA) (Eskicioglu et al., 2006). Studies conducted to determine the effect of MW irradiation on sludge pre-treatment and its influence on AD are summarised in Table 2.11.

Table 2.11 Overview of MW-irradiation pre-treatment studies

| Reference | Treatment conditions | Findings |
|---------------------------|-------------------------|--|
| Hong et al., 2004 | 57-68°C | <ul style="list-style-type: none">• Loss of viability of all bacteria above 68°C |
| Park et al., 2004 | 91.2°C, 15 minutes | <ul style="list-style-type: none">• 22% COD solubilisation• 23.2% VS reduction• 64% TCOD removal• 79% CH₄ production• 7 days reduction in HRT |
| Eskicioglu et al., 2006 | 96°C | <ul style="list-style-type: none">• 143% increase in SCOD• 17% increase in biogas production |
| Pino-Jelcic et al., 2006 | 1000 W, 2540 MHz, 65°C | <ul style="list-style-type: none">• 4.2, 2.9 and 1.5 log reduction of faecal coliforms in sludge pre-treated with MW, conventional heating and control respectively• 2.0±0.3, 1.9±0.2, 1.1±0.3 log reduction of <i>Salmonella</i> Spp |
| Eskicioglu et al., 2007 a | 96°C | <ul style="list-style-type: none">• 15% increase in COD solubilisation• 16% rise in biogas yield |
| Eskicioglu et al., 2007 b | 96°C | <ul style="list-style-type: none">• 28% increase in biogas production• 39% increase in dewaterability of digestate |
| Eskicioglu et al., 2008 a | 175°C | <ul style="list-style-type: none">• 31% increase in biogas yield• 75% enhancement in sludge dewaterability |
| Eskicioglu et al., 2009 | 120°C 150°C 175°C | <ul style="list-style-type: none">• 15 and 11% increase in COD solubilisation and VS/TS ratio respectively• 19 and 13% rise in COD solubilisation and VS/TS ratio respectively• 26 and 24% enhancement in COD |

| Reference | Treatment conditions | Findings |
|-----------------------|--|--|
| Toreci et al., 2009 | 175°C, 3.75°C min ⁻¹ MW intensity | <p>solubilisation and VS/TS ratio respectively</p> <ul style="list-style-type: none"> Improvement of COD solubilisation (TS 6%) from 10-46% (TS 11.85%) from 7-57% |
| Zhou et al, 2010 | 700 W till boiling point | <ul style="list-style-type: none"> 1.8-4.0 fold increase in SCOD 1.1-1.8 and 3.2-14.1 fold rise in soluble protein and carbohydrate concentration respectively. |
| Beszedes et al., 2011 | 5 W g ⁻¹ for 1800 seconds | <ul style="list-style-type: none"> 57% increase in COD solubilisation 60% increase in the methane content of biogas |

Advantages

- Energy efficient alternative to conventional heating by providing non-contact, quick and highly specific heating.
- No hazardous substances are generated.
- Effective sludge sanitation.
- Relatively environment friendly.

Disadvantages

- Unavailability of sufficient research data.
- Not yet tested at full-scale (Tyagi and Lo, 2011).

2.6.3 Mechanical pre-treatment techniques

These methods focus on sludge disintegration via the application of external force in the form of stress or pressure. Microbial cells are disrupted as a result of shear forces (Odegaard, 2004). Various types of mechanical pre-treatment techniques are described below:

2.6.3.1 High Pressure Homogenisation (HPH)

This technique employs a multistage high pressure pump to pressurise the sludge to several hundred bar. Pressurised sludge is then slowly pumped into a homogeniser. The velocity of sludge rapidly increases to 480 km per hour and the pressure is reduced as it approaches the impact ring. The result is intense energy release as a consequence of extreme turbulence and localised pressure difference, leading to the generation of cavitation bubbles. This rises the sludge temperature to several hundred degrees Celsius resulting in the disruption of microbial cells and release of intracellular material (Tyagi and Lo, 2011). An overview of research conducted using high pressure homogenisation is given in Table 2.12.

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Table 2.12 Overview of research using HPH

| Reference | Conditions | Findings |
|-------------------------|---|--|
| Harrison, 1991 b | 30-50 Mega Joule m ⁻³ | <ul style="list-style-type: none">• 85% cell disintegration |
| Engelhart et al., 1999 | 300 Bar (750 kJ kg ⁻¹ TS) | <ul style="list-style-type: none">• 60% rise in CH₄ production |
| Camacho et al., 2002 | 500-700 bar | <ul style="list-style-type: none">• 60% COD release from sludge• 20% reduction in sludge volume |
| Koppolow et al., 2004 | 600 bar, 60 kJ l ⁻¹ | <ul style="list-style-type: none">• 25% increase in COD solubilisation• 18% rise in biogas production• 7% rise in VS destruction |
| Stephenson et al., 2005 | Combined high pressure homogenisation and alkaline pre-treatment (Microsludge TM) | <ul style="list-style-type: none">• 80% sludge solubilisation |
| Froud et al., 2005 | 1200 PSI (Pounds per Square Inch), pH 10 for 1 hour | <ul style="list-style-type: none">• 78% increase in VS reduction• Shortening of HRT by 5 days |
| Onyeche, 2006 | | <ul style="list-style-type: none">• 30 % increase in biogas production• 23% reduction in excess sludge production |

Advantages

- Easy implementation.
- No foul odour generation.
- Improvement in sludge dewaterability.

Disadvantages

- Inefficient for pathogen removal.
- High energy consumption.
- Clogging issues due to coarse and fibrous substances.
- High tension and erosion in the pump and homogenising valve.

2.6.3.2 Ultra sonication

Ultrasound is one of the most powerful tools for disintegration of bacterial cells (Harrison, 1991a). Cavitation is the basic mode of action of ultrasonic treatment, which generates periodic compressions and rarefactions in very short time intervals (milliseconds). High temperature (several thousand °C) and pressure (up to 500 Bar) gradients are generated in the liquid phase of sludge as a consequence of this cavitation (Dewil et al., 2006), resulting in the disintegration of cell membranes and release of intracellular material. (Gogate, 2002). The most effective frequency range is 20-200 kHz (Hua and Thompson, 2000). Treatment conditions and the findings of research conducted employing ultrasound pre-treatment have been summarised in Table 2.13.

Table 2.13 Overview of ultrasound pre-treatment studies

| Reference | Conditions | Findings |
|--------------------------------|---|--|
| Shimizu et al., 1993 | 200 Watt (W), 1 Hour | <ul style="list-style-type: none"> • 60 % sludge solubilisation |
| Tiehm et al., 1997 | 31 kHz, 64 seconds | <ul style="list-style-type: none"> • 1.5 % increase in VS reduction |
| Wang et al., 1999 | 200 W, 20 minutes | <ul style="list-style-type: none"> • 75% increase in CH_4 yield |
| Jean et al., 2000 | 0.33 W ml^{-1} , 40 minutes | <ul style="list-style-type: none"> • 93% reduction of total coliforms in 10 minutes and only 1% survival ratio after 40 minutes • 82% reduction of heterotrophic bacteria |
| Chu et al., 2001 | 0.33 W ml^{-1} , 20-60 minutes | <ul style="list-style-type: none"> • Effective reduction of heterotrophic bacteria and total coliforms |
| Kim et al., 2003 | 42 kHz, 120 minutes | <ul style="list-style-type: none"> • 18.4 % increase in VS reduction • Increase in CH_4 production ($756 \text{ l}^{-1} \text{ m}^3$ WAS) |
| Andreottola and Foladori, 2006 | 30000 kJ kg^{-1} TS | <ul style="list-style-type: none"> • 24% of TCOD was converted in to colloidal COD while 20% was S COD |
| Mao et al., 2006 | 0.52 W ml^{-1} | <ul style="list-style-type: none"> • 20-39 % improvement in TS removal • 1.6-3.1 % increase in biogas production • 2-17% increase in CH_4 content of biogas |
| Khanal et al., 2007 | | <ul style="list-style-type: none"> • 11-39% increase in SCOD removal • 54% improvement in VS destruction |
| Moonkhum, 2007 | 1.9 W ml^{-1} , 20 kHz, 150 seconds | <ul style="list-style-type: none"> • 28% removal of Dissolved Organic Carbon (DOC) • 50% increase in VS removal |
| Damodar et al., 2009 | 397 kJ kg^{-1} sewage sludge, 30 minutes | <ul style="list-style-type: none"> • 44.4% increase in COD solubilisation |
| Feng et al., 2009 | 26000 kJ kg^{-1} TS | <ul style="list-style-type: none"> • 26% increase in COD solubilisation |
| Laurent et al., 2009 | $163300 \text{ kJ kg}^{-1}$ TS | <ul style="list-style-type: none"> • 22.4% increase in COD solubilisation • 39.9% increase in TSS solubilisation • 48.8% rise in VSS solubilisation |
| Salsabil et al., 2010 | $108000 \text{ kJ kg}^{-1}$ TS | <ul style="list-style-type: none"> • 22.3% removal of TSS • 29.7% removal of TSS • $43 \text{ ml g}^{-1} \text{ VSS d}^{-1}$ biogas yield |
| Wang et al., 2010 | 4.8 W cm^{-2} , 15 minutes (Temperature rise 70°C) | <ul style="list-style-type: none"> • Increase in concentration of S COD from $50 - 2500 \text{ mg l}^{-1}$ • 24.6 % increase in VS destruction |
| Braguglia et al., 2010 | 2500 kJ kg^{-1} TS | <ul style="list-style-type: none"> • 19% increase in VS degradation • 26% rise in cumulative biogas production |
| Erden et al 2010 | 9690 kJ kg^{-1} TSS | <ul style="list-style-type: none"> • 36% VS destruction |
| Foladori et al., 2010 | 53000 kJ kg^{-1} TSS | <ul style="list-style-type: none"> • 45% increase in SCOD concentration |
| Kim et al., 2010 | 45000 kJ kg^{-1} TS | <ul style="list-style-type: none"> • 50% rise in SCOD and TCOD solubilisation |

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| Reference | Conditions | Findings |
|-----------------------|-------------------------------|--|
| Salsabil et al., 2010 | 200000 kJ kg ⁻¹ TS | <ul style="list-style-type: none"> • 30% TSS removal in aerobic digestion • 20 % TSS removal in anaerobic digestion |
| Wang et al., 2010 | 200 W, 40 minutes | <ul style="list-style-type: none"> • 46% improvement in AD • 75% increase in CH₄ yield |
| Xu et al., 2010 | 0.12-1.5 W ml ⁻¹ | <ul style="list-style-type: none"> • 10.78 to 15.11 % increase in SCOD following 30 minute treatment • 15.96 to 20.76 % increase in SCOD after 60 minute treatment |

Advantages

- Operational reliability due to extensive research.
- No generation of bad odour.
- No clogging issues
- Improved dewaterability and VS destruction.
- Improvement in biogas yield.

Disadvantages

- High capital and operating cost
- High energy consumption
- Limited data available for long term performance of full scale ultrasound pre-treatment systems.

2.6.3.3 Gamma irradiation

Gamma irradiation has strong ionising properties. It can be generated either through the ionisation of particles or via ionising electromagnetic radiation obtained from radionuclides. Ionising radiation affects the structure and functioning of bacterial cells resulting in cell disruption (Show et al., 2010). Gamma irradiation has mainly been studied for its sanitation properties (Etzel et al., 1969, Yeager and O'brien, 1983, Mustapha and Forster, 1985, Müller, 2001); however, it is also capable of releasing soluble carbohydrates from bacterial cells. Table 2.14 gives a summary of research conducted on pre-treatment of sludge by gamma irradiation.

Table 2.14 Overview of gamma irradiation pre-treatment studies

| Reference | Conditions | Findings |
|--------------------|------------------------|---|
| Etzel et al., 1969 | 200000 rad, 60 minutes | <ul style="list-style-type: none"> • 2-3 fold increase in sludge settle ability • 2 fold rise in COD • 9.999% removal of coliforms |

| Reference | Conditions | Findings |
|----------------------|----------------------|---|
| Lafitte-Trouque 2002 | Forster, 500 K rad | <ul style="list-style-type: none"> 555% increase in SCOD concentration MAD of pre-treated sludge produced digestate with < 100 faecal coliforms g⁻¹ TS Thermophilic AD of pre-treated sludge produced digestate having < 1 faecal coliforms g⁻¹ TS |
| Yuan et al., 2008 | 0-30 kilo Gray (kGy) | <ul style="list-style-type: none"> 178 ml increase in biogas production from reactors fed with sludge pre-treated with gamma irradiation at 1124 kGy |

Advantages

- High level of sludge sanitation.

Disadvantages

- Lack of sufficient research.
- High energy consumption.
- No significant improvement in biogas yield.

2.6.3.4 Stirred ball mills

This technique can be employed to rupture the cell wall and cell membrane of sludge microorganisms. Two frequently used methods employing the stirred ball mill technique are the Kaddy mill and wet mill. A Kaddy mill generates shear by the counter-rotation of two plates (Springer et al., 1996). A Wet mill consists of a cylindrical grinding chamber filled with beads which are forced into rotational movement through an agitator. Shear and pressure generated through the movement of beads mechanically crushes the sludge (Lajapathi Rai et al., 2008). Table 2.15 provides a summary of studies conducted using this technique.

Table 2.15 Overview of stirred ball mills pre-treatment studies

| Reference | Conditions | Findings |
|-----------------------------|--|--|
| Harrison, 1991 b | High speed shaker ball mill | <ul style="list-style-type: none"> Enhanced cell disintegration due to energy dissipation upon heating the sludge |
| Baier and Schmidheiny, 1997 | Wet mill (Bead diameter 0.2-0.25 mm, Energy input 1-1.25 kW m ⁻³ TS day ⁻¹) | <ul style="list-style-type: none"> 47% SCOD release after 9 minutes milling 1.2-1.5 factor improvement in COD degradation and biogas production in MAD |
| Kopp et al., 1997 | Ball diameter 0.35 mm, ball velocity 6 ms ⁻¹ , energy input 2000 kJ kg ⁻¹ TS | <ul style="list-style-type: none"> 42-47% increase in VS removal |
| Muller et al., 1998 | Stirred ball mill | <ul style="list-style-type: none"> Best results obtained at high agitation speed, longer grinding time using small sized grinding |

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| Reference | Conditions | Findings |
|-------------------------|------------|-----------------------------|
| Springer A, and J, 1999 | Kaddy mill | beads • 25% rise in SCOD |

Advantages

- Operational stability due to high level of research and development.
- No foul odour generation.
- Effective sludge disintegration (Pérez-Elvira et al., 2006).

Disadvantages

- Energy loss due to friction.
- Erosion of grinding chamber.
- Clogging issues.
- Energy intensive (90% sludge disintegration requires 60 MJ energy kg⁻¹ DS) (Tyagi and Lo, 2011).

2.6.3.5 Jetting and colliding

This method involves the pressurisation of sludge to 5.50 bar followed by rapid depressurisation and smashing and jetting against a smash flat. Treatment conditions and findings of studies conducted employing this technique are given in Table 2.16.

Table 2.16 Overview of jetting and colliding pre-treatment studies

| Reference | Conditions | Findings |
|-------------------|------------|---|
| Choi et al., 1997 | 30 bar | • 50% VS removal • 610 l biogas generated per kg VS • 6 times increase in SCOD • 9 times increase in TOC |
| Nah et al., 2000 | 30 bar | • 30% increase in VS removal • 5-7 times increase in SCOD • Biogas production 790-850 l kg ⁻¹ VS |

Advantages

- Significant improvement in VS destruction and biogas production.

Disadvantages

- High installation cost
- Scarcity of research

2.6.3.6 Electric pulse power technique

This technique involves the passage of high voltage electric current (about 10 kV) in pulse durations of 10 microseconds through the two electrodes submerged in sludge. The shockwaves generated as a result of this pulsed electric current causes the disruption of sludge flocs and of microbial cells (Weise and Jung, 2001, Müller, 2001). Table 2.17 summarises the research conducted using this technique for sludge solubilisation.

Table 2.17 Overview of studies involving electric pulse power pre-treatment

| Reference | Findings |
|----------------------|---|
| Kopplow et al., 2004 | <ul style="list-style-type: none"> • 20% sludge solubilisation • 9% improvement in AD of sludge • 20% increase in biogas production |
| Choi et al., 2006 | <ul style="list-style-type: none"> • 4.5 times increase in SCOD/TCOD ratio • 4.4 times increase in SCOD • 6.5 times increase in extracellular polymeric substances |

Advantages

- Significant improvement in biogas production.
- Increase in SCOD.

Disadvantages

- High energy input.
- Erosion of electrodes.
- Lack of research.

2.6.3.7 Lysate thickening centrifugal technique

This technique employs an impact gear or ring incorporated in to the centrifugal thickener for the dissipation of kinetic energy: the force generated in the centrifuge is purposely employed for cell lysis following sludge thickening. Table 2.18 summarises studies conducted on this technique.

Table 2.18 Overview of lysate thickening centrifugal pre-treatment

| Reference | Findings |
|---------------------------|---|
| Dohanyos et al., 1997 | <ul style="list-style-type: none"> • 31.8 % increase in CH_4 yield • Increase in SCOD from 1 to 5 % of TCOD • 10-15% sludge disintegration • Ability to handle high sludge volume |
| Zabranska et al., 2006 | <ul style="list-style-type: none"> • 15-26% increase in biogas yield • Sludge age, type, organic matter content and HRT of anaerobic digester governed the extent of increase in biogas yield |
| Elliott and Mahmood, 2007 | <ul style="list-style-type: none"> • 15-26% increase in specific biogas production |

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| Reference | Findings |
|-----------|---|
| | <ul style="list-style-type: none">• 50% decrease in organic matter during MAD of pre-treated sludge |

Advantages

- Relatively simple installation and setup.
- No foul odour generation.

Disadvantages

- Low sludge disintegration in contrast to high energy consumption.
- Wear and tear of equipment plates.
- Lack of sufficient research.

2.6.3.8 High speed rotary disc

This process employs a rotary disc device which draws the sludge through two discs with the help of centrifugal force generated from a bottom fitted disc. The sludge is crushed due to the high rotational speed of the disc. A 30% increase in cumulative biogas production, 20% increase in CH_4 yield, three times more VSS removal, about 10 days reduction in SRT of anaerobic digesters and a reduction of excess sludge volume was observed after high speed rotary disc treatment at 500 RPM with a disc gap of 10 mm for 45 minutes (Imai et al., 2010).

2.6.3.9 Rapid non-equilibrium decompression

This technique utilises CO_2 to pressurise the sludge. Upon decompression, CO_2 dissolved in sludge increases in volume (about 1800 %) and results in high shear forces leading to the rupture of microbial cell walls (Sheppard. and Rigden., 2005). This technology is marketed by Eco-Solids International with the trade name of 'Cell Ruptor' (initially 'Bug Buster') who claim 80% VS destruction and significant reduction in the number (<10 MPN) of faecal coliform bacteria. A commercial-scale prototype of the 'Bug Buster' was installed at Yorkshire Water's Old Whittington WwTW in Chesterfield, Derbyshire UK. 30-40 % improvement in biogas production has been reported (Spooner et al., 2007).

2.6.3.10 Electron beam pre-treatment

This technique was initially employed for the disinfection of sludge. Electron beam irradiation causes the generation of extremely reactive chemical species such as ions, free radicals and excited atoms and molecules. These species have the capability of decomposing organic substances (Kuruez, 1991). Shin and Kang (2003) observed 30-52%

COD solubilisation, leakage of proteins, carbohydrates and SCOD from cells and reduction in SRT of an anaerobic reactor fed with sludge pre-treated by electron beam irradiation at the intensity of 0.5-10 kGy for 24 Hrs.

2.6.4 Hybrid pre-treatment techniques

These techniques involve the coupling of a chemical method with a physical method e.g. thermal or mechanical as an alternative to a single method. Sludge disintegration is enhanced as a result of the synergistic effect of two methods. Frequently used hybrid pre-treatment techniques are described below.

2.6.4.1 Thermo-chemical pre-treatment techniques

a. Conventional heating- alkali/acid treatment

Thermal pre-treatment together with the addition of strong chemicals (alkali/acid) can be applied for sludge disintegration at ambient pressure (Alsop and Conway, 1982, Hiraoka et al., 1985). Addition of acid or alkali raises the temperature of sludge, therefore reducing the amount of fuel required for subsequent heating. Table 2.19 presents the findings of studies on thermo-chemical pre-treatment for sludge disintegration.

Table 2.19 Overview of conventional heating - alkali/acid treatment

| Reference | Conditions | Findings |
|--|---|---|
| Tanaka et al., 1997 | 0.3 g NaOH g ⁻¹ VSS, 5 minutes, 130°C | <ul style="list-style-type: none"> • 50% improvement of sludge solubilisation • 50% increase in CH₄ production |
| Cassidy, 1998 Recktenwald and Karlson, 2003 Odegaard, 2004 | Krepro; pH 1-2 using H ₂ SO ₄ at 140°C, 3.5b, 30-40 minutes | <ul style="list-style-type: none"> • 40% organic matter solubilisation |
| Penaud et al., 1999 | 26.1 g l ⁻¹ NaOH, 140°C, 30 minutes | <ul style="list-style-type: none"> • 71% COD solubilisation |
| Kim et al., 2003 | 7-21 g l ⁻¹ NaOH, pH 12, 121°C, 30 minutes | <ul style="list-style-type: none"> • 34.3% increase in CH₄ production • 67.8% COD removal • 30% VS removal |
| Neyens et al., 2003 a | 100°C, pH 10, Ca(OH) ₂ , 60 minutes | <ul style="list-style-type: none"> • 100% pathogen removal |
| Valo et al., 2004 | 170°C, pH 12 | <ul style="list-style-type: none"> • 71% COD removal • 59% TS reduction • 54% rise in biogas yield |
| Vlyssides and Karlis, 2004 | pH 11, 90°C | <ul style="list-style-type: none"> • 46% VSS reduction • 0.28 l CH₄ produced kg⁻¹ VSS |
| Park et al., 2005 | 12°C, 30 minutes, 7 g l ⁻¹ , NaOH | <ul style="list-style-type: none"> • 88.9% TCOD removal • 77.5% VS reduction • 79.5% increase in CH₄ production |
| Andreottola and Foladori, 2006 | pH 12, 60-80°C, 30 minutes | <ul style="list-style-type: none"> • 30% release of SCOD |
| Liu et al., 2008 | | <ul style="list-style-type: none"> • 61.6% VS solubilisation |

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Advantages

- Low chemical dose can enhance sludge volume reduction.
- High phosphate concentration in digestate make it beneficial for land application.
- Solid residue can be used as a biofuel.

Disadvantages

- High operational and maintenance cost due to the consumption of reagents and energy required for heating.
- Generation of foul odour.

b. Microwave-alkali treatment

Several researchers have investigated the use of MW heating in combination with alkali treatment, as summarised in Table 2.20.

Table 2.20 Overview of MW-alkali pre-treatment

| Reference | Conditions | Findings |
|-----------------------|---|--|
| Qiao et al., 2008 | 0.005 g NaOH g ⁻¹ Dry solids, 170°C for 1 minute, 5 minute reaction time | <ul style="list-style-type: none">• 50% VSS solubilisation |
| Dogan and Sanin, 2009 | 160°C, pH12 using NaOH | <ul style="list-style-type: none">• 34% COD solubilisation• 16.3% rise in biogas production• 24.9% increase in TS removal• 35.4% increase in VS degradation• 30.3% improvement in TCOD removal |
| Park et al., 2010 | 70°C, 5 minutes | <ul style="list-style-type: none">• 63% VSS reduction• Complete eradication of faecal coliforms and <i>E. coli</i> |
| Chang et al., 2011 | pH 12, 1.5 g l ⁻¹ NaOH, 60W, 2 minutes | <ul style="list-style-type: none">• 45.5% increase in COD solubilisation• 93% reduction of SCOD• 63% VSS reduction |

c. Microwave enhanced advanced hydrogen peroxide oxidation / Advanced Oxidation Process (MW/H₂O₂AOP)

As the name indicates, this process involves the use of MW irradiation coupled with H₂O₂ oxidation. Given below is a summary of studies conducted using this technique (Table 2.21).

Table 2.21 Overview of AOP studies

| Reference | Conditions | Findings |
|---------------------------|---|--|
| Eskicioglu et al., 2008 a | 1 g H ₂ O ₂ g ⁻¹ TS, >80°C | <ul style="list-style-type: none">• 11-34% solubilisation of TS, TCOD and biopolymers such as proteins, sugars and humic acids |
| Lo et al., 2008 | 5 minute exposure to MW | <ul style="list-style-type: none">• Disintegration of majority of |

| Reference | Conditions | Findings |
|-----------------|---|---|
| Yu et al., 2010 | irradiation at 80°C | suspended solids |
| | 70°C, 0.04% H ₂ O ₂ | <ul style="list-style-type: none"> • 759.2 mg l⁻¹ increase in SCOD • Concentration of faecal coliforms was dropped below detection limit (1000 CFU l⁻¹) • Complete elimination of faecal coliforms |
| | 70°C, 0.08% H ₂ O ₂ | |

d. Wet oxidation

This involves the chemical oxidation of sludge by addition of O₂ at high temperature (150-330°C) and pressure (6-20 MPa). Studies conducted employing this technique are summarised in Table 2.22.

Table 2.22 Overview of wet oxidation studies

| Reference | Conditions | Findings |
|--------------------------------|--|--|
| Weemaes and Verstraete, 1998 a | ZIMPRO process (Netherlands 1960s), 260 °C, 10 MPa | <ul style="list-style-type: none"> • Complete oxidation of organic compounds to CO₂ and CH₄ |
| Stendahl and Jafverstrom, 2004 | Aqua Reci process | <ul style="list-style-type: none"> • 99.9% decomposition of organic substances |

2.6.4.2 Mechanical Chemical methods

a. Alkali - ultrasonic treatment

Table 2.23 Overview of alkali ultrasound pre-treatment

| Reference | Conditions | Findings |
|-------------------------|---|---|
| Chiu et al., 1997 | NaOH and ultra-sonication (20 kHz) | <ul style="list-style-type: none"> • High rate of sludge hydrolysis (211.9 mg l⁻¹ minute⁻¹) |
| Stephenson et al., 2005 | | <ul style="list-style-type: none"> • NaOH was found to weaken the cell walls making them easy target of lysis by sonication |
| Liu et al., 2008 | 28 kHz, 60 minutes, pH 12 (NaOH) | <ul style="list-style-type: none"> • 60% solubilisation of VS |
| Jin et al., 2009 | NaOH (100 g ⁻¹ kg DS), 30 minutes, 7500 kJ ⁻¹ kg DS | <ul style="list-style-type: none"> • 50.7% degradation of organic matter for combined alkali/ultra sound treatment • 42.5% degradation in case of only ultrasound • 43.5% degradation using NaOH |

b. Ultra sonication - Ozonation

Transfer of ozone in solution is enhanced due to the cavitation created by ultrasonic waves. Thermolytical ozone decomposition takes place in the cavitation bubbles created by ultrasonication resulting in the release of oxygen and enhanced activity of free radicals (Jyoti and Pandit, 2004). Combined ultrasound (0.26 W m⁻¹) and ozone treatment (0.6 g O₃ hour⁻¹) for 60 minutes was found to increase SCOD from 83 (control) to 3040 mg l⁻¹

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as compared to 2483 mg l⁻¹ in case of ozone treatment followed by ultrasound (Xu et al., 2010).

c. Alkali - High Pressure Homogeniser

Stephenson et al. (2005) found that HPH and alkaline treatment (pH 10, 8.27 MPa for 1 hour) resulted in 80% solubilisation of suspended organic matter, 60% improvement in VS reduction and 5 days reduction in the HRT for an anaerobic digester. This technology is marketed with the trade name Micro-sludgeTM.

d. Thermo - mechanical method

Combination of heat and mechanical force was applied for enhanced sludge disintegration by Rivard and Nagle (1996). COD solubilisation was increased to 90% as a result of pressurising the sludge and mixing it with steam to raise the temperature to 90°C followed by explosive decompression and application of shear force for 10 minutes.

e. Chemical - chemical

Alkali-Hydrogen peroxide (H₂O₂) oxidation may be effective for the reduction of sludge volume. The advantage of using H₂O₂ over conventional oxidising agents such as chlorine and hypochlorite is that it does not generate harmful chemicals since its only by-products are water and oxygen (Ayling, 1981).

2.6.5 Biological/ Enzyme hydrolysis

This method of sludge stabilisation depends on the enzymatic activity of bacteria or added enzymes (enzymatic hydrolysis) to disintegrate the bacterial cell walls (Appels et al., 2008). An increase in biogas production of about 21% was reported due to the protease activity of *Geobacillus* sp (Miah et al., 2004). Bacterial hydrolysis is also known as partial hydrolysis. It is mostly applied to primary sewage sludge (PSS) for generating an in-situ carbon source (Weemaes and Verstraete, 1998a). Although this is a relatively easy and cost-effective technique for producing easily degradable in-situ carbon, its application for sludge solubilisation is still limited.

All of the pre-treatment technologies described above have both advantages and drawbacks and it is difficult to choose a method which is both highly efficient and cost effective. Cost-benefit analysis and laboratory-scale trials are thus highly recommended as pre-requisites prior to large-scale installation of a specific pre-treatment technique.

2.7 Carbon dioxide Pressurisation

2.7.1 History

Experimental evidence for the impact of CO₂ on bacteria has been gradually building up since the work of Pasteur and Joubert (1877). A structured experimental study to determine the antimicrobial potential of CO₂ was first conducted by Valley and Rettger (1927), who investigated the impact of CO₂ on bacterial cell viability in carbonated ice cream. Fraser (1951) attempted to rupture bacterial cells by the rapid release of applied gas pressure using either Nitrogen (N₂), Nitrous oxide (N₂O) or CO₂. When CO₂ was applied to *Escherichia coli* culture at a pressure of 3447 kPa, although a significant level of cell rupture was achieved, the viable cell counts were always less than the direct counts obtained by the Petroff Hauser method. This was attributed to the killing of bacteria as a consequence of the acidification of solution due to the dissolution of CO₂ in it under pressure. Foster et al. (1962) achieved 59% cell rupture of *Serratia marcescens* in the presence of N₂ under a pressure of 11997 kPa, while only 10 - 25 % of *Staphylococcus aureus* and *Brucella abortus* cells were ruptured under the same treatment conditions. These studies demonstrated the ability of gases under pressure to kill bacteria.

Kamihira et al. (1987) were the pioneers in investigating the sterilising effect of CO₂ under pressure. They compared the sterilising effect of CO₂ in supercritical (SC), gaseous (G) and liquid (L) states on *E. coli*, *Staphylococcus aureus*, and conidia of *Aspergillus niger*. Complete sterilisation was achieved with SC CO₂ at 20265 kPa and 35°C. Following this work, there was a series of extensive studies to determine the effect of CO₂ under pressure on the survival of microorganisms. The use of P CO₂ is now considered as one of the most favourable sterilisation techniques, possessing the capability to replace traditional thermal sterilisation (Spilimbergo and Bertucco, 2003).

2.7.2 Microbicidal potential of pressurised carbon dioxide

Comprehensive research on the antimicrobial characteristics of P CO₂ has demonstrated several advantages of using it for sterilisation. Some features rendering CO₂ an attractive sterilising agent are: it is non-toxic, non-flammable and physiologically (Zhang et al., 2006) and environmentally safe (Hong et al., 1999) and most importantly, it is converted in to a supercritical fluid above 31.1°C and 7380 kPa. In the SC state, it is neither a liquid nor a gas and it possesses features such as enhanced dissolving power, low viscosity and

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high diffusivity which make it an ideal solvent for effective inhibition of microorganisms (Kim et al., 2007b).

An extensive study by Haas et al. (1989) determined the effect of P CO₂ on the inactivation of a wide variety of microorganisms in food, model systems and natural flora. The effect of pressure, pH, moisture, treatment time and temperature was considered on the antimicrobial efficiency of CO₂. About 4 log reduction was achieved by P CO₂ treatment of pure cultures of *E. coli*, *Staphylococcus aureus* and *Salmonella senftenberg* at 6205 kPa and 2 Hrs exposure time. Water content of the sample was found to play a significant role in enhancing the antimicrobial potential of CO₂. An important observation was that this antimicrobial property was found to be unique to CO₂ since N₂ under same treatment conditions had no influence on the microbial counts. Two mechanisms were proposed for the antimicrobial action of CO₂ under pressure. Either the penetration of CO₂ in cells reduces the intracellular pH which may be fatal, or the elevated intracellular concentration of CO₂ might inhibit the decarboxylase enzymes thus interrupting the metabolic chain.

Wei et al. (1991) studied the impact of P CO₂ on the survival of *Listeria monocytogenes* and *Salmonella typhimurium* spiked on food samples such as pink shrimp, chicken meat, orange juice and egg mixture. Initial experiments conducted at 5490 kPa and 5850 kPa for 2 Hrs did not show complete inactivation of *L. monocytogenes* suspended in distilled water; however at 6180 kPa complete inactivation was achieved in 2 Hrs. No change was observed in bacterial numbers under the same treatment conditions using N₂ instead of CO₂. An important observation was the reduction in pH of the cell suspension by 1.5 units after P CO₂ treatment at 6180 kPa, which was considered a possible cause of microbial inactivation. In order to verify this, 0.1 N HCl was added to the cell suspension of *L. monocytogenes* to lower the pH from 4.84 to 3.02 (1.82 unit reduction) and from 5.12 to 3.83 (1.29 unit reduction), but no change in the bacterial number was observed after 2 Hrs incubation time. Moreover, blowing CO₂ through a bacterial suspension at ambient pressure for 2 Hrs had no effect on bacterial number. P CO₂ treatment at 13700 kPa for 2 Hrs caused inactivation of 94 – 98% *Salmonella* and 79 – 84% *Listeria* spiked on chicken meat. At the same pressure and treatment time, 99% reduction was achieved in shrimps and orange juice spiked with *Listeria*. P CO₂ treatment at 13700 kPa for 2 Hrs was not very effective in sterilising egg mixture spiked with bacteria. Only 64% of *Salmonella* was

inactivated while no change was observed in the number of *Listeria*. Experiments conducted using N₂ to sterilise the food samples spiked with bacteria at 13700 kPa for 2 Hrs showed no pH drop nor any influence on bacterial viability. It was therefore considered that the sterilisation achieved by using P CO₂ was linked to the pH drop of the reaction medium as a result of dissolution of CO₂. It was also clear that the lowering of pH of the reaction medium by adding acid and blowing of CO₂ through the reaction medium at ambient pressure had no influence on bacterial number, hence confirming the synergistic relationship between pH reduction under pressure and antibacterial action of CO₂.

Lin et al. (1992 a) investigated the potential of P CO₂ as a sterilising agent and compared it with N₂ and high hydrostatic pressure under the same treatment conditions. Starting with a yeast (*S. cerevisiae*) culture of 10⁸ Colony Forming Units per millilitre (CFU ml⁻¹), CO₂ under a pressure of 6895 kPa resulted in 7 log reduction in 15 minute (min) at 35°C. At the same temperature, an increase in pressure to 13789 kPa and 20684 kPa reduced the time required for 7 log reduction to 10 and 7 min respectively. It was reported that yeast cells could be inactivated at higher temperature (45°C) without P CO₂ treatment but the elevated temperature would adversely affect the quality of the product to be sterilised. At subcritical temperature (25°C), P CO₂ treatment at 6895 kPa resulted in 4 log reduction in 45 min whereas the same log reduction was achieved in 35 min at 13789 kPa and 30 min at 20684 kPa. One hour was required for 7 log reduction at 20684 kPa and 25°C. The mechanism of microbial inactivation was reported to be similar at both subcritical and supercritical temperatures. The only major difference was the rate of diffusion of CO₂ in microbial cells, considered as a limiting step in the whole process. Intracellular diffusion of CO₂ was faster in the SC state, providing a possible explanation for the reduced time needed to achieve the same level of inactivation compared to that required at a subcritical state. After the diffusion of CO₂ into microbial cells, lipids and other vital intracellular substances were extracted upon the rapid release of applied pressure therefore causing the loss of cell viability. Experiments carried out using high pressure N₂ showed that 90% of the yeast cells were viable after treatment at 6895 kPa for 20 and 40 min. N₂ lacked the power to extract vital cell constituents, a characteristic that is specific to P CO₂ making it an ideal solvent for microbial inactivation. A survival ratio of 80% was observed in experiments conducted with high hydrostatic pressure at 20684 kPa, 35°C and 40 min, whereas 6895 kPa at the same temperature and treatment time had no effect on the cell

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numbers. Mixing of the reaction medium and rapid release and recharge of CO₂ in the pressure vessel were identified as important factors that could enhance the antimicrobial potential of P CO₂ by increasing its solubility in the reaction medium and enhancing its ability to extract vital cell components.

In their subsequent work (Lin et al., 1992) examined the effect of repeated pressure cycling of CO₂ on the inactivation of yeast (*S. cerevisiae*) cells. This study aimed to verify the hypothesis put forward in the previous study that pressure cycling could enhance the mass transfer capability of CO₂ and therefore increase the inactivation rate. Experiments were conducted at 6895 kPa, 25 and 35°C with the repeated release and recharge of CO₂ in the pressure vessel (once and twice), whereas the controls were run without any pressure cycling. Pressure cycling (once) significantly reduced the time required for inactivation of 80% cells at 35°C from 7 to 3.5 Hrs. This was further reduced to 2 Hrs when pressure cycling was done twice. At 25°C, one-time pressure cycling caused inactivation of 80% of cells in 4 Hrs (7 Hrs without pressure cycling), while with two cycles the same degree of inactivation was attained in 2.5 Hrs. It was therefore concluded that the cells were ruptured due to the expansion of dissolved intracellular CO₂ upon sudden release of applied pressure.

Inactivation of *Leuconostoc dextranicum* by P CO₂ was studied at supercritical (35 and 45 °C) and subcritical (25°C) temperatures by Lin et al. (1993) at 6895 and 20684 kPa. Control experiments were conducted at the same conditions using N₂ and hydrostatic pressure instead of CO₂. Treatment of a culture of 10⁹ CFU ml⁻¹ yielded a 5 log reduction in 7 min at 35°C and 6895 kPa while complete inactivation was achieved in 20 min. Increase of pressure to 20684 kPa reduced the time required for 5 log reduction to 2 min while complete inactivation was achieved in 15 min. When the temperature was raised to 45°C the time for complete inactivation was reduced to 10 min at 6895 kPa, since high temperature facilitates the penetration of CO₂ in microbial cells by relaxing the cell wall. At subcritical temperature (25°C), 40 min and 35 min were required for complete inactivation at 6895 kPa and 20684 kPa respectively. Control experiments conducted using hydrostatic pressure showed no effect on cell viability.

Research on the antimicrobial effect of P CO₂ on *Listeria monocytogenes* (Lin, 1994) showed that two distinct phases were involved in inactivation at a constant temperature; a

slow initial phase and a subsequent faster phase. The slow initial phase was assumed to correspond to the penetration of CO₂ in microbial cells. After the concentration of dissolved CO₂ reached to a critical point, the second phase was initiated which involved disturbance in the membrane structure, imbalance of enzymatic reactions and extraction of important intracellular constituents resulting in the loss of cell viability. Hence the diffusion of CO₂ in microbial cells was identified as a limiting factor. This hypothesis was supported by the experimental results. One log reduction of *L. monocytogenes* (initial concentration 10⁹ CFU ml⁻¹) required 10 min at 35°C and 6894 kPa while in the next 20 min, 8 log reduction was achieved causing complete inactivation. At 20682 kPa and 35°C, one log reduction took 2 min while complete inactivation took 14 min. Rapid inactivation at high pressure suggested that the enhanced solubility of CO₂ in the reaction medium at elevated pressure leads to quick diffusion across the cell wall, effectively overcoming the limiting factor and resulting in the instant loss of cell viability. Elevated temperature (45°C) caused one log reduction in 4 min at 6894 kPa while complete inactivation was attained in another 4 min, possibly due to the cell wall being relaxed at high temperature allowing rapid intracellular penetration of CO₂. Presence of water was also identified as a vital factor as it not only helped in the dissolution of CO₂ but also caused the swelling of cell walls thus relaxing them for easier penetration of CO₂. The presence of oil or fat in the suspending medium was found to interfere with the sterilising capability of P CO₂ by affecting the structure of the cell wall and cellular membranes.

Nakamura et al. (1994) investigated the effect of rapid release of P CO₂ on *Saccharomyces cerevisiae* (baker's yeast) under various process parameters such as temperature, pressure, treatment time and water content of cells and compared it with N₂ under pressure. The survival ratio of yeast cells was found to drop rapidly with an increase in pressure (2026 – 4053 kPa), temperature (20 to 40°C) and treatment time (0.5 to 3 Hrs). Complete inactivation of wet yeast cells (>80%) was achieved at 4053 kPa, 40°C and more than 3 Hrs treatment time. Under the same treatment conditions N₂ could inactivate only 8% of wet yeast cells thus indicating that the enhanced solubility of CO₂ in water as compared to N₂ is an important factor contributing to its antimicrobial properties. Sudden release of applied pressure was observed to reduce the survival ratio compared to slow pressure release. This observation was linked to the absorption and desorption of gas by yeast cells. When the cells were exposed to P CO₂, they became slowly perforated and filled by gas and upon sudden release of pressure, they were mechanically ruptured. This was

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confirmed by Scanning Electron Micrographs (SEMs) of cells treated at 4053 kPa, 40°C and 5 Hrs as shown in Figure 2.3. Some cells were completely ruptured while others showed clear changes in their morphology in the form of holes and wrinkles; untreated cells had a smooth surface. Thus it was concluded that the sterilisation was a result of mechanical cell rupture and disturbance of physiological processes upon the rapid release of applied pressure of CO₂.

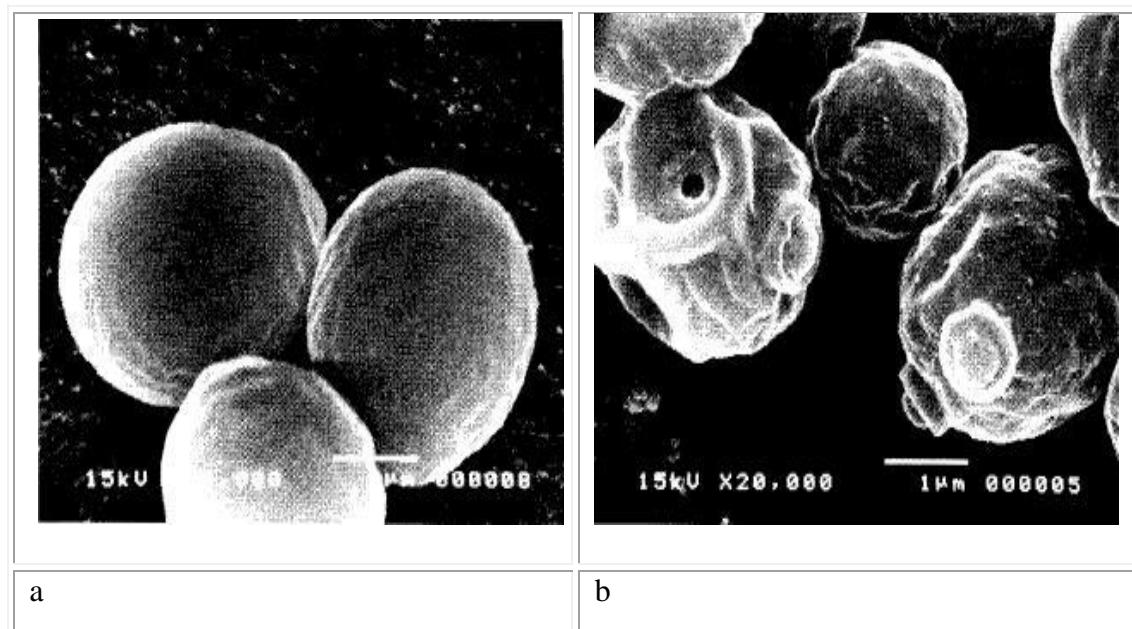


Figure 2.3 Scanning Electron Micrographs (SEMs) of *S. cerevisiae*. Untreated (a) and treated (b) at 4053 kPa, 40°C for 5 Hrs.

(Nakamura et al., 1994)

Antimicrobial potential of P CO₂ on *E. coli* was investigated by Ballestra et al. (1996) at 1200, 2500 and 5000 kPa and 25, 35 and 45°C. *E. coli* was selected in the context of its importance as an indicator of faecal contamination and due to the extensive knowledge available on cell and molecular biology of *E. coli* which might help in understanding the mechanism of action of P CO₂ on microbial inactivation. Experimental results depicted an increase in inactivation rate and decrease in treatment time with increasing pressure. About 4 log reduction was attained in 80 min at 2500 kPa and 45°C while the same result was achieved in 14 min at 5000 kPa. Two distinct phases were observed in the survival curves of *E. coli*, an earlier slow phase followed by an accelerated phase. The duration of first phase was found to decrease with an increase in applied pressure. At the pressure of 1200 kPa, the duration of first phase was 30 min while it was reduced to 15 min and 5 min at 2500 and 5000 kPa respectively. On the basis of these results it was assumed that the

initial phase involved the diffusion of CO_2 in microbial cells followed by rapid inactivation in later phase. Intracellular diffusion of CO_2 was therefore identified as the controlling factor and enhanced solubility of CO_2 at constant temperature and elevated pressure was considered as the possible explanation of shortening of initial phase with an increase in pressure. Inactivation of *E. coli* at 25, 35 and 45°C was studied at 5000 kPa. A sharp decrease in time required for earlier phase of inactivation was observed (from 22 to 12 min) with an increase in temperature from 25 to 35°C. However, the inactivation rate was not much affected with the rise of temperature above 35°C. At low pressure, the effect of temperature on the inactivation rate of *E. coli* was more pronounced. SEMs of *E. coli* treated at 5000 kPa, 35°C for 15 min depicted that the cell walls were distorted pointing towards the possibility of cytoplasmic leakage. However, although the cell walls of 25% cells were undamaged the cell viability was only 1% indicating the involvement of complex phenomenon like deformation of membrane structure, loss of protein function and enzymatic activities in the microbial inactivation rather than just cell rupture. This was further confirmed by the complete loss of activities of Alkaline Phosphatase and β Galactosidase after P CO_2 treatment at 5000 kPa, 35°C and 15 min while the activities of some enzymes such as Acid Phosphatase were only slightly affected. This selective enzyme inhibition was attributed to intracellular acidification due to the dissolution of CO_2 in cytoplasm resulting in the precipitation of enzymes with acidic isoelectric points such as Alkaline Phosphatase and β Galactosidase whereas the enzymes with basic isoelectric points were unaffected. Enzyme inactivation was identified as one of the major factor causing loss of viability of intact cells.

Using a specially designed continuous flow system, Shimoda et al. (1998) were successful in achieving high concentration of dissolved CO_2 in the treatment medium which enhanced its antimicrobial property. The treatment vessel had a stainless steel mesh filter having pore size of 10 μm attached at the bottom which converted P CO_2 pumped through it in to micro bubbles. The surface area was increased when these micro bubbles travelled upwards and enhanced the dissolution of CO_2 (Figure 2.4).

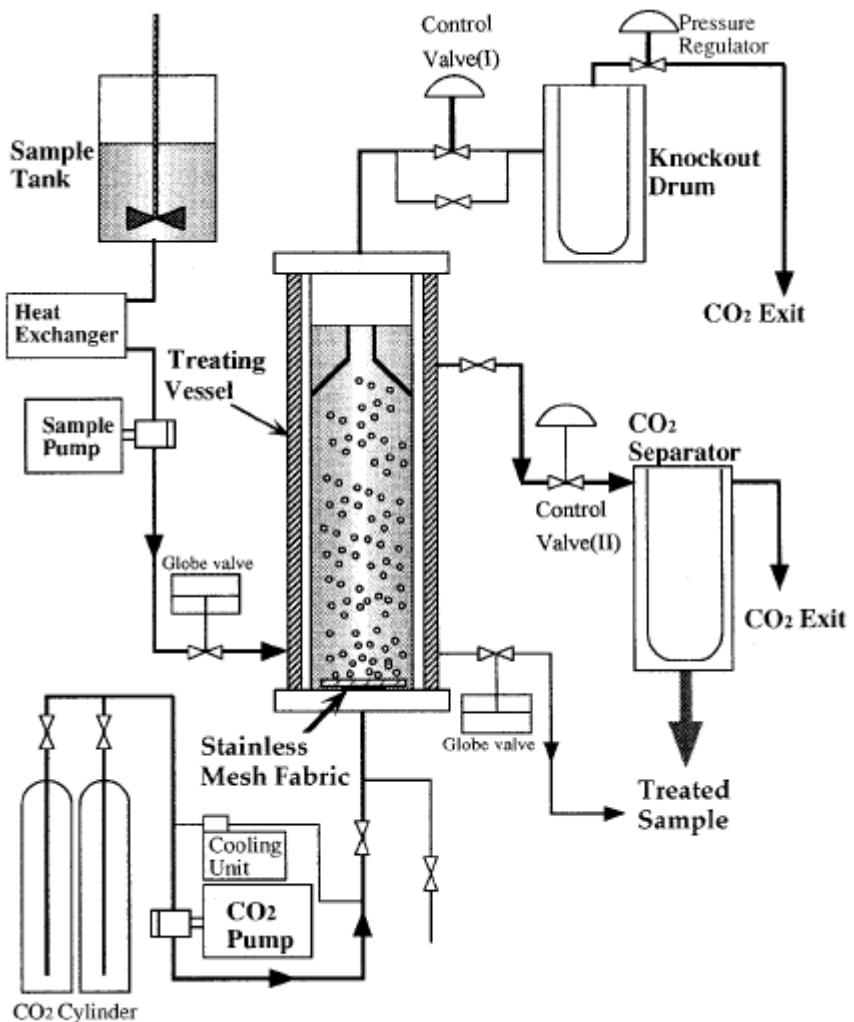


Figure 2.4 Schematic diagram of continuous flow system with microbubbles of pressurised CO_2
(Shimoda et al., 1998)

The efficiency of this system was compared with a batch system having a stainless steel mesh filter through which P CO_2 was fed. Major difference between two systems was the time required for depressurisation which was a few micro seconds in the continuous system while in the batch system it was about 5 min. Different depressurisation rates were expected to cause microbial inactivation in different modes. *E. coli*, *S. cerevisiae*, *Lactobacillus brevis*, *Torulopsis versatilis* and *Zygosaccharomyces rouxii* were selected as test microorganisms. A cell suspension of 10^6 CFU ml⁻¹ of each microorganism in Physiological Saline (PS) was used in P CO_2 treatment. 5.7 log reduction of *E. coli*, *S. cerevisiae* and *L. brevis* was achieved at 6000 kPa, 35°C, sample flow rate 20 Kg hr⁻¹ and CO_2 flow rate 0.5 – 2.0 Kg hr⁻¹. Complete inactivation of *E. coli* and *S. cerevisiae* was achieved at CO_2 flow rate of 1.0 Kg hr⁻¹. Calculation of dissolved CO_2 concentration in

treatment medium showed positive impact of CO_2 flow rate on the concentration of dissolved CO_2 . Furthermore, it was found that the amount of dissolved CO_2 in the absence of mesh filter was less than one half of the value in its presence pointing towards the role of filter in elevating the dissolved CO_2 concentration. SEMs of *S. cerevisiae* (Figure 2.5) treated in a continuous flow system at 6000 kPa, 35°C and CO_2 flow rate of 1.0 Kg hr⁻¹ depicted peculiar signs of cell rupture with a possibility of cytoplasmic leakage linked to enhanced UV absorption by cell suspension after treatment. However, it was suggested that the slow release of pressure in a batch system failed to rupture the cells (not verified by SEM) hence microbial inactivation in batch system was attributed to the enzyme inactivation.

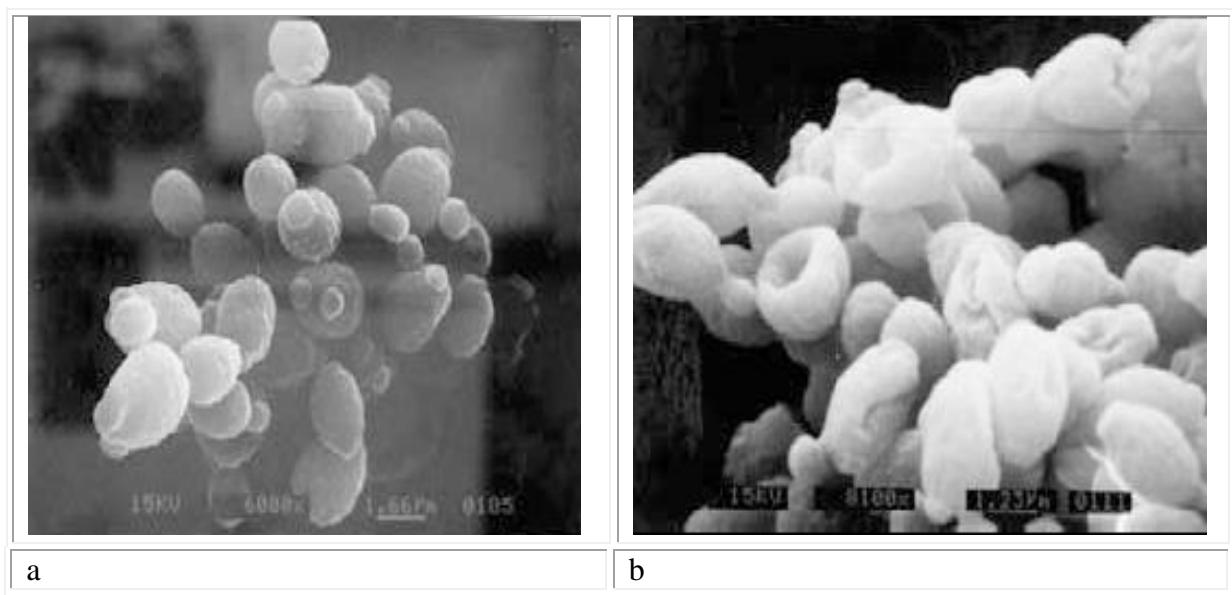


Figure 2.5 SEMs of *S. cerevisiae* treated in a continuous flow system at 6000 kPa, 35°C and CO_2 flow rate of 1.0 Kg hr⁻¹. Control (a) and Treated (b) (Shimoda et al., 1998)

Influence of P CO_2 treatment on the viability of *E. coli* and *S. aureus* in Phosphate Buffer (PB) and Ground Beef (GB) was studied by Sirisee et al. (1998) at a temperature range of 35, 42.5 and 50°C and pressure range of 1034, 2067 and 3103 kPa. About 10^9 CFU ml⁻¹ of *E. coli* and 10^8 CFU ml⁻¹ of *S. aureus* in PB were subjected to P CO_2 treatment at the selected temperature and pressure range. Viability was found to decrease with an increase in pressure and exposure time. Treatment at 35°C and pressure range of 1034, 2067 and 3103 kPa resulted in 1.2, 3.1 and 3.5 log reduction of *E. coli* respectively. The effect of P CO_2 treatment on *E. coli* was apparent after 40 min at 35°C while at high temperature there was a significant loss of viability in less time since 7 log reduction of *E. coli* was

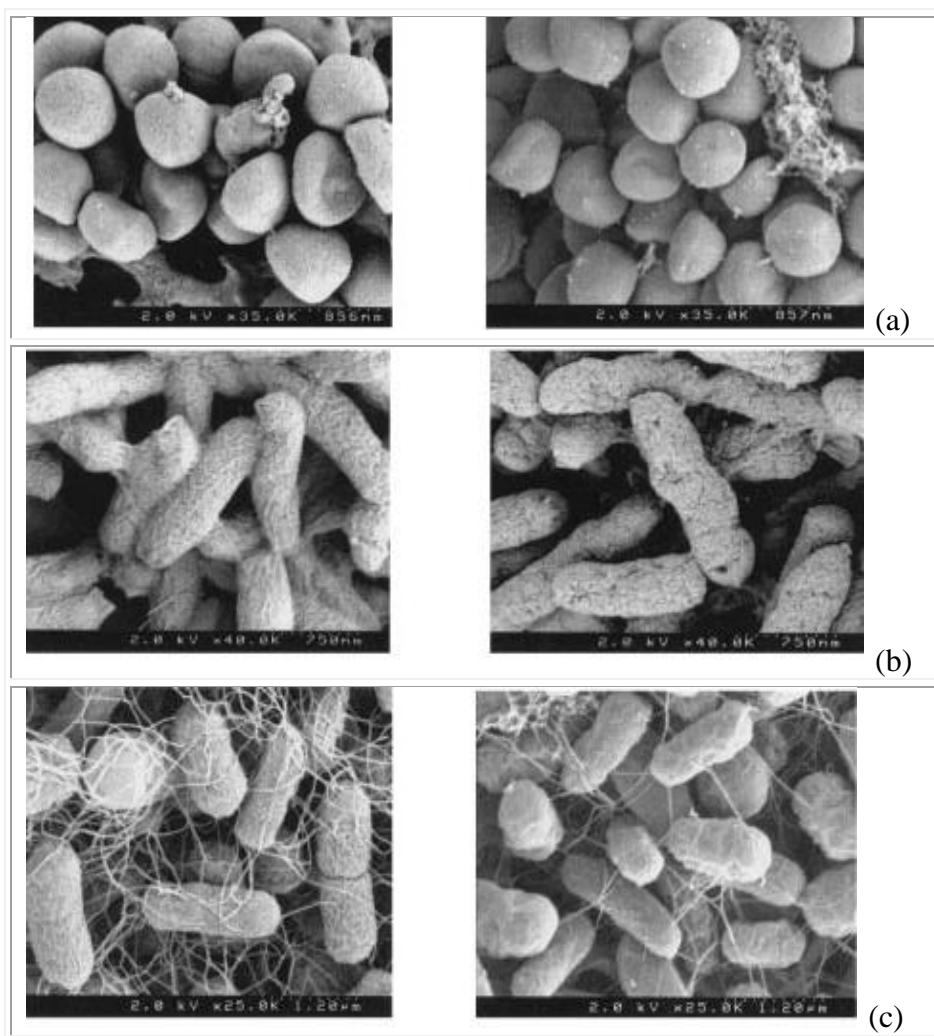
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achieved in 10 min at 3103 kPa and 42.5°C. Viability of *S. aureus* in PB was reduced by 5 log in 40 min at 1034 kPa and 35°C while 4 and 7 log reduction was achieved at 2067 and 3103 kPa. No viable cell was detected after 15 min treatment at 1034 and 2067 kPa, 42.5°C. Effect of pressure on the viability of *S. aureus* was apparent after 30 min at 35°C and 10 min at 42.5°C. Treatment condition of 3103 kPa and 42.5°C was selected for the inactivation of these microorganisms in GB. One log reduction of *E. coli* took 178 min in GB as compared to only 1.7 min in the case of PB. Similarly 25 min were needed for 1 log reduction of *S. aureus* in GB in contrast to only 2.8 min in PB. This was explained by low moisture and high fat and protein content of GB which interfered with the inactivation mechanism. To investigate the effect of P CO₂ on the pH of treatment medium, cell suspensions of both microbes in PB were treated at 1034, 2067 and 3103 kPa, 35°C for 60 min and the pH difference before and after treatment was noted. Furthermore, the pH was monitored continuously for 6 Hrs after treatment at ambient temperature and pressure. At each pressure, a drop of about 3 units in the initial pH (6.7) was observed after P CO₂ treatment (end pH about 3.6) which showed a slight variation during the monitoring period of 6 Hrs. This pH drop was due to the dissolution of CO₂ in treatment medium which might also cause intracellular acidification due to penetration of dissolved CO₂ in cells causing the inhibition of metabolic activities. An interesting observation was the complete recovery of total viable and uninjured cells of *S. aureus* treated at 3103 kPa and 42.5°C for 10 min after an incubation time of 18 and 24 Hrs respectively. *E. coli* treated at 3103 kPa and 42.5°C showed no sign of recovery after 30 Hrs incubation time indicating the complete loss of viability.

In contrast to most of the previous studies focusing on the inactivation of microorganisms in liquid medium, Debs Louka et al. (1999) investigated the efficiency of P CO₂ for inactivating microorganisms on a solid hydrophilic medium (filter paper) over a pressure range of 1500 – 5500 kPa, treatment time of 7 Hrs and ambient temperature. *E. coli*, *S. cerevisiae* and *Enterococcus faecalis* were the test microorganisms. Survival curves of P CO₂ treatment at 5000 kPa showed that *E. coli* was most sensitive as 3.6 log reduction was achieved in 60 min while in this time, *S. cerevisiae* and *E. faecalis* were reduced by only 1.5 and 0.2 logs respectively. Two distinct stages were observed in the survival curves a rapid earlier stage followed by a slow one. Microbial inactivation accelerated with an increase in pressure and exposure time. Significant drop in the pH of treatment medium (about 1.6 units) was observed after P CO₂ treatment which was considered as the cause of

inhibition of important biochemical reactions leading to microbial inactivation. Water was identified as a vital factor for enhancing the antimicrobial action of P CO_2 . Inactivation rate declined sharply in the absence of water however, rate of decompression was not found to influence the inactivation rate as observed in some of the previous studies.

Broadening the scope of P CO_2 , Dillow et al. (1999) successfully sterilised biodegradable polymers Poly Lactic co Glycolic Acid (PLGA) and Poly Lactic Acid (PLA) contaminated with *E. coli* without any variation in the physical and chemical properties of polymer. Pure cultures ($10^7 - 10^9 \text{ CFU ml}^{-1}$) of eight bacteria widespread in medical waste i.e. *Staphylococcus aureus*, *Bacillus cereus*, *Listeria innocua*, *Salmonella salford*, *Proteus vulgaris*, *Legionella dunnifii*, *Pseudomonas aeruginosa* and *E. coli* were subjected to SC CO_2 treatment. All microorganisms except *B. cereus* were completely sterilised at 20500 kPa, between 0.6 – 4 Hrs treatment time and temperature range of 25 – 40°C in a customized super critical fluid sterilisation apparatus. A peculiar feature of experimental process was the partial depressurisation and re-pressurisation of sterilisation unit, mechanical shaking throughout the exposure time and the addition of glass beads in sample for boosting agitation and improving the mass transport capability of CO_2 . Complete inactivation of *E. coli* (initial concentration $10^7 - 10^9 \text{ CFU ml}^{-1}$) at 20500 kPa took 45 min at 25°C and 20min at 42°C. No inactivation was achieved when N_2 was used instead of CO_2 under the same conditions. Inactivation kinetics of *E. coli* was not affected by the presence of PLGA and PLA. Solid microspheres of PLGA and PLA inoculated with 10^9 CFU ml^{-1} *E. coli* were sterilised in 45 min at 20500 kPa and 25°C while at 34°C and same pressure, treatment time was reduced to 30 min. No chemical change in the polymers was observed after treatment. SEMs of *S. aureus*, *P. aeruginosa* and *E. coli* showed that the cell walls were unaffected during P CO_2 treatment (Figure 2.6). The cell walls of gram negative bacteria *P. aeruginosa* and *E. coli* however showed some signs of deformation although they were not completely ruptured. Inactivation mechanism was therefore thought to involve intracellular acidification due to the rapid diffusion of dissolved CO_2 which lethally damaged the biological system.



**Figure 2.6 SEMs of *S. aureus* (a) *P. aeruginosa* (b) and *E. coli* (c) before (left) and after (right) P CO₂ treatment at 25°C and 20500 kPa for 1 hour
(Dillow et al., 1999)**

Efficiency of P CO₂ for the inactivation of *Salmonella typhimurium* suspended in Physiological Saline (PS) was explored by Erkmen, (2000b) with reference to the process parameters such as pressure, temperature, treatment time, initial cell number and physical and chemical properties of treatment medium. About 10⁷ CFU ml⁻¹ of *S. typhimurium* was treated at 1520, 3040 and 6079 kPa at 25, 35 and 45°C. Cell viability decreased sharply with an increase in pressure therefore, less time was required for the same level of inactivation at high pressure. Complete inactivation was attained in 150, 120 and 30 min at 1520, 3040 and 6079 kPa respectively. Bactericidal potential of P CO₂ was enhanced with the rise of temperature. Complete loss of cell viability at 6079 kPa was attained in 30 min at 25°C while 15 and 12 min were needed for complete inactivation at 35 and 45°C

respectively. Two discrete phases were observed in the survival curves of *S. typhimurium*, an initial slow stage followed by a quicker one as shown in Figure 2.7.

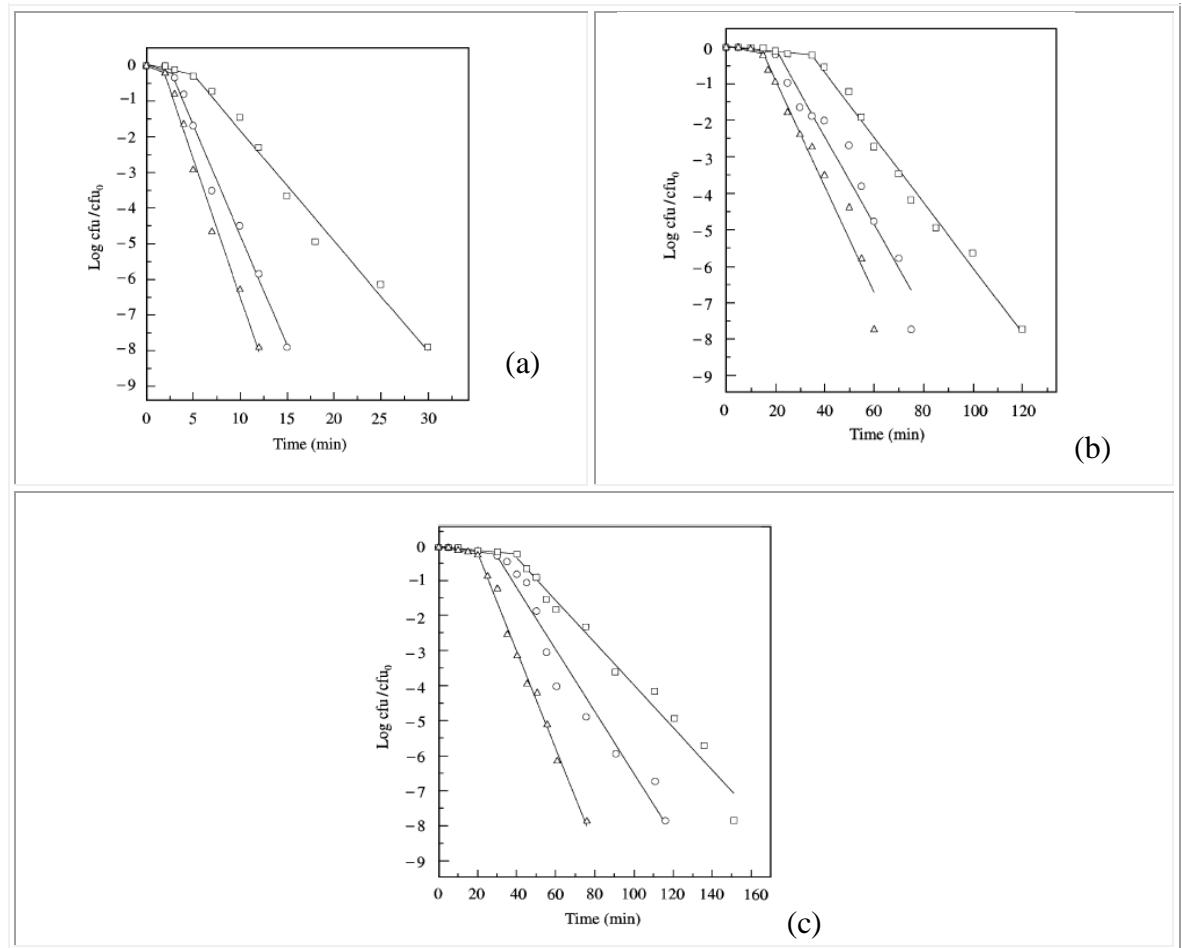


Figure 2.7 Inactivation of *Salmonella typhimurium* in PS as a function of temperature during $P\text{ CO}_2$ treatment at 6079 kPa (a), 3040 kPa (b) and 1520 kPa (c). (25°C \square ; 35°C \circ ; 45°C Δ)
(Erkmen, 2000b)

The length of first stage was reduced with an increase in pressure. At 1520 and 3040 kPa, duration of initial stage was 30 and 20 min respectively and was reduced to 3 min at 6079 kPa. It was explained by the stressing of *S. typhimurium* in initial phase due to the drop of intracellular pH as a result of the penetration of dissolved CO_2 in cells followed by their instantaneous inactivation in the later stage. High concentration of cells in the treatment medium adversely affected the inactivation rate at all of the selected temperatures and pressures. The pH of cell suspension was found to drop sharply after $P\text{ CO}_2$ treatment due to the dissolution of CO_2 . Average pH values after treatment at 1520 and 6079 kPa were 4.74 and 4.84 respectively.

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Antimicrobial effect of P CO₂ on *E. coli* in nutrient broth and milk was studied by Erkmen, (2001) at 2500, 5000, 7500 and 10000 kPa and 20, 30 and 40°C. *E. coli* suspended in nutrient broth was inactivated in 140, 100, 65 and 50 min at 2500, 5000, 7500 and 10000 kPa respectively. Less time required for inactivation at high pressure was linked to the enhanced solubility of CO₂ in reaction medium at elevated pressure. pH of suspending medium also had a remarkable effect on the rate of inactivation. *E. coli* suspension in nutrient broth with initial pH of 6.75, 5.50 and 4.50 was reduced by 7.5 logs in 50, 80 and 90 min respectively when treated with P CO₂ at 10000 kPa for 30 min. Low initial pH had no influence on cell viability when the culture was incubated at ambient pressure. Fat content of milk interfered with the antimicrobial efficiency of P CO₂ and the inactivation was not much effective as in the nutrient broth. *E. coli* was found to resist P CO₂ treatment due to the high fat content of whole milk. Average pH values of treated whole and skimmed milk were 5.98 and 5.92, respectively. Acidification of treatment medium was found to play a major role in inactivation by increasing the intracellular penetration of dissolved CO₂ causing the loss of pH gradient and extraction of important intracellular substances resulting in the loss of cell viability.

Mazzoni et al. (2001) made an attempt to explore antimicrobial potential of SC CO₂ for the sterilisation of Alfa alfa seeds artificially contaminated with *E. coli* (approximately 10⁴ CFU g⁻¹). Spiked Alfa alfa seeds were treated at 13789, 20684 and 27579 kPa for 15, 30 and 60 min. Inactivation rate enhanced with an increase in pressure and treatment time. Reduction of the initial concentration of *E. coli* was 26.6%, 68.1% and 81.3% after 15 min treatment at 13789, 20684 and 27579 kPa respectively. Percentage of inactivation increased from 81.3% to 92.8 % at 27579 kPa by extending the treatment time from 15 to 60 min. There was no effect of P CO₂ treatment on seed viability as no difference was observed in the germination rate of untreated and treated seeds. Therefore the study successfully demonstrated the possibility of using this technique for improving seed security.

Sophisticated analytical techniques such as scanning and transmission electron microscopy, Confocal Laser Scanning Microscopy (CLSM) and Fluorospectrometry (FSM) were used by Goel et al. (2003b) to study the cellular damage caused to *E. coli* as a consequence of P CO₂ treatment. Selected process parameters were pressure (10000-30000 kPa), temperature (37-57°C) and exposure time (30-75 min). Minor changes were

observed in the structure of *E. coli* following P CO₂ treatment at 10000 kPa, 37°C for 30 min (Figure 2.8 B). However, elongated treatment time of 75 min resulted in obvious morphological changes in the cells of *E. coli* in the form of holes and wrinkles visible in the SEMs (Figure 2.8 C).

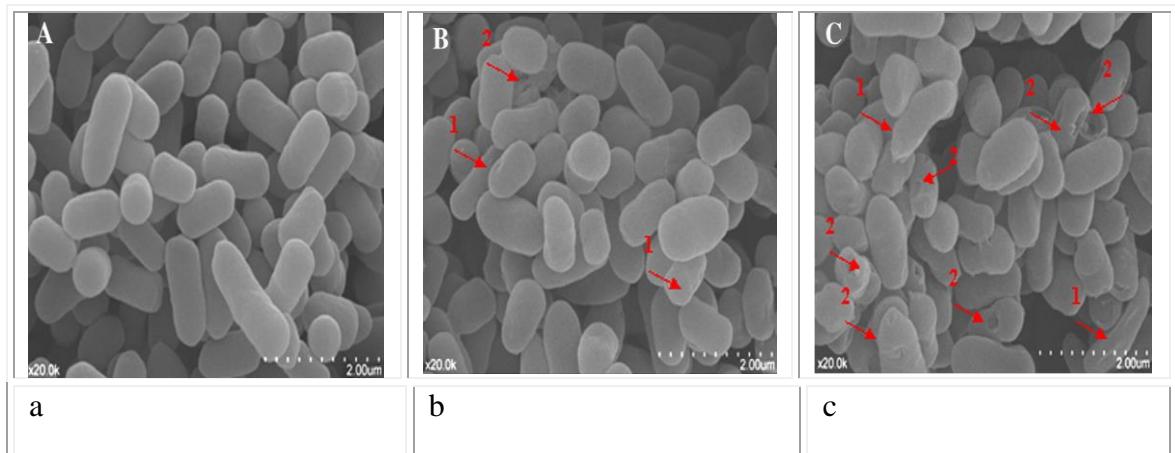


Figure 2.8 SEMs of *E. coli*. Untreated (a) and treated at 10000 kPa, 37°C, 30 min (b) and 75 min (c)
(Goel et al., 2003b)

Healthy cells of *E. coli* exhibited uniformly distributed cytoplasm. P CO₂ treatment (10000 kPa, 37°C for 30 and 75 min) caused evenly distributed cytoplasm to coagulate resulting in the formation of dark and blank areas and segregation of cell membrane from cytoplasm (Figure 2.9 B). This phenomenon was more obvious in the TEMs of samples treated for longer time period (75 min) (Figure 2.9 C).

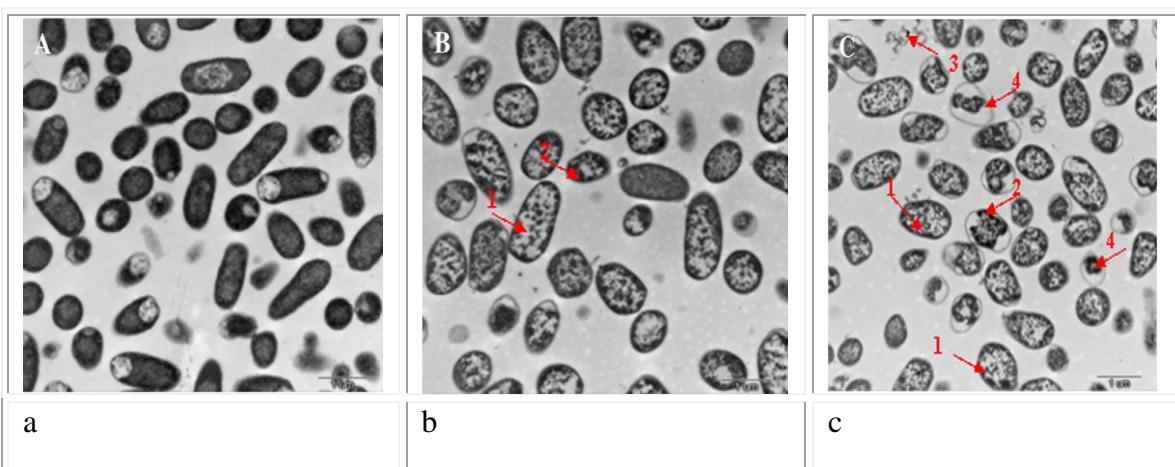


Figure 2.9 TEMs of *E. coli*. Untreated (a) and treated at 10000 kPa, 37°C, 30 min (b) and 75 min (c).
(Goel et al., 2003b)

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CLSM was used to determine the effect of P CO₂ on membrane permeability with the aid of fluorescent stains SYTO 9 and PI. SYTO 9 stains all cells whether their membranes are intact or damaged whereas PI can only penetrate the cells with damaged membranes. CLSM images depicted that healthy cells exhibited green fluorescence which gradually became darker after 5 min treatment at 10000 kPa, 37°C. Green fluorescence continued to become brighter with the elongation of treatment time (15 min) pointing towards the rupture of outer membrane resulting in the easier penetration of SYTO 9. However, after 30 min, small fraction of cells showed red fluorescence reflecting the permeability of cell membrane facilitating the penetration of PI. Prolonged exposure resulted in gradual increase in the proportion of cells emitting red/orange fluorescence until after 75 min very few cells were left which displayed green fluorescence. Similar results were achieved from the fluorescence intensity and spectral patterns of *E. coli* treated with P CO₂ (10000 kPa, 37°C for 5-75 min) and stained with SYTO 9 and PI. Intact cells did not uptake SYTO 9 significantly resulting in a weak green fluorescence. Gradual increase of treatment time caused damage to outer membrane easing the penetration of SYTO 9 and resulting in enhanced green fluorescence. Elongated exposure time increased the permeability of cell membrane resulting in the penetration of PI and exclusion of SYTO 9 causing enhanced red fluorescence and decrease in green fluorescence. Another important observation was the decrease in membrane fluidity rendering it unable to perform vital biological functions. This was attributed to the change of physical state of lipid bilayer due to the strong dissolution power of SC CO₂.

Efficiency of CO₂ under pressure to serve as a non-thermal sterilisation technique for the inactivation of 10⁵ CFU ml⁻¹ of *S. typhimurium*, *E. coli* O157:H7 and *L. monocytogenes* inoculated in orange juice was explored by Kincal et al. (2005) at ambient temperature, 21000, 38000 and 107000 kPa pressure and variable treatment time. All three pressures successfully resulted in 5 log reduction of *S. typhimurium* and *L. monocytogenes*. Inactivation of *E. coli* O157:H7 followed a gradual increase with the elevated pressure. Cell viability was reduced by 2, 3 and 5 log at 21000, 38000 and 107000 kPa respectively. No change in viable cell count was observed in orange juice treated at 21000 and 38000 kPa and stored at ambient temperature (22°C) for 14 days thus indicating the disability of injured cells if present in the sample to repair and regain viability. Microbial inactivation was attributed to the lowering of pH during P CO₂ treatment and mechanical disruption

during flash depressurisation of pressure vessel however more sophisticated techniques such as SEM and TEM were not used to verify this assumption.

Cui and Jahng (2006) attempted to develop a safe potable water disinfection technique using subcritical CO_2 (300, 400 and 600 kPa) for the inactivation of bacteria (*E. coli*) and viruses (Bacteriophage T4). Longest exposure time was 30 min since it is the normal retention time of water in a chlorine tank. Three different concentrations; low, medium and high of both test organisms were checked for their resistance to P CO_2 treatment. Rate of inactivation of *E. coli* was found to be directly proportional to the increase of pressure and treatment time and it followed a linear pattern. High concentration samples were found to resist the treatment due to the clustering of cells which was overcome with the increase in applied pressure. Proposed inactivation mechanism of *E. coli* was the uptake of dissolved CO_2 by microorganisms and their rupture upon the extraction of treated water from pressure vessel. This assumption was supported by the ruptured cells and holes in cell walls visible in the SEMs of P CO_2 treated cells (Figure 2.10 c and d) in contrast to the smooth appearance of normal cells (Figure 2.10 a). Molecular CO_2 instead of pH drop of reaction medium was considered as the major culprit of cell death.

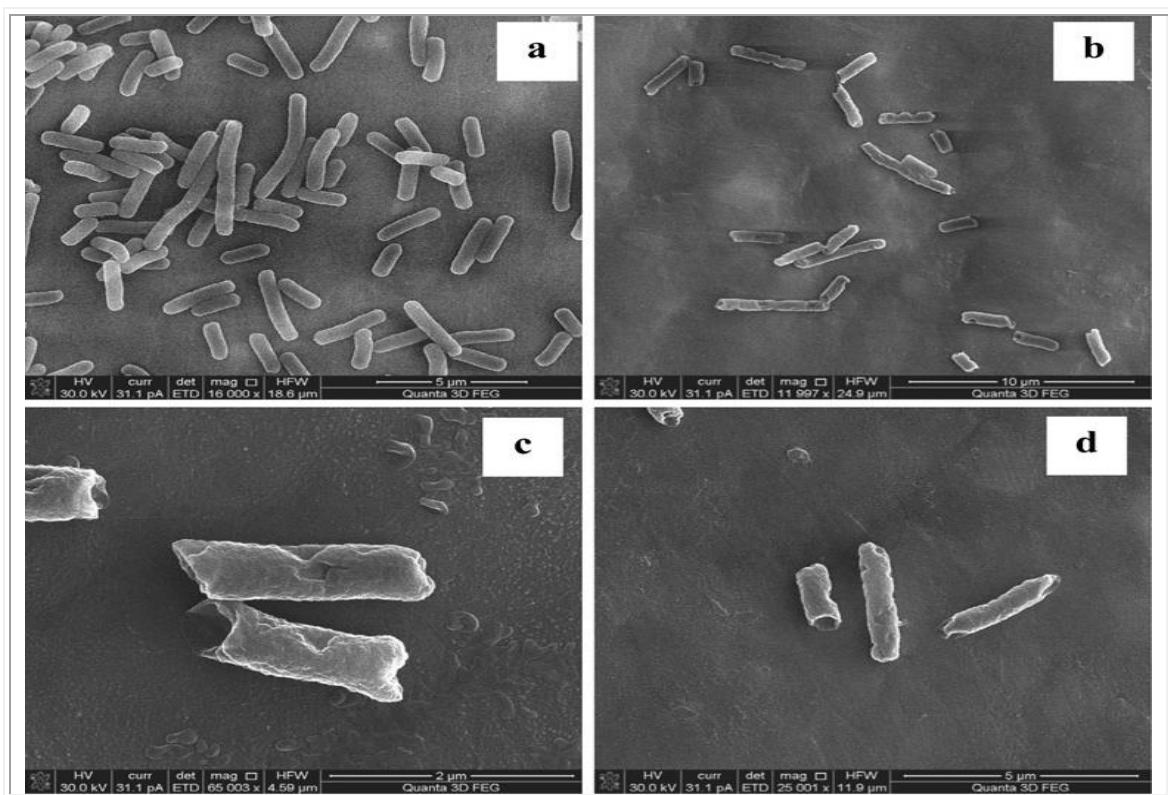


Figure 2.10 SEMs of *E. coli* (a) untreated and treated (b, c and d) with P CO_2 (Cui and Jahng, 2006)

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Gunes et al. (2006) explored the bactericidal potential of P CO₂ on the survival of *E. coli* in apple cider which was treated in a continuous system over a pressure range of 6900 to 48300 kPa, temperature range of 25 to 45°C and CO₂/product ratio of 70g kg⁻¹ and 140g kg⁻¹. Experimental conditions were adjusted such that the effect of three physical states of CO₂; gas (6900 kPa at 35 and 45°C), liquid (6900, 27600 and 48300 kPa at 25°C) and supercritical (27600 kPa at 35°C and 27600 and 48300 kPa at 45°C) could be studied on the survival of 10⁶ CFU ml⁻¹ *E. coli* in apple cider. Complete inactivation was achieved at all three physical states of CO₂. Generally, inactivation rate increased with a rise of temperature and/or pressure however, none of these factors had any influence on cell viability in the absence of CO₂. Both CO₂/product ratios i.e. 70g kg⁻¹ and 140g kg⁻¹ effectively inactivated the initial cell density of *E. coli*. No viable cell was detected in the treated sample after one week of storage at 6°C indicating the disability of injured cells if present in the treated sample to recover and re-grow. Moreover, the soluble solid content and pH of apple cider were unaffected by the treatment process. The mode of inactivation was attributed to cell rupture as a result of rapid depressurisation as indicated by some previous investigators (Shimoda et al., 1998) however no specific technique such as electron microscopy was used to confirm this assumption.

Textile disinfection was another possible application of P CO₂ as demonstrated by Cinquemani et al. (2007) who achieved complete sterilisation of medical fabric infected with *E. coli* and *Micrococcus leuteus* while the physical and chemical properties of the fabric were unaltered. Medical fabric spiked with both microbes (about 10⁶ CFU ml⁻¹) was treated at a pressure range of 5000 to 10000 kPa, temperature range of 20-65°C over a treatment time of 0.5-2 Hrs in the presence or absence of water. *E. coli* was completely inactivated at 20°C, 5000 kPa in 1 hour while *M. leuteus* was inactivated at 65°C. Water was identified as a critical factor influencing the antimicrobial potential of P CO₂ since the increase in temperature from 10 to 65°C did not show a considerable effect on the loss of cell viability in the absence of water. Possible explanation was the dissolution of CO₂ in water to form carbonic acid which interacts with the microbial cell walls to initiate the inactivation.

Kim et al. (2007a) studied the inactivation of about 10⁸ CFU ml⁻¹ of generic *E. coli* and pathogenic *E. coli* O157:H7 suspended in Physiological Saline (PS) by SC CO₂ in a temperature and pressure range of 35 - 45°C and 8000 – 15000 kPa respectively. Influence

of suspending medium on the effectiveness of SC CO₂ was determined by comparing the inactivation rate of both species under same treatment conditions suspended in Physiological Buffered Saline (PBS). Residence time of 20 min was found sufficient to cause 8 log reduction of either strain of *E. coli* at 40°C and all of the tested pressures (8000, 10000, 12000 and 15000 kPa). However, much longer treatment time was required for the same log reduction in PBS. For instance, the time required for 8 log reduction of *E. coli* O157:H7 and generic *E. coli* increased from 11.6 and 12.2 min to 23.3 and 23.8 min respectively at 10000 kPa and 35°C when the treatment medium was switched from PS to PBS. Longer time required for inactivation in PBS was explained by its buffering capacity which helps to resist the changes in pH while PS lacked this capacity. It was further confirmed by the average pH drop of PS from 5.8 to 4.0 (1.8 units) as compared to 7.0 to 5.3 (1.7 units) in case of PBS followed by the SC CO₂ treatment at the selected temperature and pressure range. SEMs of *E. coli* O157:H7 treated at 10000 kPa, 35°C for 30 min showed some deformation however the cells were intact and no signs of cell rupture were apparent (Figure 2.11).

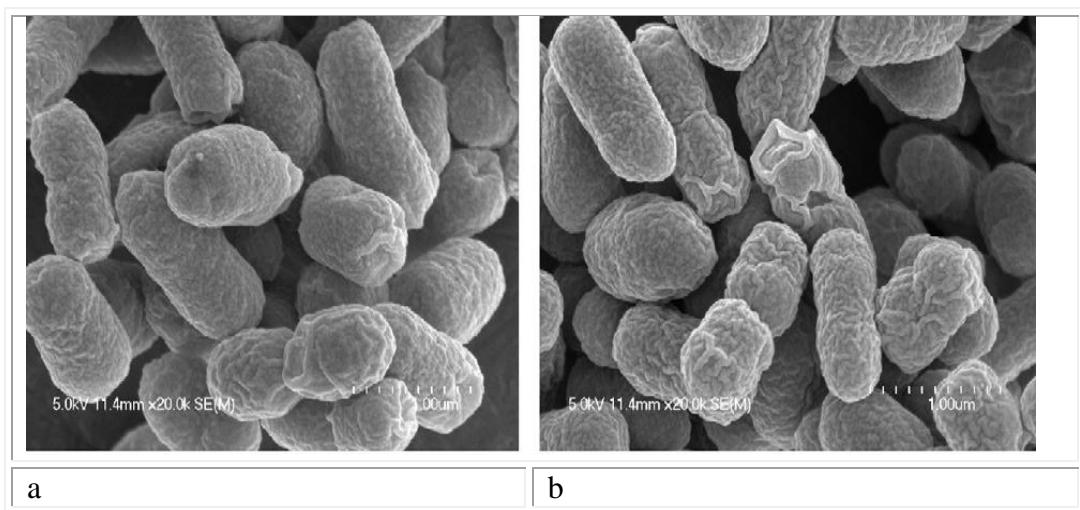


Figure 2.11 SEMs of *E. coli* O157:H7 in PS, untreated (a) and treated (b) by SC-CO₂ at 35 °C and 10000 kPa for 30 min
(Kim et al., 2007a)

However, the Transmission Electron Micrographs (TEMs) revealed a significant difference between treated and untreated cells. Agglomeration of cytoplasm was apparent in treated cells and the cell membrane was also segregated from the cytoplasm (Figure 2.12) pointing towards the possibility of extraction of vital cell components.

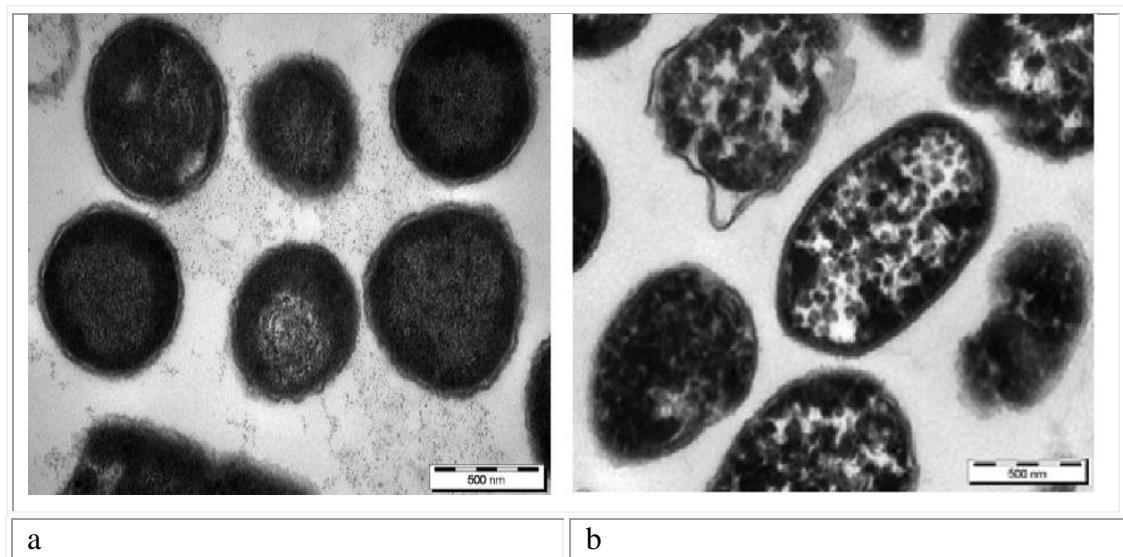


Figure 2.12 TEMs of *E. coli* O157:H7 in PS, untreated (a) and treated (b) by SC-CO₂ at 35°C and 10000 kPa for 30 min
(Kim et al., 2007a)

Possibility of leakage of cytoplasmic substances during SC CO₂ was verified by the absorbance of Ultraviolet (UV) light by untreated and treated cell suspension at wavelengths of 260 and 280 nm. Enhanced UV absorbance by the cell suspension treated by SC CO₂ as compared to the untreated one pointed towards the extraction of vital intracellular substances in treatment medium. Among the six enzymes identified in untreated cells, most of them were found to lose their activities following the SC CO₂ treatment. Hence the inactivation mechanism was considered as the physiological imbalance resulting in the loss of biological activities rather than cell rupture.

Inactivation of *S. typhimurium* by SC CO₂ was explored in another study by Kim et al. (2007b) alongside the development of a mathematical model for inactivation. About 10⁸ - 10⁹ CFU ml⁻¹ *S. typhimurium* in PS or PBS was subjected to SC CO₂ treatment at a pressure and temperature range of 8000-15000 kPa and 35-45°C respectively. Unlike as observed in previous studies, (Erkmen, 2000b, Erkmen and Karaman, 2001) the survival curves of *S. typhimurium* depicted three distinct phases named as ; lag, exponential and stationary phase as shown in Figure 2.13.

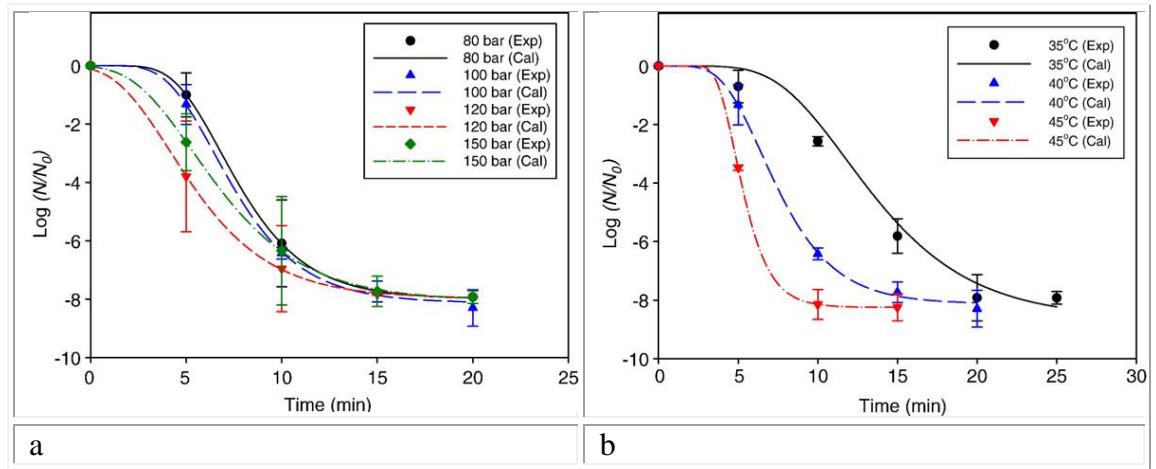


Figure 2.13 Effect of pressure variation at 40°C (a); and temperature variation at 10000 kPa on the inactivation of *S. typhimurium* suspended in PS (N₀: initial cell counts, N: viable cell counts after treatment, Exp: experimental data, Cal: calculated values by fitting the modified Gompertz equation.
(Kim et al., 2007b)

Lag phase was considered as the limiting step as it involved the dissolution of CO_2 in cell suspension followed by intracellular penetration. The duration of lag phase was found to decrease with an increase in pressure due to the enhanced solubility of CO_2 at elevated pressure. Time required for complete inactivation reduced with an increase in temperature at a constant pressure. Cell viability was lost by 8 log in 20, 15 and 10 min at 35, 40 and 45°C respectively at 10000 kPa. A slower inactivation rate was observed when *S. typhimurium* was suspended in PBS due to the resistance in pH change offered by its buffering capacity. Since PS lacked this buffering capacity, the cells lost their viability more quickly and easily when suspended in PS rather than PBS which was confirmed by the end point pH values of 4.0 and 5.5 respectively following SC CO_2 treatment. SEMs of *S. typhimurium* treated at 10000 kPa, 35°C for 30 min showed some wrinkles and vesicles on their cell wall as compared to a relatively smoother outer covering of untreated cells (Figure 2.14) however, the cells were intact.

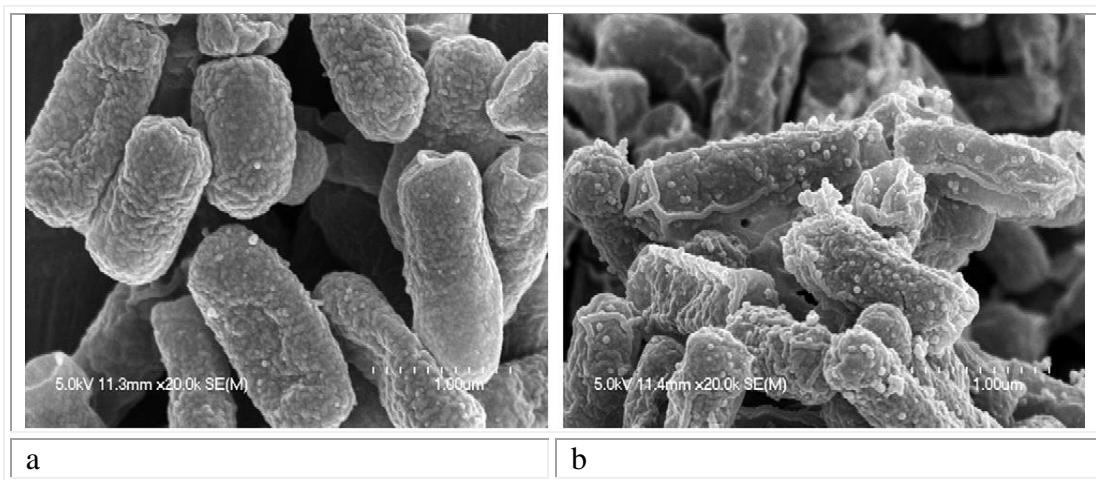


Figure 2.14 SEMs of *S. typhimurium* untreated (a) and treated (b) by SC-CO₂ at 35°C and 10000 kPa for 30 min
(Kim et al., 2007b)

TEMs revealed cytoplasmic disruption, coagulation of cytoplasm and membrane deformation pointing towards the possibility of extraction of intracellular substances in the cell suspension (Figure 2.15). This assumption was further supported by increased U.V absorbance at 260 and 280 nm by the supernatant of treated cell suspension. However enzymatic activities were found to be only moderately affected. Analysis of protein profiles of treated and untreated cells by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) depicted that untreated cells had a high ratio of soluble to insoluble proteins which was completely reversed after SC CO₂ treatment probably due to the denaturation of proteins which might contribute to the loss of cell viability.

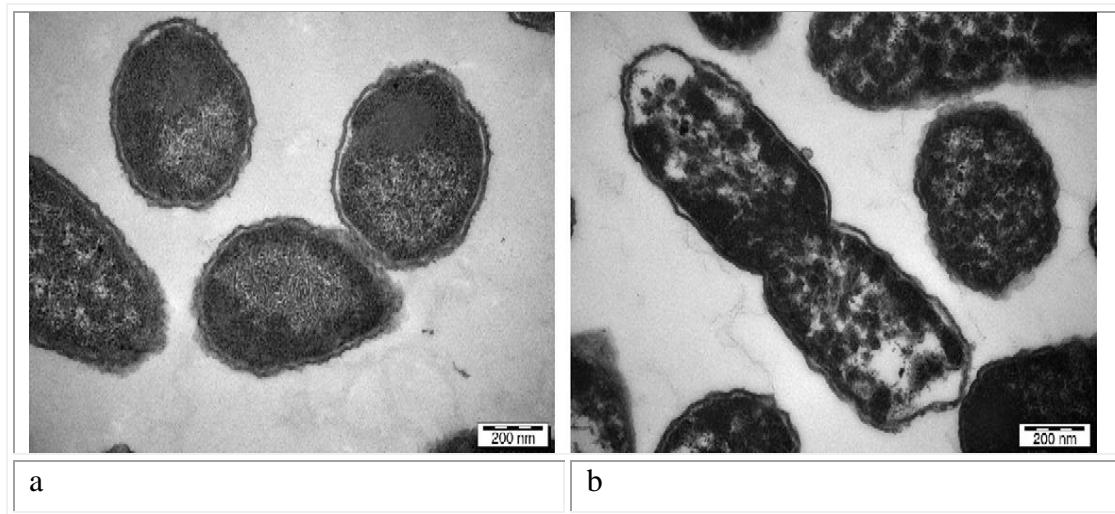


Figure 2.15 TEMs of *S. typhimurium* untreated (a) and treated (b) by SC-CO₂ at 35°C and 10000 kPa for 30 min (Kim et al., 2007b)

Use of SC CO₂ for the inactivation of bacteria in drinking water was attempted by Kobayashi et al. (2007). *E. coli* was selected as the test microorganism. Experiments were conducted on sterile distilled water inoculated with 10⁸ CFU ml⁻¹ of *E. coli* and water from a municipal water filtration plant with about 10² MPN 100 ml⁻¹ coliform bacteria. The SC CO₂ treatment was carried out in a continuous flow system at 10000 kPa, 35°C for 6.7 and 13.3 min with a CO₂/sample flow rate of 3.8 to 10.6 min⁻¹ respectively. Concentration of dissolved CO₂ was determined by Kuenen gas absorption coefficient. Cell viability decreased sharply with an increase in dissolved CO₂ concentration. The survival curves of *E. coli* and coliform bacteria were comparable indicating that the mechanism of action of SC CO₂ is independent of the type of coliform bacteria. Complete inactivation of *E. coli* and coliforms was attained in 13.3 min at 10000 kPa and 35°C. Further detailed studies were considered as a pre-requisite for the practical application of this technique in a water filtration plant.

Liao et al. (2007) investigated the efficacy of dense phase CO₂, a term used for P CO₂ to sterilise cloudy apple juice inoculated with *E. coli*. Effect of various parameters on cell viability was taken in to account such as pressure, temperature, treatment time, purity of CO₂ and initial microbial count. *E. coli* suspended in cloudy apple juice was treated at a temperature range of 32, 37, 42, 47 and 52°C, pressure range of 20000, 30000 and 45000 kPa and exposure time of 30 min to determine the effect of temperature and pressure on survival rate. At each of the selected pressure, inactivation rate was found to increase with

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an increase in temperature and vice versa. Since pressure controls the solubility of CO₂, hence the dissolution of CO₂ in apple juice rapidly increased at elevated pressure causing a drop of pH moreover, the fluidity of cell wall increased at high temperature facilitating the penetration of CO₂. That is why these factors had a profound impact on cell viability. Inactivation rate was found to increase with an increase in exposure time from 8 to 75 min at 20000 kPa and 37°C or 30000 kPa and 42°C. Survival curves showed a fast to slow two stage kinetics (Figure 2.16) and the values of regression coefficients were higher than 0.85. Since CO₂ was in the supercritical state, it swiftly diffused across the cell wall overcoming the limiting factor of inactivation followed by a slow step involving the leakage of cell contents resulting in the complete loss of viability. Antimicrobial potential of CO₂ was directly related to its purity as the inactivation rate dropped when the purity of CO₂ was reduced from 99% to 95%. If the initial cell density was high the inactivation was slow due to the clumping of cells which rendered them resistant to P CO₂ treatment.

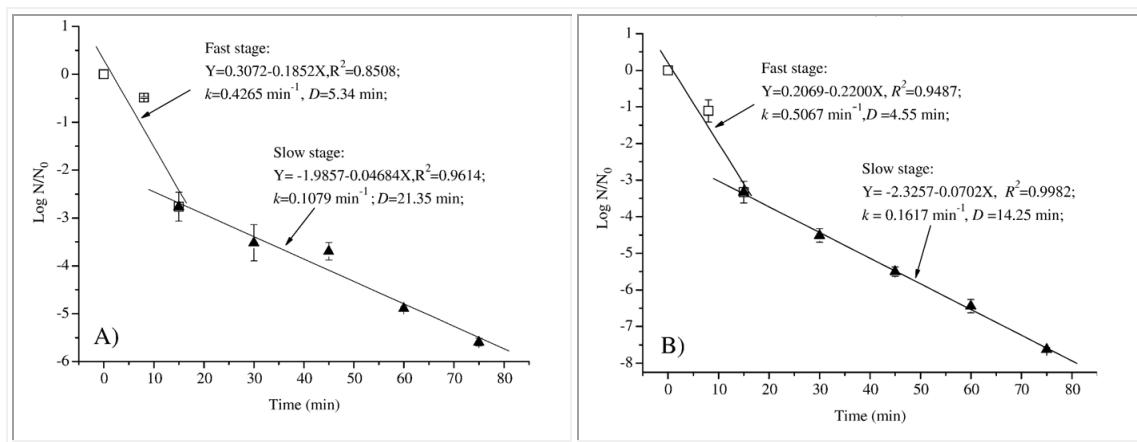


Figure 2.16 Fast-to-slow two-stage inactivation kinetics for *E. coli* in cloudy apple juice exposed to P CO₂ with data fitted using a first-order reaction (Liao et al., 2007)

Kim et al. (2008) investigated the influence of co-solvents on the survival rate of *Listeria monocytogenes* treated with SC CO₂. About 10⁸ to 10⁹ CFU ml⁻¹ of *L. monocytogenes* in PS (pH 7) was subjected to SC CO₂ treatment at selected pressure range (8000, 10000, 12000 and 15000 kPa) at 40°C for 5 to 20 min. Cell viability dropped with an increase in pressure and treatment time. Approximately 8 log reduction was achieved in 15 min at all selected pressures. Temperature variation was found to affect the inactivation rate more effectively as compared to change in pressure. Viability of *L. monocytogenes* subjected to SC CO₂ treatment at 10000 kPa and variable temperature (35, 40 and 45°C) was found to

decrease significantly with the increase of temperature. Shorter exposure time was sufficient to achieve the same level of inactivation at elevated temperature. Starting with a same initial pH and cell density, longer exposure time was required to attain the same log reduction in PBS as compared to PS which was attributed to the resistance in pH change due to the buffering capacity of PBS. This phenomenon emphasizes the role of pH reduction as a triggering factor for microbial inactivation. Water was identified as a facilitating medium for enhanced microbial inactivation however high water content may affect the bactericidal action of SC CO₂ adversely. Oleic acid and Sucrose monolaurate were added to the cell suspension as representative fatty acid and surfactant respectively and the effect of SC CO₂ on cell viability was studied in their presence. Antimicrobial efficiency of SC CO₂ was totally lost in the presence of oleic acid as it might have interfered with the dissolution of CO₂ and resisted the change in membrane structure by forming an external protective coating on cells. However, the presence of a small amount of surfactant was found to enhance the sterilising capability of SC CO₂ but higher concentration might be toxic. Increased UV absorbance by cell suspension at 260 and 280 nm was linked to the release of nucleic acids and proteins due to the alteration of bacterial cell wall and cell membrane structure. Analysis of cellular fatty acid profiles of SC CO₂ treated cells compared with the untreated ones showed that the quantity of each fatty acid decreased with treatment which could be justified by the lipophilic nature of SC CO₂ making it capable of dissolving and extracting the fatty acids. Surprisingly, the SEMs and TEMs did not show a remarkable change in cell morphology and cytoplasm (Figure 2.17) after SC CO₂ treatment at 10000 kPa, 35°C for 30 min. This observation was justified by the thick peptidoglycan layer of gram positive bacterial cell wall which offers more resistance to SC CO₂ treatment. However, although the disruption of cell membrane was not apparent it was significantly damaged to release the cell contents as depicted by the increased UV absorbance.

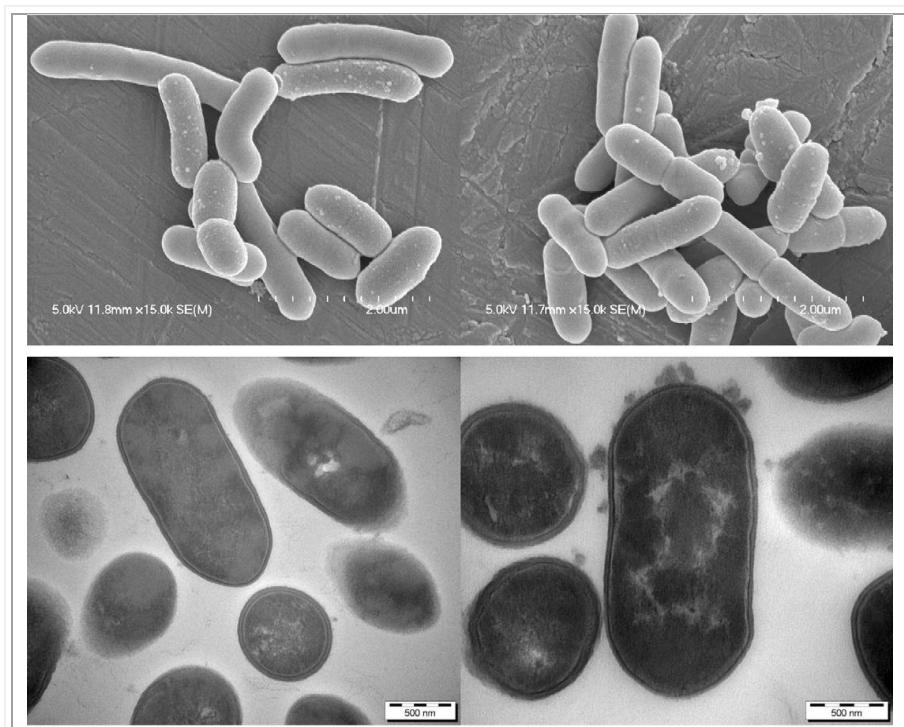


Figure 2.17 SEMs (upper) and TEMs (lower) of *Listeria monocytogenes* untreated (left) and treated (right) by SC-CO₂ at 35°C and 10000 kPa for 30 min (Kim et al., 2008)

Choi et al. (2009) investigated the efficiency of SC CO₂ for the sterilisation of soy sauce and hot pepper paste marinades and marinated pork. Generic *E. coli*, *L. monocytogenes*, *S. typhimurium* and *E. coli* O157:H7 were selected as the test microorganisms. Experimental conditions were; pressure (10000, 12000 and 14000 kPa), temperature (40 and 45°C) and exposure time (20, 30 and 40 min). Higher temperature, pressure and exposure time gave maximum inactivation of selected microorganisms. About 2.5 to 3.4 log reduction was attained at 14000 kPa, 45°C in 40 min when the microorganisms were suspended in soy sauce marinade (Generic *E. coli* 2.5 log CFUcm⁻², *S. typhimurium* 3.4 log CFUcm⁻², *L. monocytogenes* 3.1 log CFUcm⁻² and *E. coli* O157:H7 2.7 log CFUcm⁻²). When the microorganisms suspended in hot pepper paste marinade were subjected to the same treatment conditions, the inactivation range was 2.1 to 2.7 log CFUcm⁻² (Generic *E. coli* 2.1 log CFUcm⁻², *S. typhimurium* 2.7 log CFUcm⁻², *L. monocytogenes* 2.6 log CFUcm⁻² and *E. coli* O157:H7 2.1 log CFUcm⁻²). Profound inactivation in the marinade with high water content (soy sauce) depicted vital role of water as a medium for the dissolution of CO₂ to form carbonic acid and cause a pH drop which played a key role in microbial inactivation. All selected bacteria except *L. monocytogenes* were effectively inactivated by

SC CO₂ treatment in marinated pork at 14000 kPa, 45°C and 40 min. Higher resistance of *L. monocytogenes* was linked to the thicker cell wall, a characteristic of gram positive bacteria as compared to a relatively thin cell wall of gram negative bacteria which makes them more susceptible to pressure treatment resulting in quick inactivation. Temperature and water content were identified as the vital factors influencing microbial inactivation.

Dehghani et al. (2009) applied the P CO₂ technique for sterilising Ginseng, a traditional Chinese medicine which is cultivated in wild soil for about 6 years resulting in the high level of contamination of product with bacteria and fungi. Commonly used sterilisation methods such as autoclaving, gamma irradiation and exposure to ethylene oxide render undesirable impacts on the quality of medicine by inactivating the active compounds responsible for the beneficial properties of Ginseng. Efficiency of P CO₂ for sterilising Ginseng powder contaminated with 10⁷ bacteria g⁻¹ was studied with reference to operating pressure, temperature, retention time and presence of additives. Using pure CO₂, only 2.7 log reduction of bacteria was achieved at 10000 kPa after a long retention time of 15 Hrs at 60°C. However the addition of a very small amount (0.02mlg⁻¹) of either of these liquids; water, hydrogen peroxide or ethanol as additives resulted in a remarkable increase in the inactivation rate of bacteria and fungi at a relatively short exposure time. Bacterial viability was lost by 4.3 log in 6 Hrs at 30°C and 10000 kPa in the presence of these additives. Complete inactivation of bacteria and fungi was achieved at 17000 kPa, 30°C and 2 Hrs exposure time in the presence of 0.1 ml g⁻¹ of water, hydrogen peroxide or ethanol.

Jung et al. (2009) probed in to the efficacy of SC CO₂ for sterilising alfalfa seeds artificially contaminated with *E. coli* O157:H7, *S. typhimurium* and *L. monocytogenes* (about 10⁶ – 10⁸ CFUg⁻¹). The germination percentage of seeds treated at 10000, 15000, 20000 kPa; 30, 40, 45°C for 5, 10 or 15 min was determined after three days. Reduction of *E. coli* O157:H7 and *L. monocytogenes* below detection limit (< 0.3 CFUg⁻¹) was achieved at 15000 kPa, 45°C for 15 min or 20000 kPa, 45°C for 10 min. Whereas 7 log reduction of *S. typhimurium* was achieved at the treatment conditions of 15000 kPa, 45°C for 10 min or 20000 kPa, 40°C for 10 min and at 45°C for 5 min. The germination percentage was not affected by treatment at 10000 kPa, 35 or 40°C for 5, 10 or 15 min. However, a remarkable decrease in germination percentage was observed in the seeds treated at 20000 kPa, 35, 40 and 45°C for 10 and 15 min. Hence it was recommended to treat the seeds at

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15000 kPa, 35°C for 10 min to achieve 3.5 log reduction of *E. coli* O157:H7 and 10000 kPa, 45°C for 5 min to reduce *L. monocytogenes* and *S. typhimurium* by 2.6 and 2.4 logs respectively without any adverse effect on the seed germination.

Li et al. (2009) investigated the correlation between increase in membrane permeability and loss of viability of *E. coli*, *L. monocytogenes* and *S. cerevisiae* as a result of P CO₂ treatment at the selected pressure (10500-21000 kPa), temperature (35-45°C) and treatment time (0-60 min). Spectrophotometry was used to determine the membrane integrity by measuring the uptake of fluorescent dye Propidium Iodide (PI). This dye consists of a small hydrophilic molecule and it could only enter the cells whose membranes are damaged. After entering the cells, PI infuses in to the Deoxyribo Nucleic Acid (DNA) resulting in enhanced fluorescence (Yan et al., 2009). Enhanced fluorescence due to the increased uptake of PI upon increasing the severity of process parameters (pressure, temperature and treatment time) was linked to the membrane damage and was directly proportional to the rate of inactivation. DNA was found to be denatured as a result of P CO₂ treatment. This was based on 15% less fluorescence displayed by P CO₂ treated DNA extracted from *E. coli*. Since PI only binds with double stranded DNA, it was assumed that P CO₂ treatment caused the segregation of strands of double helix. Reduction of pH of reaction medium due to the formation of carbonic acid was considered to be the cause of denaturation of DNA. Negative staining also confirmed the loss of cell membrane integrity of treated cells. This technique involved the use of heavy metal dye which could only percolate the membrane compromised cells resulting in their dark appearance in TEMs in contrast to relatively lighter colour of control cells (Figure 2.18).

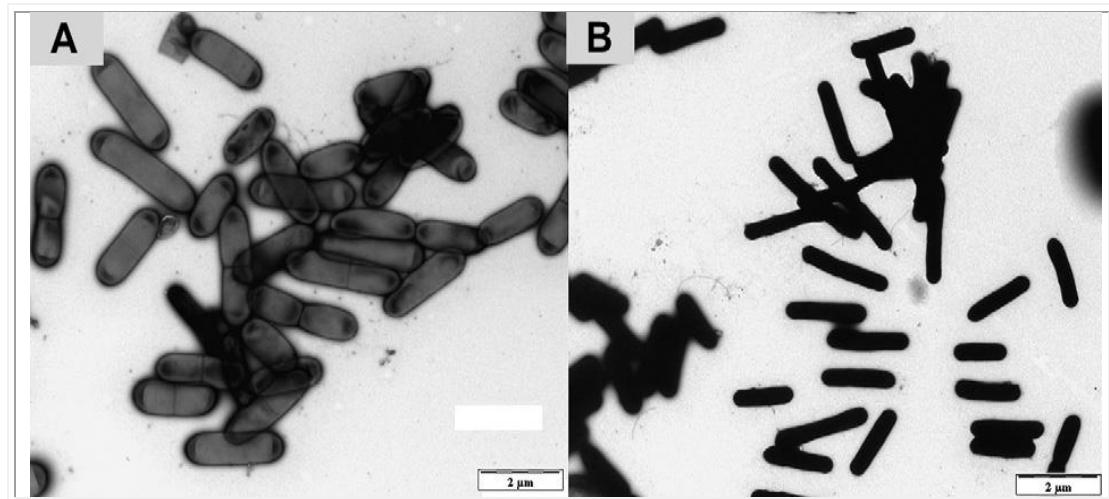


Figure 2.18 TEMs of negatively stained *L. monocytogenes* (a. untreated, b. treated with P CO₂ at 21000 kPa, 45°C

(Li et al., 2009)

Effect of P CO₂ treatment on the internal structure of microorganisms was determined with the aid of TEMs of ultrathin sections. Before treatment the cytoplasm of *E. coli* was uniformly distributed (Figure 2.19, a) however after treatment, it appeared to be coagulated and blank areas were visible in addition to tiny holes in the cell wall and cytoplasm protruding out of them (Figure 2.19, b and c).

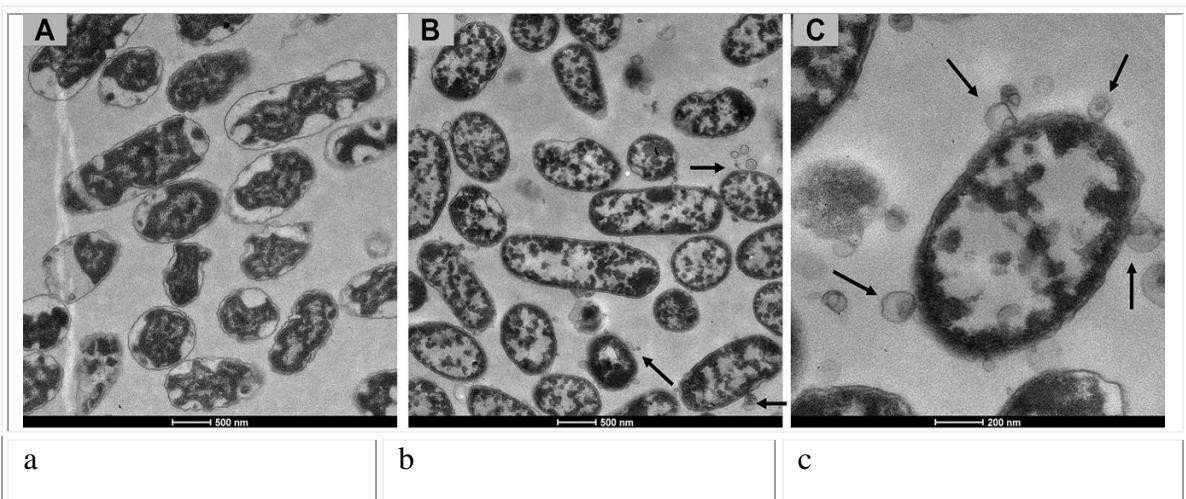


Figure 2.19 TEMs of *E. coli* (a) untreated (b and c) treated at 21000 kPa, 45°C and 60 min

(Li et al., 2009)

However, no drastic difference was observed between the control and treated cells of *L. monocytogenes* and *S. cerevisiae* which was attributed to the relatively thick cell walls of

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gram positive bacteria and fungi respectively as compared to the thin cell walls of gram positive bacteria (*E. coli*).

Relationship between the increase in permeability and fluidity of cell membrane and its subsequent effect on cell viability of *S. cerevisiae* was studied by Lin et al. (2009) using the techniques of Flow Cytometry (FCM) and Flourospectrometry (FSM). Moreover, structural and anatomical changes in cells following P CO₂ treatment were determined by the SEM and TEM respectively and the influence on enzyme activity was studied using the APIZYM kit. Rate of inactivation was found to be enhanced with the increase in pressure at constant temperature and vice versa. Maximum log reduction was 4.87 at 30000 kPa and 25°C. Progressive increase in the membrane permeability as a result of P CO₂ treatment at 10000 kPa, 35°C with the elongation of exposure time was determined by FCM. Exposure time of 30, 75 and 120 min caused 95.97%, 99.71% and 99.99% cells permeable and the respective inactivation rates were 80%, 99.95% and 99.99%. Higher percentage of permeable cells as compared to the ones inactivated after 30 min exposure time pointed out that some of them were still alive. However, with the elongation of exposure time, the viability was eventually lost. Similar pattern was observed in the FSM values which gradually increased with the elongation of exposure time. This trend was linked to the reduction of membrane fluidity with the increase in treatment time. Eleven enzymes were identified in untreated cells out of which eight lost their activities after 30 min and one more was inactivated after 75 min. This inhibition of enzyme activity was identified to be selective as observed by some earlier researchers (Ballestra et al., 1996). There was no obvious change in the morphology of *S. cerevisiae* treated at 10000 kPa for 30 min. However, with the increase in treatment time (75, 120 min) some signs of deformation, a few lines and crinkles and some cell debris was visible in the SEMs.

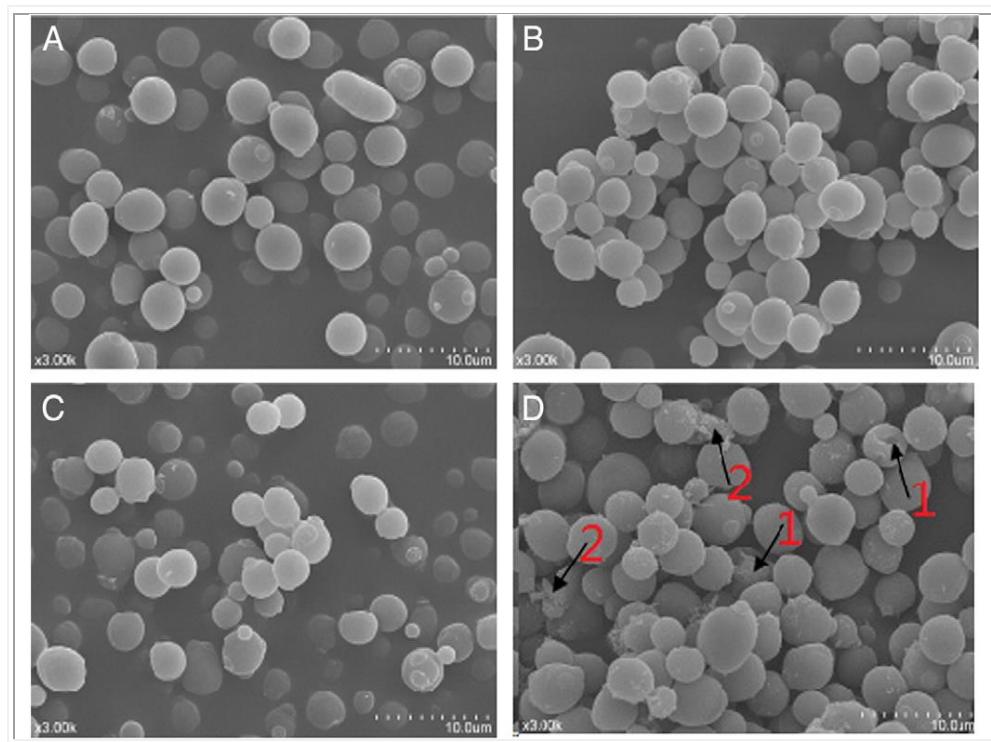


Figure 2.20 SEMs of *S. cerevisiae* untreated (a) and treated with P CO₂ at 10000 kPa, 35°C for 30 (b), 75 (c) and 120 min (d). (Arrow # 1 wrinkles, Arrow # 2 debris) (Lin et al., 2009)

TEMs also narrated the same story i.e. with the elongation of exposure time; regularly distributed cytoplasm of *S. cerevisiae* gradually lost its density leading to the appearance of blank areas. Few cells were ruptured resulting in the extraction of intracellular substances corresponding to the debris visible in the SEMs of cells treated for 120 min.

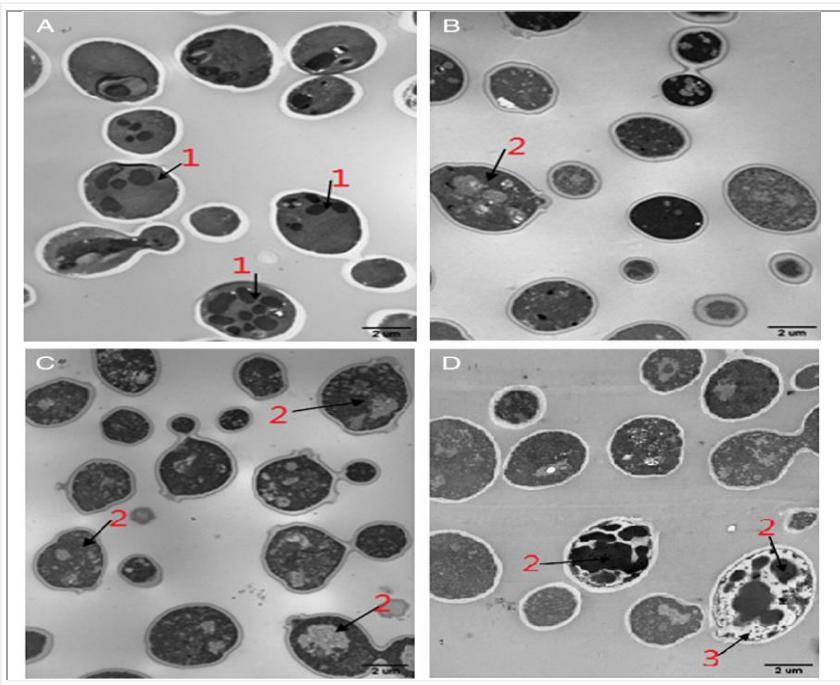


Figure 2.21 TEMs of *S. cerevisiae*. Untreated (a) and treated with P CO₂ at 10000 kPa, 35°C for 30 (b), 75 (c) and 120 (d) min. (Arrows # 1 Dark areas, Arrows #2 Reduction of density of cytoplasm and Arrows # 3 rupture of cells)
(Lin et al., 2009)

It can be deduced from the above described account that there has been a gradual progress in the research aimed to assess the potential of P CO₂ as a means of achieving cold sterilisation. However, the main focus was to investigate the utilization of this technique for food sterilisation in order to replace the traditional thermal sterilisation. A few studies have been executed to sterilise the medical fabrics, biodegradable polymers, drinking water and herbal medicines using this technique which showed encouraging results. However, there is still an unexplored area where the use of this technique might be beneficial i.e. the sanitisation of waste water. There exists a strong possibility for the development of a novel technique for pre-treatment of SS before AD using P CO₂ for enhanced sanitation and minimising the bio security issues associated with the agricultural utilisation of digested SS.

2.8 Factors affecting the efficiency of pressurised carbon dioxide

Growing interest in the application of P CO₂ for the sterilisation of a range of substances such as fresh and processed food products, biodegradable polymers, pharmaceuticals and

medical fabrics, has intensified the need to identify the factors which directly or indirectly affect its efficiency as a sterilising agent and understand the relationship between them in order to enhance its microbicidal action and procure the maximum benefits of this technique. Given below is an account of various factors that have a significant influence on the efficiency of P CO₂.

2.8.1 Pressure and Temperature

Almost every investigation has identified the profound impact of pressure and temperature on the inactivation efficiency of CO₂. This can be attributed to the capability of these parameters to affect the mass transfer properties and biochemical activities of CO₂. Generally, an increase in pressure enhances the efficiency of P CO₂ (Debs-Louka et al., 1999, Erkmen and Karaman, 2001) resulting in a high level of inactivation in a relatively shorter exposure time (Lin et al., 1993, Hong et al., 1997, Hong and Pyun, 1999, Hong et al., 1999). This can be attributed to the fact that solubility of CO₂ is influenced by the pressure, so an increase in pressure results in increased dissolution of CO₂ in the reaction medium thus lowering the pH. Moreover, at high pressure, CO₂ has a high solvating power which facilitates microbial inactivation (Garcia-Gonzalez et al., 2007). However, the increased dissolution of CO₂ as a consequence of rise in pressure is limited by the saturation level (Sims and Estigarribia, 2003).

Temperature is also a vital factor affecting the antimicrobial potential of P CO₂. Generally, an increase in temperature enhances the inactivation rate by stimulating the diffusivity of CO₂ and making the cell membrane more penetrable. It is not recommended to use P CO₂ at temperature far above its critical temperature (31.1°C), however, since there is a rapid decrease in the solvent density as a result of which its solubilisation capacity is reduced (Lin et al., 1993, Hong et al., 1997, Hong and Pyun, 1999, Hong et al., 1999).

2.8.2 Exposure time

Exposure time was found to have a profound impact on the inactivation efficiency of P CO₂ as reported by many investigators. The majority of them observed a strong increase in inactivation with an increase in exposure time (Hong and Pyun, 1999, Erkmen, 2000a, Erkmen and Karaman, 2001, Spilimbergo et al., 2002).

Erkmen (2000b) attained the same level of microbial inactivation in a shorter exposure time by increasing the applied pressure. Hong and Pyun (1999) achieved a same log

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reduction of microbial cells in a decreased exposure time by increasing the applied pressure of CO₂. Recently, Dehghani et al. (2009) were successful in reducing the long treatment time of 15 Hrs to 2 Hrs by adding a very minute amount (0.1 ml) of water, ethanol or H₂O₂ mixture to powdered ginseng for inactivating pathogenic microorganisms.

2.8.3 Physical state of carbon dioxide

A slight variation in temperature and pressure can change the physical state of CO₂ from subcritical (gaseous/liquid) to supercritical. The change in physical state brings about a drastic change in the properties of CO₂. Only a few studies have compared the effectiveness of different physical states of CO₂ in inactivating microbes (Kamihira et al., 1987, Lin et al., 1992 a, Dillow et al., 1999, Gunes et al., 2006). These show a consensus on the enhanced effectiveness of SC CO₂ for inactivating microbes as compared to subcritical CO₂. This enhanced effectiveness is attributed to the physico-chemical properties that lie between a liquid and gas (Mazzoni et al., 2001, Sirisue et al., 1998). Some important features that render SC CO₂ an excellent solvent are its high diffusivity, low viscosity and high dissolving power (Kim et al., 2007b) which enhance its mass transport properties. These features enhance the penetration and dissolution of SC CO₂ across the microbial cells to lower the intracellular pH and affect the physiological activities of biological systems, eventually resulting in a high degree of sterilisation (Garcia-Gonzalez et al., 2007). The mechanism of action was considered to be same in both subcritical and supercritical states, however, except that the intracellular penetration of SC CO₂ was faster than in subcritical conditions, resulting in quicker inactivation in the former case (Lin et al., 1992 a).

2.8.4 Water content

The water content of the suspending medium was found to have a considerable influence on the inactivation rate of microorganisms (Kamihira et al., 1987, Debs-Louka et al., 1999, Schmidt et al., 2005, Dillow et al., 1999). Generally, inactivation of microorganisms was enhanced in the presence of water. In the earlier period of P CO₂ development, it was suggested that sterilisation of dry substances by this technique would not be feasible (Hass et al., 1989). Contrary to this, quite a few researchers (Cinquemani et al., 2007, Dillow et al., 1999, Schmidt et al., 2005) were able to achieve sterilisation in the absence of water, although complete disinfection was obtained by only one researcher (Dillow et al., 1999);

however, the addition of only a very small amount of water can result in a large increase in the inactivation rate.

Enhanced microbial inactivation in the presence of water can be attributed to the dissolution of CO₂ in the water to produce carbonic acid (H₂CO₃) which dissociates to produce H⁺ ions thus lowering the pH of suspending medium. This causes an increase in cell wall permeability allowing easier penetration of CO₂ through the lipoprotein layer of cell membrane. The resulting intracellular pH drop eventually leads to the inactivation of important enzymes. The outcome of this disruption of biochemical reactions vital for the continuity of life is a high rate of microbial inactivation (Garcia-Gonzalez et al., 2007).

2.8.5 Rate of depressurisation

Pioneering researchers in the field of P CO₂ (Fraser, 1951, Foster et al., 1962) were of the view that explosive depressurisation caused the microbial cells to rupture. It was believed that at high pressure, CO₂ penetrated into the microbial cells; and on the sudden release of pressure there was a rapid expansion of the gas absorbed in cells, which resulted in their rupture (Garcia-Gonzalez et al., 2007)

Disruption of microbial cells by explosive decompression of CO₂ was initially observed by Fraser (1951) whose main aim was to collect the cell contents of *E. coli* by cell rupture. Carrying his work forward, Foster et al. (1962) achieved 31-59% rupture of *Serratia marcescens* following N₂ saturation at 11997 kPa using an improved cell rupture apparatus. They suggested the use of other suitable germicide gases such as CO₂. Following this work, various other investigators attributed the enhanced disruption of bacterial cells to rapid decompression (Lin et al., 1991, Nakamura et al., 1994, Shimoda et al., 1998). In order to verify this hypothesis, Enomoto et.al (1997) compared the inactivation of baker's yeast *Saccharomyces cerevise* using different gases (CO₂, N₂O, N₂ and Ar) at 4053 kPa, 40°C for 4 Hrs followed by explosive decompression. Sterilisation was achieved only with water soluble gases i.e. CO₂, N₂O, but interestingly inactivation was not always dependent on explosive decompression alone. In fact, most of the microbial cells were found to be inactivated in the CO₂ pressurisation step as supported by the work of many researchers (Arreola et al., 1991, Hong and Pyun, 1999, Debs-Louka et al., 1999).

2.9 Mechanism of action of pressurised carbon dioxide

Ever since the possibility of utilising CO₂ as an effective sterilising agent was demonstrated (Fraser, 1951), scientists had been curious to determine the exact mechanism by which it inactivated the microorganisms. Several hypotheses have been proposed to explain the microbicidal action of P CO₂ (Spilimbergo and Bertucco, 2003) but there is no consensus on any specific mechanism yet. It seems as if the biocidal property of P CO₂ can be attributed to a series of interrelated mechanisms. Generally, the mechanisms of microbial inactivation by P CO₂ can be divided in two broad categories; mechanical cell rupture and physiological inactivation (Zhang et al., 2006). Figure 2.22 schematically represents the proposed mechanisms of inactivation and their interrelationship.

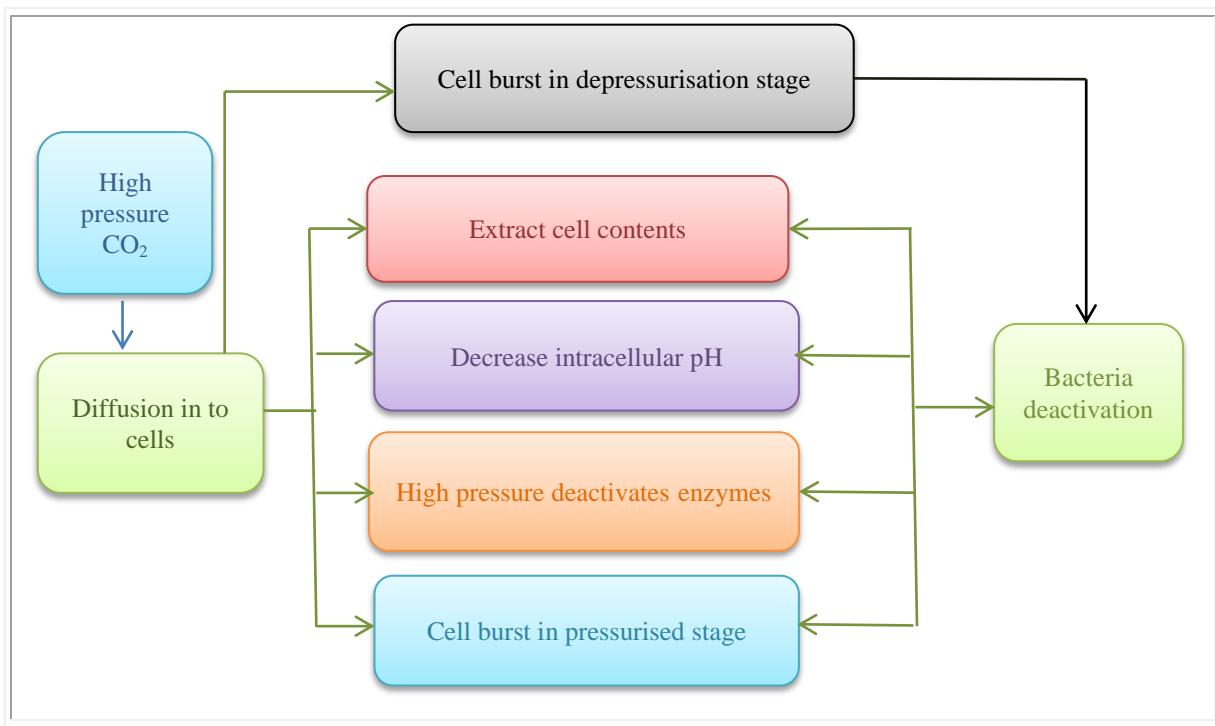


Figure 2.22 Interrelationship between the proposed mechanisms of action

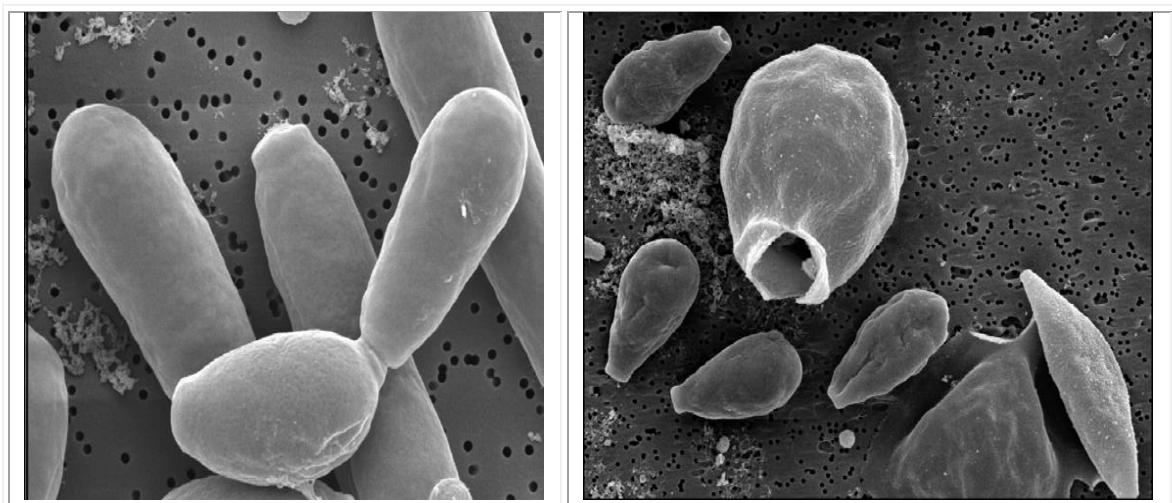
(Zhang et al., 2006)

2.9.1 Mechanical cell rupture

First proposed mechanism for the antimicrobial action of P CO₂ was the mechanical rupture of microbial cells as a consequence of explosive decompression (Fraser, 1951). It is difficult, however, to ascertain that the cells were actually ruptured on the basis of simple microscopic images without employing SEM and TEM. Following this work, a number of researchers tried to investigate whether the cells were ruptured as a

consequence of P CO_2 treatment using the techniques of SEM and TEM to observe the changes in cell morphology and internal structure, and UV absorbance and protein analysis to determine the extraction of cell components.

Nakamura et al. (1994) reported complete disruption of yeast cells (supported by SEMs Figure 2.3) due to rapid depressurisation and attributed it to either physical cell rupture and/or physiological disturbance. Cells may be biologically inactive even if they are physically intact as observed by Ballestra et al., (1996) found that a degree of cell rupture was achieved by sudden release of pressure but viability was less than 1% even though 25% of cells were intact. Erden et al. (2010) also reported the rupture of some yeast cells treated at 2700 kPa, 21°C for 5 min (Figure 2.23).



**Figure 2.23 SEMs of yeast cells untreated (left) and treated (right) with P CO_2 at 27600 kPa, 21°C for 5 min
(Erden et al., 2010)**

Most of the cells had a contracted appearance, however, with signs of shrinking. The mode of cell death was therefore attributed to the extraction of cell components due to the lipophilic nature of P CO_2 rather than to explosive cell rupture.

Hong et al. (1997) were unable to enhance the inactivation rate by mechanical cell rupture as a consequence of sudden release of pressure, thus placing a large question mark in front of the 'explosive cell rupture' hypothesis. This observation was in agreement with Arreola et al. (1991) who found that microbial inactivation was not related to the rapid release of applied pressure. Moreover, similar conclusions were drawn by Isenschmid et al. (1995)

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who attributed microbial death to the 'anaesthesia effect' on the cell membrane due to the lipophilic nature of CO₂ rather than mechanical cell rupture.

SEM studies conducted by Dillow et al. (1999) and Hong and Pyun (1999) depicted intact cell walls following P CO₂ treatment (Figure 2.6 and Figure 2.24 respectively). The TEM studies showed some fractures in the cell membrane and empty spaces in cytoplasm of treated cells, pointing towards the release of vital cell components which disturbed the biological system of microbial cells eventually leading to their death (Hong and Pyun, 1999).

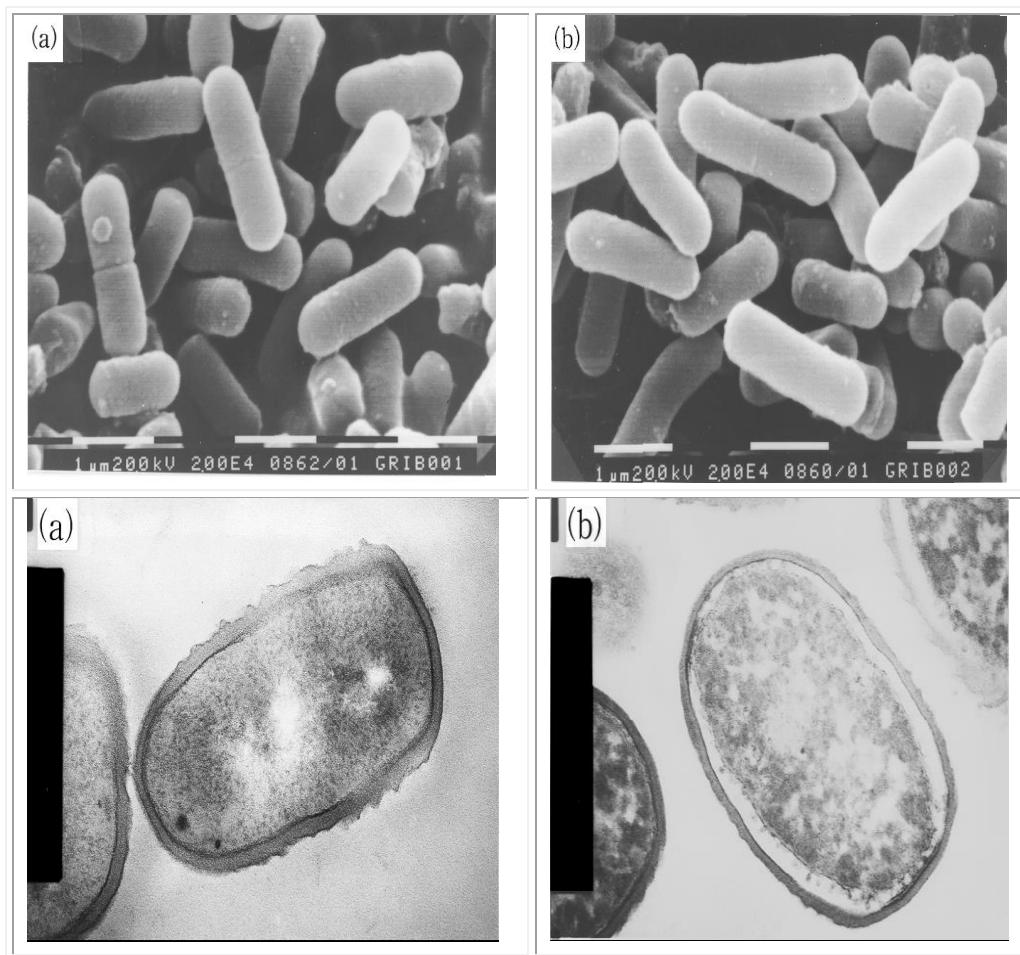


Figure 2.24 SEMs (above) and TEMs (below) of *L. plantarum* cells untreated (a) and treated (b) with CO₂ at 6865 kPa, 30°C for 1 hour (Hong and Pyun, 1999)

White et al. (2006) found that although the surface of *S. typhimurium* was unaffected by P CO₂ treatment (9652.6 kPa, 35°C), the internal structure showed some changes. The density of cytoplasm was reduced and the cytoplasmic membranes appeared rough. Kim et

al. (2007a), (2007b) also observed that the SEM images of treated cells (*E. coli* O157:H7 and *S. typhimurium*) showed no signs of rupture or damage (Figure 2.11Figure 2.12Figure 2.14 and Figure 2.15); whereas TEM images showed that cytoplasm was segregated from plasma membrane and coagulated. The presence of blank areas was linked to the extraction of intracellular substances, and this was supported by an increase in UV absorbance of the treated cell suspension. In a subsequent study, Kim et al. (2008) found that SEMs of *L. monocytogenes* treated with P CO₂ (10000 kPa, 35°C for 30 min) exhibited no obvious change in cell morphology. TEMs showed minor differences in cell anatomy such as a few empty spaces and agglomerated cytoplasm, which might be due to the extraction of intracellular substances. This was verified through enhanced UV absorbance of the treated cell suspension at 260 and 280 nm.

Later on, Cui and Jahng (2006) discovered that *E. coli* suspended in distilled water was ruptured as a result of P CO₂ treatment at subcritical pressure (300-600 kPa) for 20 min. Song et al. (1992) observed that the gradual increase in CO₂ concentration (0-10%) and temperature (34-42°C) intensified the change in morphology of *L. plantarum*, although the cells remained intact.

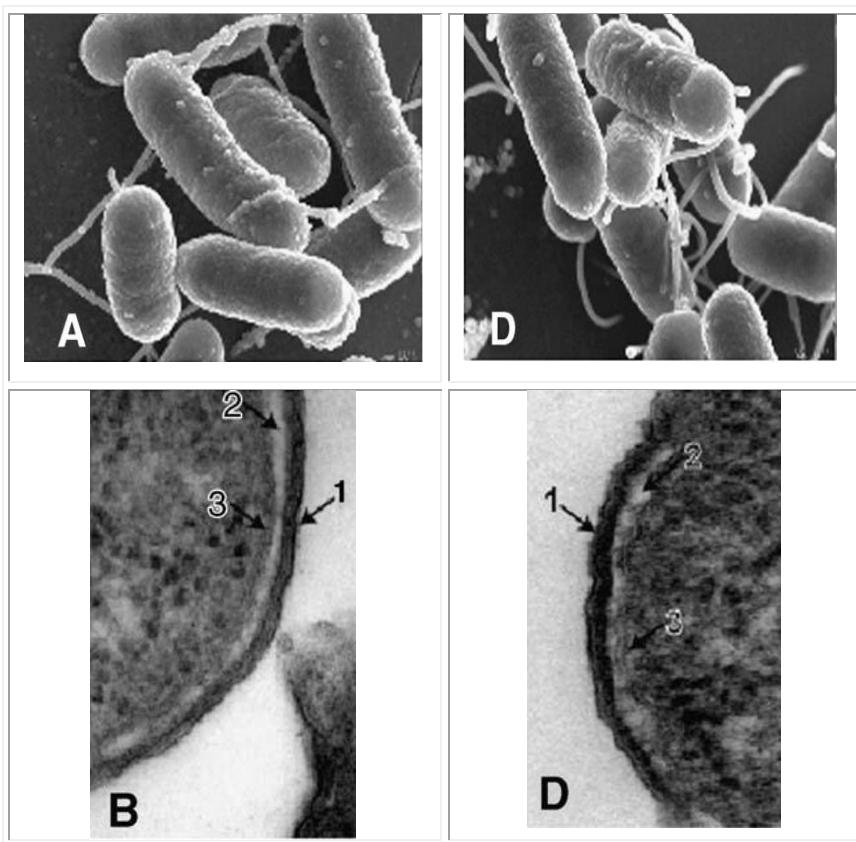


Figure 2.25 SEMs (top) and TEMs (Bottom) of *S. typhimurium*. Untreated (left) and treated (right). (Arrows # 1 cell wall, Arrow # 2 periplasmic space and Arrow # 3 lipid bilayers)

(Cui and Jahng, 2006)

Gradual accumulation of experimental corroboration ruling out the sole involvement of ‘mechanical cell rupture’ in microbial inactivation diverted researchers interest towards investigating the influence of P CO₂ on biochemical reactions and microbial cell components. This led to the development of a new theory for the antimicrobial action of P CO₂ which was termed as physiological inactivation.

2.9.2 Physiological inactivation

Physiological inactivation of microbiological systems as a consequence of P CO₂ is a complex process but can be summarised in a series of interrelated steps (listed below) which take place concurrently within and outside the microbial cells.

1. Dissolution of P CO₂ in extracellular aqueous medium.
2. Alteration of physical and chemical structure of cell membrane.
3. Intracellular acidification.
4. Loss of enzyme activity.

5. Inhibition of cellular metabolism due to molecular CO_2 and bicarbonate (HCO_3^-) ions.
6. Disturbance of intracellular electrolyte balance.
7. Extraction of important intracellular substances (Garcia-Gonzalez et al., 2007).

Figure 2.26 is a schematic representation of the physiological inactivation of bacteria by P CO_2 . The details of this process are described step by step to give a better understanding of its lethal effect on microorganisms.

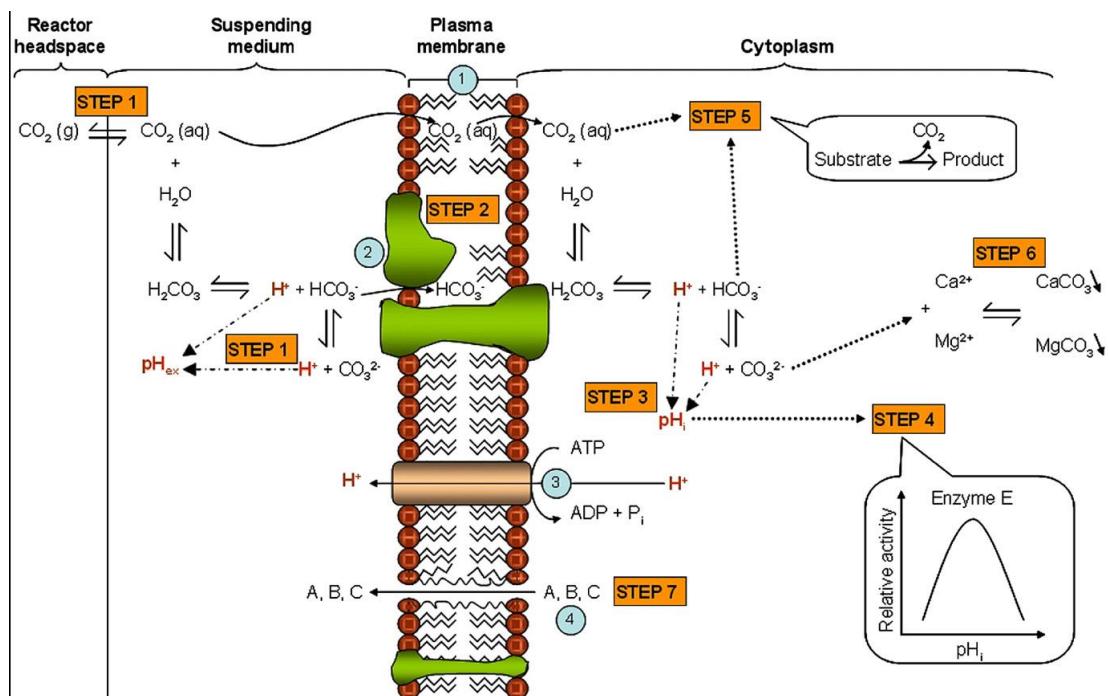


Figure 2.26 A schematic diagram of how P CO_2 may exert its lethal action on bacteria. 1. A phospholipid bilayer 2. Integral membrane proteins 3. A plasma membrane H^+ ATPase 4. Intracellular substances
(Garcia-Gonzalez et al., 2007)

2.9.2.1 Dissolution of P CO_2 in extracellular aqueous medium

CO_2 is slightly soluble in water at room temperature but there is a significant increase in its solubility in water as well as in lipids with an increase in temperature and/or pressure (Hong et al., 1999). These hydro and lipophilic properties of CO_2 make it an ideal solvent of choice for sterilisation (Isenschmid et al., 1995). When the microorganisms in various treatment media (Foods, Broths) are subjected to P CO_2 treatment, CO_2 dissolves in the

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aqueous phase of the treatment medium to produce carbonic acid (H_2CO_3) which eventually dissociates into bicarbonate H_2CO_3^- , carbonate CO_3^{2-} and hydrogen H^+ ions (as shown in the following equations) (Damar and Balaban, 2006) resulting in the drop of pH of reaction medium.



This pH drop could be considered as one of the causes of inactivation and elimination of the microbial resistance to P CO_2 treatment (Isenschmid et al., 1995, Hong et al., 1999). Some authors have linked this pH reduction with the inhibition of microbial growth (Daniels et al., 1985). However, it cannot be considered as the only lethal effect of P CO_2 on microorganisms (Haas et al., 1989, Wei et al., 1991, Meyssami et al., 1992). It was suggested by Lin et al. (1993) that acidification of the treatment medium leads to an increase in cell permeability that enhances the intracellular penetration of CO_2 which can then directly interfere with the biochemical systems.

2.9.2.2 Alteration of physical and chemical structure of cell membrane

Due to its lipophilic nature, solubility of CO_2 is higher in organic solvents as compared to water. For instance it is 4.5 times more soluble in ethanol and 9.9 times in acetone as compared to water. This explains the rapid diffusion of CO_2 across plasma membrane and its subsequent accumulation in the internal layer of cell membrane, which is rich in phospholipids (Isenschmid et al., 1995). Spilimbergo et al. (2002) were able to theoretically confirm the high affinity between CO_2 and the plasma membrane by calculating the amount of CO_2 dissolved in plasma membrane phospholipids, specifically phosphoditietanol amines and phosphatilglycerol. Accumulation of molecular CO_2 in the plasma membrane increases its fluidity making it easily penetrable. This accumulated CO_2 has a strong potential to damage the plasma membrane both structurally and functionally, a phenomenon referred to as the 'anaesthesia effect' that causes the order loss of the lipid chain of cell membrane (Isenschmid et al., 1995, Garcia-Gonzalez et al., 2007).

2.9.2.3 Intracellular acidification

As a consequence of the increase in plasma membrane permeability due to 'anesthesia effect', there is a strong possibility of intracellular accumulation of a large number of CO₂ molecules which consequently lower the intracellular pH to an extent which is out of the range of the buffering capacity of cytoplasm, resulting in the collapse of pH gradient across the membrane (Hutkins and Nannen, 1993).

Spilimbergo et al. (2005) determined the intracellular pH of *Bacillus subtilis* cells subjected to P CO₂ treatment at 8000 kPa, 30°C for 5 min using flow cytometry aided by a fluorescent probe while the extracellular pH was calculated theoretically by means of the SAFT equation. Both intra and extracellular pH values were estimated as 3.3 which is in agreement with the assumption that microbial cells lose the ability to maintain a pH gradient upon P CO₂ treatment.

2.9.2.4 Loss of enzyme activity

In addition to the detrimental effect on cell structure and function, intracellular pH decrease specifically targets enzyme activity. Lowering of intracellular pH results in inhibition and/or inactivation of key enzymes necessary for carrying out biochemical activities of cell for maintaining life, such as glycolysis, active transport of ions, amino acids and peptide transport, and proton translocation (Hutkins and Nannen, 1993). Ishikawa et al. (1995a) achieved complete inactivation of lipase and alkaline protease enzymes subjected to P CO₂ treatment at 15000 kPa and 35°C. It was observed that alkaline protease was inactivated as a consequence of a pH drop by dissolved CO₂ whereas the mechanism of inactivation of lipase was not confirmed.

Hong and Pyun (2001) investigated the physiological changes in *Lactobacillus plantarum* cells following P CO₂ treatment. Surprisingly, no change was observed in the specific activity of membrane bound H⁺ ATPase even though cell viability was reduced by several log units. It was therefore deduced that membrane bound H⁺ ATPase was not involved in microbial inactivation by P CO₂ treatment. Furthermore, among the 13 enzymes detected in untreated cells, some of them lost their activities significantly while others remain unaffected. The phenomenon of selective enzyme inhibition was also confirmed by Ballestra et al. (1996) who linked intracellular acidification to the precipitation of enzymes with acidic isoelectric points while the enzymes with basic isoelectric points remain unaffected. Recently, however, Kim et al. (2007b) found that only 4 out of 9 enzymes

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detected in untreated *Salmonella typhimurium* lost their activity following P CO₂ treatment at 10000 kPa and 35°C. In spite of this, the inactivation of key enzymes as a consequence of cytoplasmic acidification due to intracellular accumulation of CO₂ still remains a strong explanation for the microbicidal property of P CO₂.

2.9.2.5 Inhibition of cell metabolism due to molecular CO₂ and bicarbonate ions (HCO³⁻)
Cell metabolism does not depend solely on enzymatic activity; the intracellular concentration of co-factors, substrates and products also plays a vital role. Intracellular concentration of HCO₃⁻ ion which is controlled by internal pH buffering plays an important role in controlling cell metabolism either by stimulating or inhibiting a specific enzyme. The mechanism of action of HCO₃⁻ ion and dissolved (unhydrated) CO₂ on the cell membrane is due to their influence on carboxylation and decarboxylation reactions (Jones and Greenfield, 1982). Since CO₂ plays a dual role of either a substrate in carboxylation reactions or a product in decarboxylation reactions, the ratio of dissolved CO₂ to HCO₃⁻ ion influences the function of carboxylases. Both are utilised without distinction in the carboxylation reactions which are important for synthesizing amino acids and nucleic acids thus indirectly influencing the protein synthesis. On the other hand, CO₂ is the product of almost all decarboxylation reactions thus its high concentration inhibits the decarboxylation enzymes (Jones and Greenfield, 1982); however the exact mechanism of inhibition still remains unravelled. Hence the inhibitory effect of CO₂ and HCO₃⁻ ion on microbial cell metabolism can be linked to their influence on the function of carboxylases and decarboxylases.

2.9.2.6 Disturbance of intracellular electrolyte balance

Accumulation of P CO₂ in bacterial cells may pose a serious threat to their physiological activities by converting bicarbonate ions (HCO₃⁻) to carbonate ions (CO₃²⁻) which could result in the intracellular precipitation of calcium and magnesium ions (Ca⁺ and Mg⁺). Besides regulating a large number of cellular activities, these inorganic electrolytes play a vital role in maintaining the osmotic balance between cells and their external environment, thus their precipitation can be lethal for microbial cells. As a result of intracellular precipitation of these inorganic ions, certain Ca⁺ and Mg⁺ soluble proteins could also be precipitated depending on the binding site of the ion and the chemical structure of protein, thus lethally disturbing the biological system (Lin et al., 1993). Moreover, the collapse of proton motive force across the plasma membrane as a consequence of lowering of

extracellular pH can also disturb the cytoplasmic Ca^+ balance (Garcia-Gonzalez et al., 2007). Hong and Pyun (2001) observed that K^+ and Mg^+ ions were released from microbial cells treated with P CO_2 at 7000 kPa, 30°C for 10 minutes, which might disturb the intracellular concentration of these ions subsequently affecting biological functions and eventually contributing to the microbial inactivation.

2.9.2.7 Extraction of important intracellular substances

Due to its lipo and hydrophilic nature, CO_2 has a high solvating power, with the help of which it can extract important intracellular substances such as phospholipids and hydrophobic compounds (Kamihira et al., 1987, Lin et al., 1993, Lin, 1994). This extraction is enhanced by the rapid release of applied pressure which causes the extraction of intracellular materials from biological systems into the extracellular environment. It has also been suggested that the inactivation rate could be improved by repeated pressurisation and depressurisation to enhance the penetration and accumulation of CO_2 in microbial cells in the former stage followed by the extraction of intracellular substances in the later (Lin et al., 1992, Lin et al., 1993). The deformation of cell walls of *E. coli* subjected to P CO_2 at 5000 kPa and 35°C was observed by Ballestra et al., (1996) which supports the theory of extraction of intracellular substances. Shortly afterwards, Hong and Pyun (1999) further verified this phenomenon by the TEM study of *Lactobacillus plantarum* cells treated with P CO_2 at 6865 kPa, 30°C for 1 hour. The TEMs depicted peculiar features such as enlarged periplasmic space between the cell wall and cytoplasmic membrane and empty spaces in the cytoplasm, which pointed towards the possible leakage of plasma membrane and/or cytoplasm (shown in Figure 2.24)

In their later studies Hong and Pyun (2001) observed irreversible changes in cell membranes including release of intracellular ions (such as K^+ and Mg^+ ions), leakage of UV absorbing substances and uptake of Phloxine B dye (which intact viable cells are able actively to exclude) by *Lactobacillus plantarum* cells treated with P CO_2 at 7000 kPa, 30°C for 10 min. Kim et al. (2007a, 2007b) further confirmed this hypothesis by observing the increased UV absorbance by the supernatant of *E. coli* and *Salmonella* subjected to P CO_2 and cytoplasmic disruption, membrane deformation and agglomeration of intracellular substances depicted in the TEMs of treated cells.

Thus it can be concluded that microbial inactivation is not the consequence of only one of the above described steps. In fact, these steps take place synergistically to account for the

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antimicrobial action of P CO₂. However, depending on the treatment conditions (pressure, temperature, exposure time, nature of suspension medium etc.), some steps might dominate the others.

2.10 Conclusions and opportunities for further research

Literature review has identified some key areas where further research and interaction of AD and CO₂ pressurisation can be beneficial. An overview of AD and various stages involved in it are given in section 2.1. Whereas, section 2.2 identifies the role of AD in waste water management and the potential advantages and disadvantages of AD in waste water treatment. Pros and cons of land application of SS are discussed in section 2.3. This section highlights the beneficial aspects of land application of SS on one hand while on the other; it identifies the threats associated with it. Some disease outbreaks that occurred in past due to the pathogens in animal manure or SS applied on agricultural land have also been compiled. Bacterial pathogens and the importance of faecal indicator bacteria have been described in section 2.4. Next section (2.5) overviews the background situation for the implementation of SSM in the UK. The microbiological standards defined in SSM and 40 CFR part 503 for safe land recycling of sludge have been summarised. Discussed afterwards in detail (section 2.6) are achievements, advantages and disadvantages of various pre-treatment techniques which have been used to shorten the rate limiting step of AD i.e. hydrolysis and enhance the biogas production by solubilising the cell walls of sludge microorganisms. It is noteworthy that most of the researchers were interested in improvement of biogas production not in the inactivation of pathogens or sanitation of digestate. A novel technique ‘Bug Buster’ has been claimed to increase the biogas production by pressurising the SS with biogas prior to AD. This technique was found to be linked with the extensively studied microbial inactivation by P CO₂ in food industry. History and sequential development of P CO₂ technique has been discussed in detail in section 2.7. One point is quite evident that majority of the researchers have looked in to the effect of SC CO₂ or CO₂ at very high pressure for pathogen inactivation in food products. Factors which affect the efficiency of P CO₂ for microbial inactivation have been discussed in section 2.8. Interrelationship of these factors and the extent of their influence on microbial inactivation have been assessed. Different theories explaining the mechanism of action of P CO₂ for microbial inactivation have been explained in section 2.9. Careful analysis of these theories depicts that so far there has been no agreement on a

common mechanism of action. Although, a review study (Garcia-Gonzalez et al., 2007) has proposed a hypothetical inactivation mechanism, still lots of work needs to be done to unravel this mystery.

Present research was therefore intended to address the issue of pathogens in Co-Settled Sewage sludge by P CO₂ pre-treatment before AD, since this technology had already shown promising results in food industry. However, probably for the first time, this technique was applied for the inactivation of pathogens in SS at low pressure range (1500-2800 kPa). Since it had already been reported that the mechanism of action of super and sub critical CO₂ is the same with the exception that the inactivation is very fast in case of SC CO₂ while it might take more time to achieve the same level of inactivation using sub critical CO₂ (Lin et al., 1992 a). Initially, inactivation of FIB in pure culture was probed using P CO₂. Later on, the influence of P CO₂ pre-treatment on biogas potential of PSS in CSTR semi continuous AD and survival of FIB was studied. Mechanism of microbial inactivation was investigated using electron microscopy, enzymatic kits and UV visible spectroscopy.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and Glassware

All the chemicals used in this study were obtained from Fisher Scientific (Loughborough, UK) except where notified. The reagents were prepared using De Ionised (DI) water (Elix electrodionisation system, 97% ionic rejection, Millipore Corporation, UK) according to the pack instructions.

All reagents and apparatus used in microbiological analysis were sterilised in an autoclave (Rodwell scientific instruments. Essex, UK) at 121°C, 15 PSI for 15 minutes.

The glassware was washed with alkaline laboratory detergent (Fisher Scientific, UK) and tap water followed by rinsing with DI water and dried in a drying oven.

3.1.2 Microorganisms

The strain of *Escherichia coli* used in this study was isolated from Co-settled Sewage Sludge (CSS) obtained from Millbrook Wastewater Treatment Works (WWTW), (Southern water) Southampton, UK.

Salmonella enterica (ATCC 14028) was obtained from National Collection of Industrial Marine and Food Bacteria (NCIMB) Aberdeen, Scotland UK.

3.1.3 Carbon dioxide

Compressed carbon dioxide (99% purity) was obtained from BOC gases, UK.

3.1.4 CO₂ pressurisation unit- Design and Operation

Two CO₂ pressurisation units were used both could be pressurised to 2800 kPa, one had a maximum working volume of 100 ml while the other one had 1000 ml.

Pure cultures of bacteria were treated with P CO₂ in the smaller vessel by placing 2-3 ml of samples in a stainless steel sampling cup suspended in the vessel (Startec group, Kent, UK) (Figure 3.1). The vessel was then pressurised to a maximum pressure of 2800 kPa by connecting to the purpose manufactured gas pressurisation system (filling station) as shown in Figure 3.2. The filling station was connected to a CO₂ cylinder (BOC gases, UK) via a high pressure regulator and the pressurisation operation only took a few seconds.

Materials and Methods

Depressurisation of the vessel also only took only a few seconds and was by means of depressing a pressure relief valve/lever.



Figure 3.1 Photograph of the small capacity pressurisation unit and filling station

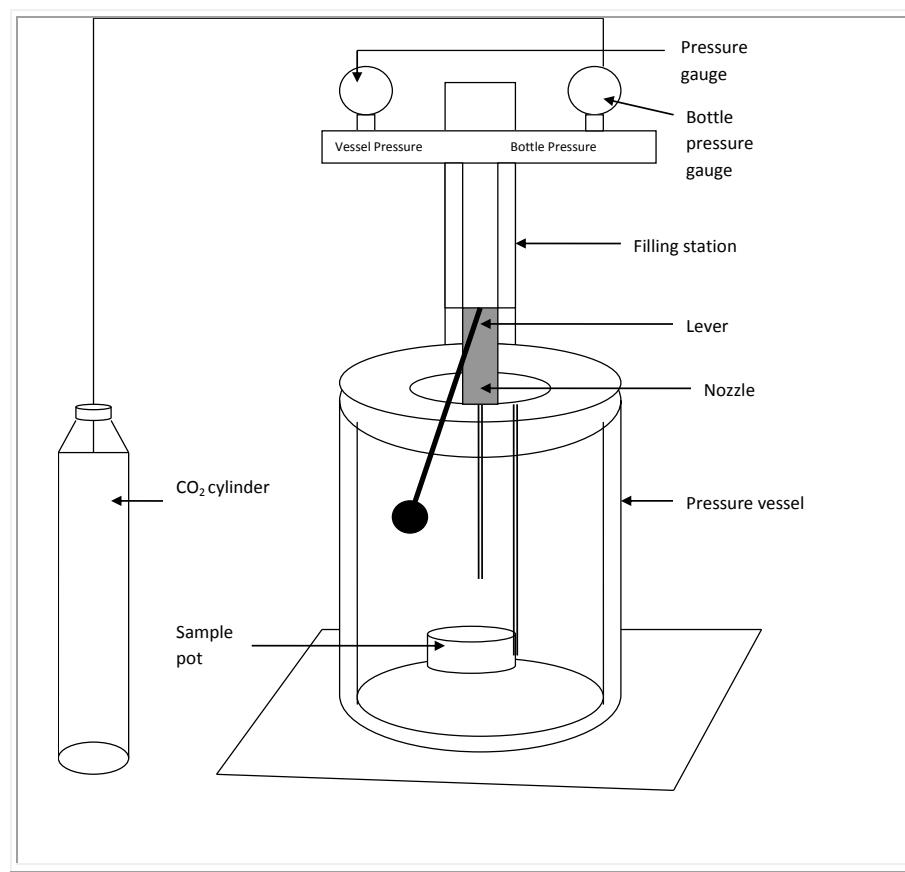


Figure 3.2 Schematic of CO₂ pressurisation unit

P CO₂ pre-treatment of CSS was carried out in a pressure vessel made of stainless steel with a capacity of 2.2 litre (l) (working volume 1 l) and maximum pressure limit of 2800 kPa (Prosep filter systems Ltd. West Yorkshire, UK, Model No. 530110XN10B10V). The

Materials and Methods

pressure vessel was mounted in a wooden casing to keep it upright (Figure 3.3 a) and then placed on an orbital shaker (Stuart scientific Ltd. Staffordshire, UK) which was operated at 100 RPM (Figure 3.3 b).

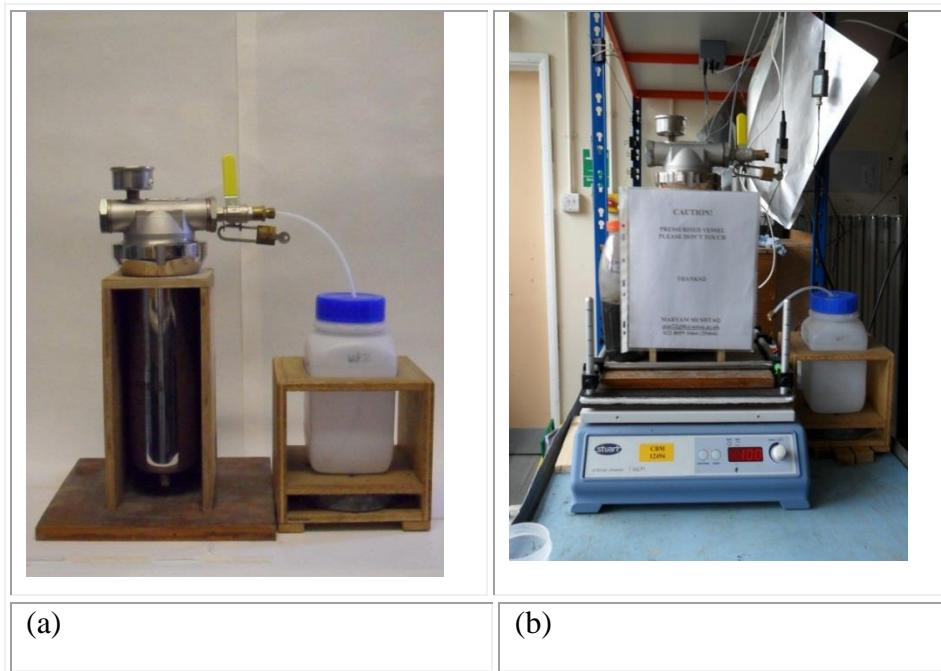


Figure 3.3 P CO₂ treatment vessel in wooden casing (a) and on the orbital shaker (b)

The vessel was fitted with a pressure gauge and an inlet/outlet valve which could either be connected to the CO₂ cylinder for pressurisation or to the gas release tubing for pressure release. This tube passed into a plastic bottle with a hole in lid so as to contain any aerosols. Usually, 1 litre of CSS was treated as a single batch, a schematic of the equipment is shown in Figure 3.4.

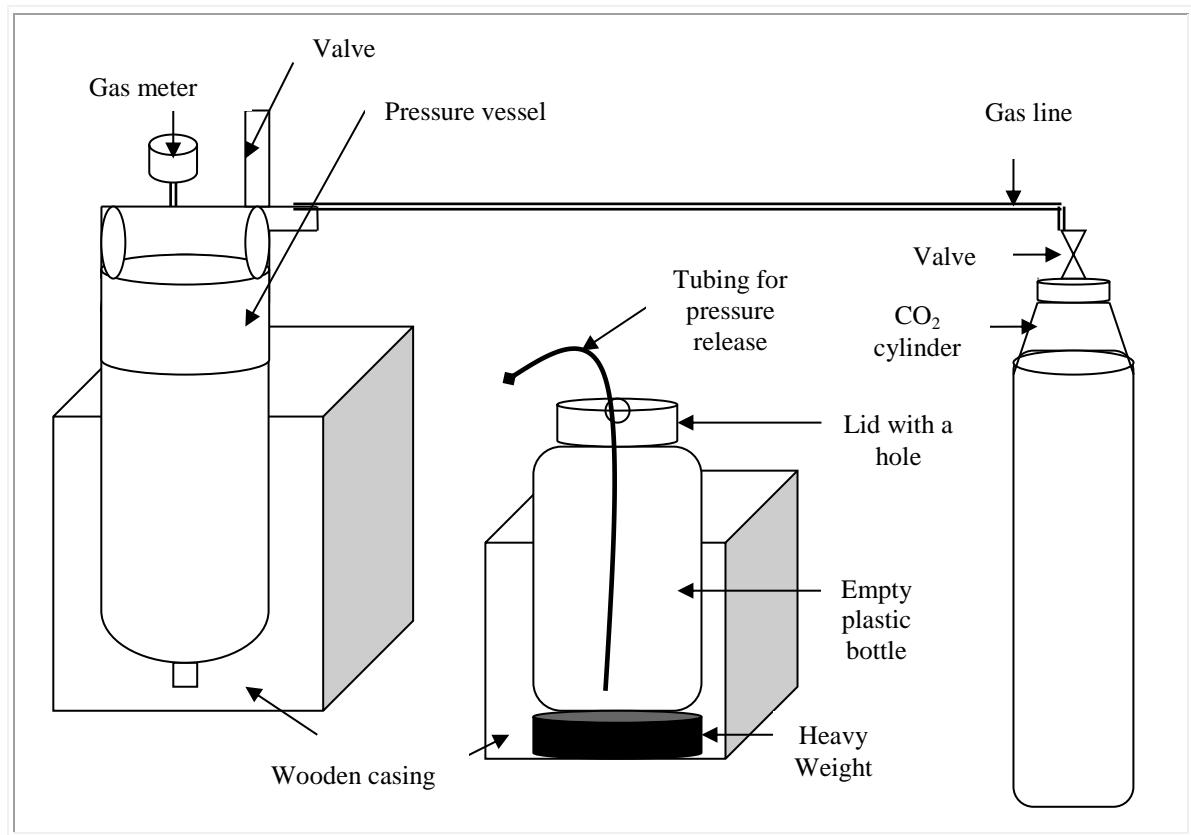


Figure 3.4 Schematic of the larger scale CO₂ pressurisation system

3.1.5 Inoculum and feed stock

Sludge taken from an anaerobic digester at Millbrook Wastewater Treatment Plant (WwTP), Southampton, UK was used as inoculum for anaerobic digesters operated in both batch and continuous mode. The inoculum was used on the day of collection and when used in the batch (BMP) it was first sieved in order to remove any large size particles.

Co-settled sewage sludge (CSS) was used as the feed stock for CSTR experiments and was collected every fortnight and stored at 4°C. Each batch collected was analysed on the day of collection for Total and Volatile solids (TS & VS) and Total faecal coliform and *E. coli* count using Most Probable Number (MPN) method.

3.1.6 Anaerobic digesters- Design and Operation

3.1.6.1 Continuous Stirred Tank Reactors (CSTR)

a. Description.

Materials and Methods

The digesters had a total volume of 5 litres and were operated at a working volume of 4 litres. A schematic drawing of a pair of digesters is shown in Figure 3.5. The digesters were constructed in PVC with a top flange to which a top plate was secured using stainless steel bolts and wing nuts. A gas tight seal between the top plate and the digester flange was maintained using a closed pore neoprene gasket. The top plate was fitted with a gas outlet connector and a feed port sealed with a rubber bung. On the top plate a DC motor was mounted which coupled to the digester stirrer through a draught tube water gas seal, the draught tube itself being secured in a gas tight compression seal. Digestate was removed from the digester via a 15mm diameter outlet port at the base of the digester. The contents of the digesters were continuously stirred by means of an asymmetric stirrer at 40rpm. Temperature was maintained at $35^{\circ}\text{C} \pm 0.5$; by water circulating through an external heating coil that surrounded the digesters. When assembled, and before filling, each digester was tested for gas leaks by applying a positive pressure to the digester and submerging in water to ensure there was no gas escape when all ports were sealed. The digesters were connected to gas counters, which continuously measured gas production throughout the digestion period; the gas counters operated by the alternate filling and discharging of a calibrated cell which logged each discharge via a labjack (labjack ltd) computer interface. (Walker et al, 2009). The calibration of each gas counter was checked twice a week by attaching a 10-litre gas collection bag (Tedlar SKC 232, SKC Ltd, Blandford Forum, UK) to the gas vent of gas counter.

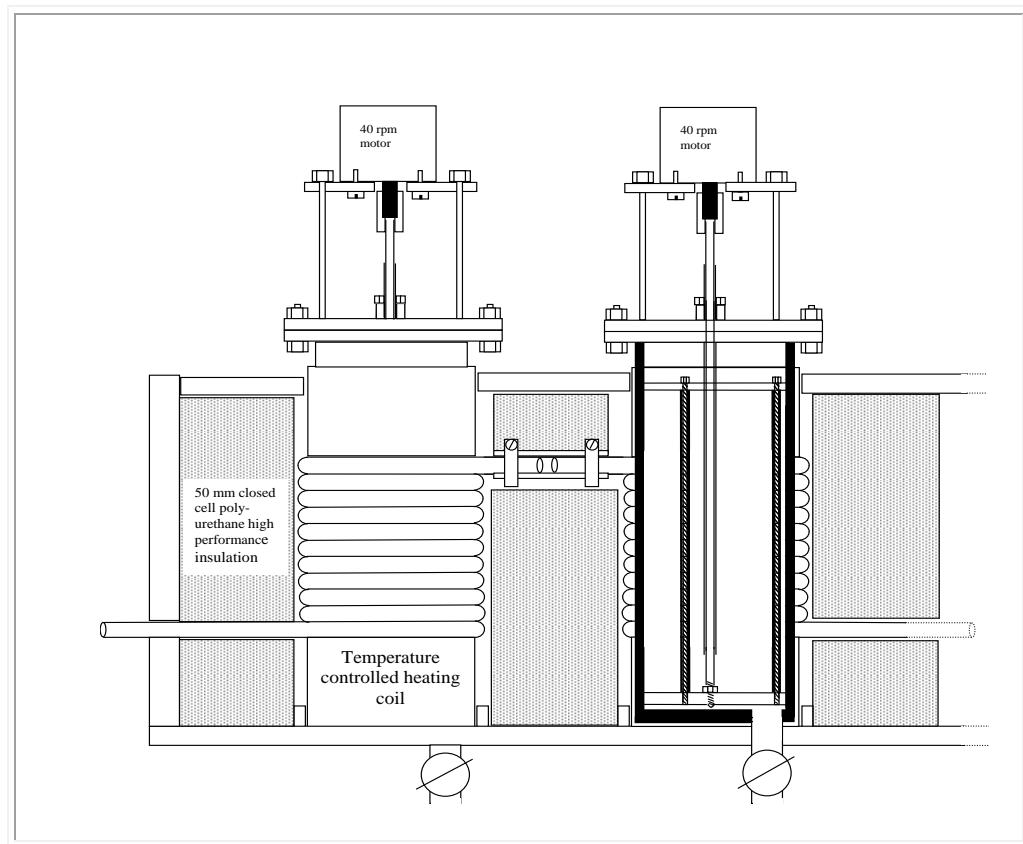


Figure 3.5 Schematic of 5 l CSTR digester

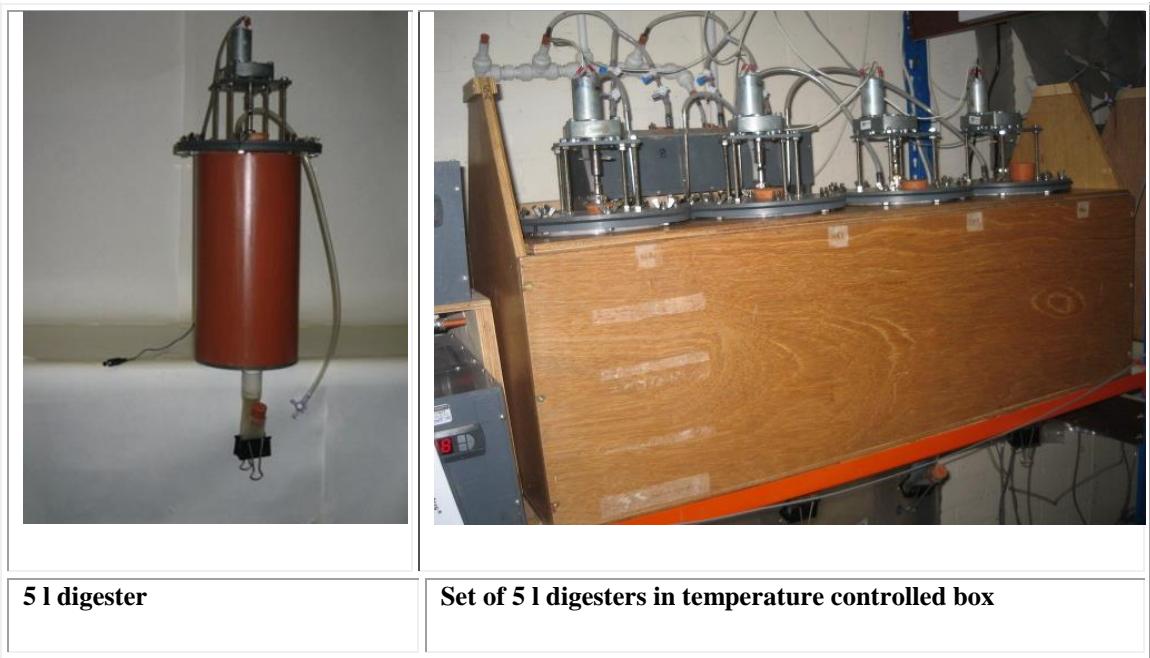


Figure 3.6. Lab scale digesters used in the research

The volume of biogas collected was then measured by a water displacement gasometer (Walker et al., 2009). Total gas volume was corrected to allow for the amount of sample removed and expressed at standard temperature and pressure (STP) of 0°C and

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101.325kPa. Biogas composition was analysed by taking 10 ml gas samples from the Tedlar bag in a syringe using a gas chromatograph.

b. Operation

The digesters were operated in a semi continuous mode i.e. fed daily with a specific amount of feedstock and digestate removed to maintain a constant volume in the digesters. The organic loading rate (OLR) was determined according to equation 3.1.

$$OLR = \frac{mVS_{substrate}}{V_{reactor}} \text{ (Unit: g VS l}^{-1}\text{d}^{-1}\text{)} \quad \text{Equation 3.1}$$

Where,

m was the mass of substrate daily added to the reactor (g d^{-1})

$VS_{substrate}$ was the volatile solid content of feedstock (% wet weight)

$V_{reactor}$ was the volume of reactor (l)

The VS content of each new batch of feedstock was measured and then adjusted to a constant by the addition of water, in this way the volume of feed stock added to the digesters was kept constant. This maintained a constant Hydraulic Retention Time (HRT) as expressed in equation 3.2.

$$HRT = \frac{V_{reactor}}{Q} \text{ (Unit: Days)} \quad \text{Equation 3.2}$$

Where:

$V_{reactor}$ was the working volume of each reactor (ml)

Q was the daily flow of material (substrate added and digestate removed) through the reactor (ml d^{-1})

The amount of substrate and digestate was measured in g but for ease of calculation it was assumed that both the substrate and digestate had a specific gravity of 1.0. Therefore, 1g of CSS and digestate was considered to be equivalent to 1ml.

c. Performance

The performance of bioreactors was monitored in terms of specific biogas and methane production and VS destruction which were calculated using equations 3.3, 3.4 and 3.5.

$$\text{Specific biogas production} = \frac{V_{biogas}}{OLR \times V_{reactor}} \text{ (Unit: } 1 \text{ g}^{-1} \text{ VS)} \quad \text{Equation 3.3}$$

Where:

V_{biogas} was the volume of biogas produced daily ($l d^{-1}$)

OLR was the organic loading rate ($g VS l^{-1} d^{-1}$)

$V_{reactor}$ was the volume of reactor (l)

$$\text{Specific methane production} = \frac{V_{CH_4}}{OLR \times V_{reactor}} \text{ (Unit: } 1 \text{ g}^{-1} \text{ VS)} \quad \text{Equation 3.4}$$

Where:

V_{CH_4} was the volume of methane produced daily ($l d^{-1}$)

OLR was the organic loading rate ($g VS l^{-1} d^{-1}$)

$V_{reactor}$ was the volume of reactor (l)

The destruction of volatile solids was calculated as follows:

$$VS_{reduction} = \frac{Feed(g) \times VS_{feed} - Digestate(g) \times VS_{digestate}}{Feed(g) \times VS_{feed}} \quad \text{Equation 3.5}$$

In making the calculations, the weight of biogas was considered as well as the weight of digestate removed daily. For this purpose, it was assumed that biogas consisted of 50% CH_4 and 50% CO_2 with the weight of 1 mole of biogas taken as 1.38 gram as in equation 3.6.

$$\text{Theoretical amount of digestate removed} = Feed_{added}(g) - Biogas_{removed}(g) \quad \text{Equation 3.6}$$

Finally, VS destruction was calculated using equation 3.7 and then expressed as a %

$$VS \text{ destruction} = \frac{VS_{added}(g) - VS_{removed}(g)}{VS_{added}(g)} \quad \text{Equation 3.7}$$

3.1.6.2 Batch (Biochemical Methane Potential) test

a. *Test equipment*

The BMP apparatus consisted of 29 digesters maintained in a temperature controlled water bath at $35^\circ C$ with each one connected to the liquid displacement gasometers filled with a

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barrier solution. Each digester was in fact a glass bottle with a capacity of 550 ml and a working volume of 400 ml sealed with a rubber bung through which a stainless steel metal tube was inserted. This was connected by PVC tubing leading to a sealed acrylic cylinder with graduated markings and partially immersed in a trough of acidified saline (75% saturated solution of Sodium Chloride (NaCl), pH 2 designed to minimise the solubility of CO₂) with a constant head overflow. Gas volume was corrected to a STP of 0°C and 101.325 kPa (Walker et al., 2009). Biogas samples were collected from the gas collection cylinder via a 3 way valve and a syringe and analysed for gas composition (3.2.2.3). The gas collection tubes were refilled with the barrier solution when required but never less frequently than every 7 days, this was done using a vacuum pump connected to the 3 way valve at the top of each cylinder.

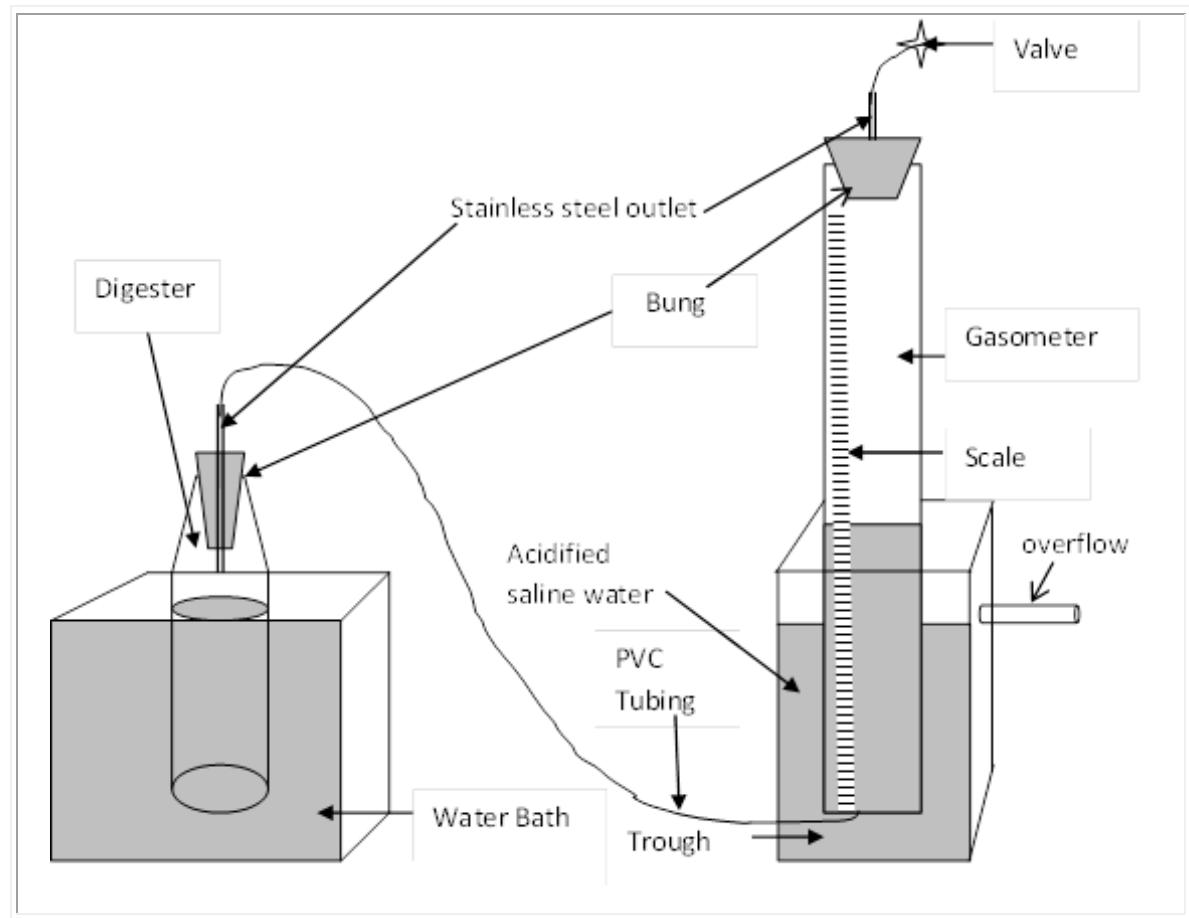


Figure 3.7 Schematic diagram of BMP apparatus

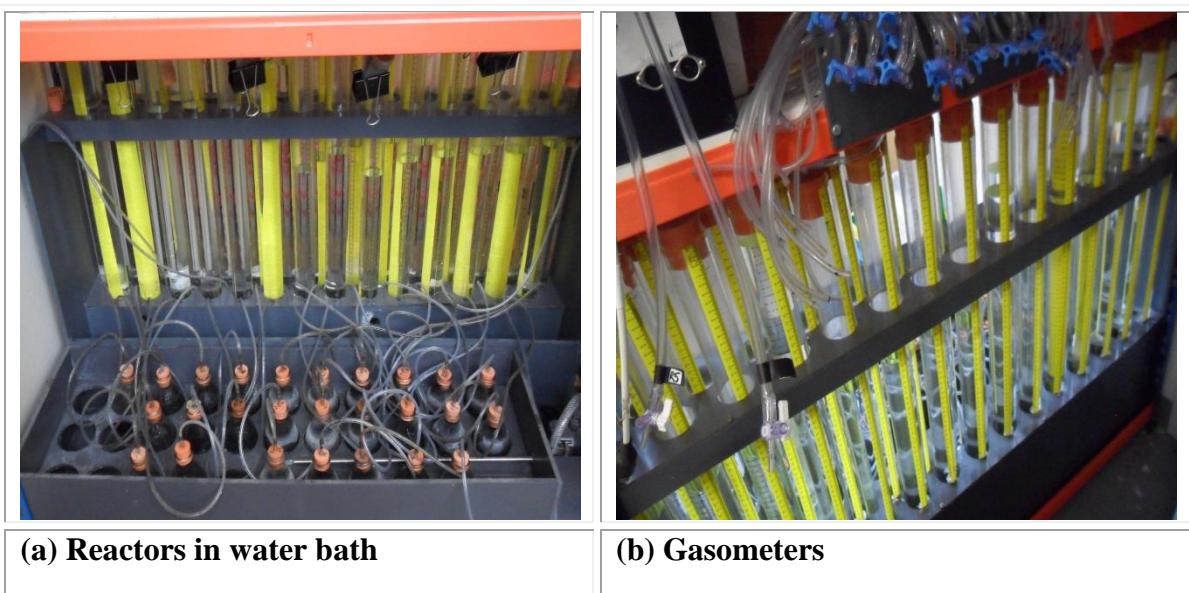


Figure 3.8 Photographs of BMP apparatus

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b. Operation

Before starting the test, the BMP apparatus was checked for leaks by filling the trough in which the gasometers were mounted, with the acidified saline solution and pulling it up in the gasometer and making sure that the level had not dropped in a 48 hour period. On the day of test, fresh inoculum and substrate were collected (3.1.5). Control reactors were filled with 400 ml inoculum whereas; the test reactors were filled with a mixture of inoculum and substrate in a ratio according to the experimental design. Temperature, pressure and level of barrier solution in the gasometers was noted during working hours every hour in the first week, every 2-3 Hrs in the 2nd and 3rd week and twice a day over the remaining period of the test. TS and VS of digestate was determined at the end of BMP test. The results were expressed in terms of cumulative biogas and specific methane yield per gram of substrate added and the VS destruction.

3.2 Analytical methods

3.2.1 Microbiological

3.2.1.1 Preparation of culture media

The microbiological culture media were obtained from Oxoid Ltd. Basingstoke, UK. Majority of them were in the form of powder and the pack provided guidance for preparation of medium. Required amount of culture media were prepared by dissolving the recommended amount of medium in DI water followed by sterilisation in an autoclave (Rodwell scientific instruments. Essex, UK) at 121°C, 15 PSI for 15 minutes. Sterilised media was brought to room temperature and either used or stored in a refrigerator.

Agar media was prepared according to the pack instructions in Erlenmeyer flask and sterilised by autoclaving. Autoclaved medium was cooled to 60°C and then poured in disposable triple vent petri plates (Sterilin UK) and cooled till agar was solidified. The petri dishes were then stored upside down in the incubator at 37°C for 24 Hrs in order to dry the agar surface properly. After 24 Hrs, the petri dishes were used as required and the surplus were stored in the refrigerator.

3.2.1.2 Isolation and identification of *E. coli* from CSS

E. coli was isolated from freshly collected CSS. The procedure was modified from ‘The isolation and enumeration of *E. coli* by a multiple tube most probable number

technique' (Standing committee of analysts, 2003). All the chemicals and reagents were the same except the selective agar used was Mac Conkey's which was used to identify and confirm *E. coli* by the characteristic colony shape and colour of *E. coli* on this medium. The detailed procedure was as follows;

1 g of CSS was added to 99 ml of Maximum Recovery Diluent (MRD) and transferred to a sterile stomacher bag followed by homogenisation in a stomacher (Lab blender 400 – Seward Medical London, UK) for 2 min. 1 ml of this homogenised suspension was diluted 5 times in 9 ml of MRD such that each dilution had 0.01 g, 0.001g, 0.0001g, 0.00001g and 0.000001g of CSS. These dilutions were denoted as 10^2 , 10^3 , 10^4 , 10^5 and 10^6 respectively. 1ml from each dilution was inoculated into each of 5 test tubes containing 10 ml of Lauryl Tryptose Broth with Bromocresol Purple (LTB+BCP) as an indicator of acid production. The inoculated test tubes were placed in an incubator (Leec compact incubator, Nottingham England) at 37°C for 18-24 Hrs after which they were examined for acid production (change of the bromocresol indicator from purple to yellow). All the tubes showing colour change were considered presumptively positive for *E. coli*. After a further incubation period of 24 \pm 2 Hrs, all positive test tubes were sub-cultured into: 10 ml Brilliant Green Bile Broth (BGBB); and 10ml of Tryptone Water (TW) both contained in screw capped test tubes. Both sets of confirmatory tubes were then incubated at 44°C for 21 \pm 3 Hrs. At the end of incubation period, BGBB was checked for turbidity and an Indole test was performed on TW. Presence of *E. coli* was confirmed by the turbidity in BGBB and the appearance of a deep red ring in TW upon the addition of Kovac's reagent. A sterile loop was used to transfer a small amount of inoculum from test tubes showing a positive result for *E. coli* onto Mac Conkey agar (MCA) in petri plates. The plates were then incubated at 37°C for 24 Hrs and the presence of *E. coli* was confirmed by the specific 'rose red' colonies which are formed on MCA. Further confirmation was carried out by sub culturing the colonies in TW and repeating the Indole test. This sub-culturing and confirmatory procedure was repeated three times to make sure that the isolated strain was *E. coli*.

3.2.1.3 Revival of *Salmonella enterica*

Salmonella enterica was obtained from NCIMB in the form of a lyophilised culture in a glass ampoule. The glass ampoule was carefully opened by making a file cut and cracking it. After that, about 0.5 ml of Nutrient Broth (NB) was added to the ampoule and mixed. This suspension was then sub-cultured in two Erlenmeyer flasks containing 100 ml of

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NB and thoroughly mixed. The suspension was incubated at 37°C for 24 Hrs in an incubator shaker. At the end of incubation period, 0.1 ml of culture from each flask was sub-cultured in fresh NB and incubated under the same conditions. This process was repeated once more to ensure that the freeze dried culture was actively viable.

3.2.1.4 Storage and maintenance of bacterial strains

E. coli was sub cultured from MCA in TW and then streaked on to NA slants using a sterile inoculating loop. The slants were incubated at 37°C for 24 ± 2 Hrs and after that stored at 4°C in a refrigerator. These slants were used to inoculate TW to grow a culture of *E. coli* in subsequent experiments. Fresh NA slants were prepared every fortnight to retain the viability.

Liquid culture of *S. enterica* prepared by subsequent sub-culturing of lypholysed culture in NB was streaked on to NA slants which were incubated at 37°C for 24 ± 2 Hrs and after that, they were stored in a refrigerator at 4°C. This sub-culturing in NB and then streaking on to NA slants was carried out every fortnight to prevent the loss of viability. These slants were used to inoculate Buffered Peptone Water (BPW) to grow a culture of *S. enterica* for the future experiments.

3.2.1.5 Preparation of bacterial cultures

In order to prepare the bacterial cultures for use in experiments 100 ml of a suitable nutrient medium (TW for *E. coli* and NB for *S. enterica*) in an Erlenmeyer flask was inoculated with a loop full of culture from a NA slant. The flask was then placed in a shaking incubator at 37°C for 24 ± 2 Hrs to grow an OC. After this time, 0.1 ml of OC culture was used to inoculate 100 ml of fresh nutrient medium in Erlenmeyer flask followed by incubation in a shaking incubator at 37°C until the desired cell density was achieved. Fresh cell suspension was prepared in the same way for each experiment. An estimation of the cell density on each occasion was based on the optical density (OD) of the growth medium which was measured using a UV- visible spectrophotometer (Cecil 3000 Series, Cecil Instruments, Cambridge, UK) at a wavelength of 400nm. Hourly increase in OD was compared to a standard graph which was prepared by carrying out a growth curve experiment (4.2) in which a culture was grown and each hour the cell density was determined by spread plate technique and plotted against the OD reading.

3.2.1.6 Enumeration of bacteria

Four different techniques were used for the enumeration of bacteria depending on the material under consideration.

a. *Most Probable Number (MPN) technique*

The MPN technique was used to enumerate *E. coli* both in CSS and pure culture. The procedure for CSS was the same as described in 3.2.1.2 with the exception of MCA inoculation step. At the end of confirmatory tests with BGBB and Indole test, the number of *E. coli* was calculated from MPN table.

In the case of pure culture, there was no need of homogenisation in stomacher. So 1 ml of pure culture was directly added in 9ml MRD (10^1) and then serially diluted seven times in 9ml of MRD (10^8). The last five dilutions (10^4 – 10^8) were then used to inoculate a series of 5 test tubes with 10 ml of LTB+BCP. Since the number of *E. coli* in pure culture was higher than in CSS so the pure culture was diluted more (10^8) than CSS (10^6) in order to achieve count in the range of MPN table.

Enumeration of *Salmonella* in the CSS was carried out using a method modified from the standard procedure for the isolation and enumeration of *Salmonella* species by a multiple tube most probable number technique as described by Neyens and Baeyens (2003b). Unlike the procedure described in this method, the pre-enrichment step was not carried out. The sludge was homogenised in an isotonic diluent and incubated in a non-selective medium to recover environmentally stressed organisms followed by selective enrichment by sub-culturing on a selective agar containing lactose and acidity indicator, the detailed procedure is as follows:

10 g of CSS was added to 90 ml of $\frac{1}{4}$ strength Ringer's Solution (RS), transferred to a stomacher bag and homogenised in a stomacher for 2-5 min. 1ml of this suspension was added to 5x9ml test tubes of Single Strength Rappaport Vassiliadis Enrichment Broth (SS RVEB) (0.1 g CSS per culture tube). At the same time a series of serial dilutions were prepared by adding 1 ml of the initial suspension to 1 x 9ml test tube of $\frac{1}{4}$ strength RS (10^1 dilution). 1 ml of 10^1 dilution was added to 9 ml of $\frac{1}{4}$ strength RS (10^2 dilution) and 1 ml of this dilution was added to 5 x 9 ml test tubes of SS RVEB (0.01 g CSS per culture tube). 1ml of 10^2 dilution was added to 9 ml of $\frac{1}{4}$ strength RS (10^3 dilution) and 1 ml of this dilution was added to 5 x 9 ml test tubes of SS RVEB (0.001 g CSS per culture tube).

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1ml of 10^3 dilution was added to 9 ml of $\frac{1}{4}$ strength RS (10^4 dilution) and 1 ml of this dilution was added to 5 x 9 ml test tubes of SS RVEB (0.0001 g CSS per culture tube). Finally, the four sets of five test tubes containing 0.1 g, 0.01 g, 0.001 g and 0.0001g CSS were incubated at 42°C for 24 - 48 Hrs. Each tube showing turbidity was confirmed for the presence of *Salmonella* by streaking on to Xylose Lysine Desoxycholate (XLD) agar followed by the incubation of petri plates at 37°C for 24 Hrs. The test tubes showing red colonies with black centres were positive for *Salmonella*. Number of *Salmonella* in the sample was determined from the MPN tables on the basis of the number of positive test tubes in each dilution. Result was expressed in the number of *Salmonella* per gram of sludge.

b. Spread plate technique

This technique was performed only with pure culture. The reason for not using this method for enumerating *E. coli* and *Salmonella* in CSS was to avoid the problems caused by the growth of other bacteria on agar petri plates which can interfere and affect the results. Pure culture (1ml) was serially diluted in 9 ml of isotonic diluent dispensed in screw capped test tubes yielding 10^1 dilution each. 0.1 ml of the appropriate dilution (which gives colonies in the countable range of 30-300) was poured on the surface of agar and spread evenly using a sterile 'hockey stick' spreader. The petri plates were incubated at 37°C for 24±2 Hrs followed by the counting of colonies and calculation of cell density per ml of the original dilution.

c. Membrane filtration technique

This technique was used with pure culture. Serial dilution was carried out in the same way as for the spread plate technique with the exception that the last dilution was such as to obtain the countable range (30-300 colonies) in 10 ml instead of 0.1 ml of the dilution medium. Using a sterile forceps, a membrane filter was placed on the porous disc of a membrane filtration unit (Millipore, UK) which was connected to a vacuum source. 10 ml of the appropriate dilution was poured on to the surface of membrane filter followed by the application of vacuum to filter out the liquid. The membrane filter was then transferred to a petri plate with well dried agar medium with the help of a forceps. The forceps was sterilised during the transfer of membrane filter by dipping in ethanol and burning in flame to avoid cross contamination. The petri plates with membrane filters were then incubated

at the required temperature and time. The colonies were counted at the end of incubation period and the number of bacteria per ml was calculated.

3.2.1.7 Pressurised carbon dioxide treatment

a. Bacterial culture

The bacterial cultures were grown to a given cell density as described in section 3.2.1.5 using OD to estimate the number which was also confirmed by serial dilution and spread plating on selective agar. From the flask 2-3 ml of the culture medium was transferred into the sample pot and pressurised to the required pressure for a specific treatment time. The remaining broth culture was kept as a control at ambient temperature and pressure. At the end of treatment time, the pressure vessel was depressurised and the sample was taken out. The number of bacteria in the treated and control samples were enumerated using the appropriate technique as described in section 3.2.1.6.

b. Co-settled primary sewage sludge

Initial concentration of Faecal Indicator Bacteria (FIB) in the CSS was determined by MPN techniques as described in 3.2.1.6 (a). The required amount of CSS was weighed, added to the pressure vessel and pressurised to the required pressure. A control sample was kept alongside the pressure vessel on the shaker. At the end of treatment time, both samples were processed to determine the concentration of FIB.

3.2.1.8 Determination of growth rate, doubling time, generation time and inactivation rate

The growth rate and doubling time were calculated as in equation 3.8 and 3.9.

$$\text{Growth rate} = \left(\log_{10} \left(\frac{N}{N_0} \right) \cdot \log_e(10) \right) / (T - T_0) \quad \text{Equation 3.8}$$

Where,

N_0 is the initial cell count

T_0 is the initial time

N is the endpoint cell count

T is the endpoint time

$$\text{Doubling time} = \log_e(2) / \text{Growth rate}$$

Equation 3.9

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$$\text{Generation time} = (\log \text{CFU}_n - \log \text{CFU}_{n0}) / \log(2)$$

Equation 3.10

Where,

CFU_n is the number of Colony Forming Units at the end of treatment

CFU_{n0} is the number of Colony Forming Units at the start of treatment

The inactivation rate of FIB following P CO₂ treatment was determined by the enumeration of viable cells in both treated and untreated samples employing either MPN; spread plate or membrane filtration technique and using equation 3.11:

$$\text{Inactivation rate} = \log_{10} N_2 - \log_{10} N$$

Equation 3.11

Where,

N₂ is the initial cell count (from control 2)

N is the cell count after treatment

3.2.1.9 Electron microscopy

a. **Transmission Electron Microscopy (TEM)**

A bacterial culture with cell density of 10⁶ CFU ml⁻¹ was prepared as described in 3.2.1.1 and 10 ml of this culture was treated with P CO₂ 2800 kPa for 24 Hrs, control sample was kept at the room temperature and ambient pressure. At the end of treatment time, both samples were centrifuged at 10000 x g, 4°C for 30 min in a high speed refrigerated centrifuge Sorvall legend XT/XTR (Thermo Fisher scientific Loughborough, UK). Following centrifugation, the supernatant was decanted and the cell pellets were re-suspended in 1 ml primary fixative comprising of 3% glutaraldehyde, 4% formaldehyde in 0.1 M PIPES buffer (pH 7.2). They were then processed according to method 4 as reported by Page et al.,(1994). The alginate embedded cells were processed as normal for TEM. Briefly, the specimens were rinsed in 0.1M PIPES buffer, post fixed in 1% buffered osmium tetroxide (1 hour), rinsed in buffer, block stained in 2% aqueous Uranyl Acetate (20 min), dehydrated in an ethanol series and embedded in TAAB resin (TAAB Laboratories, Aldermaston, UK). Gold sections were cut on an Ultra cut E ultra-microtome, stained with Reynolds lead stain and viewed on a FEI Tecnai 12 transmission electron microscope (Japan) equipped with a SIS mega view III digital camera.

b. **Scanning Electron Microscopy (SEM)**

The preparation, processing, P CO₂ treatment and re-suspension in the primary fixative were same as done for TEM. At the end of primary fixation, the samples were again centrifuged and half of the primary fixative was removed followed by the re-suspension of cell pellet in rest of the fixative. This very small amount of sample was then rested for 30 min on the surface of a 13 mm cover slip coated with 3-Aminopropyl triethoxysilane kept in a glass vial. The coated cover slip provided static charge for holding the bacterial cells. Next step was the buffer rinsing, post fixation in 1% buffered osmium tetroxide and dehydration in alcohol series. After this, the cover slip with the bacterial cells attached to it was dried in a critical point drier (Balzers - CPD 030) followed by mounting on aluminium stubs which were coated with gold palladium in a sputter coater (SEM coating unit E 5100 Polaron equipment limited). The samples were then viewed on a scanning electron microscope FEI Quanta 200 (Holland).

3.2.1.10 Determination of enzyme inactivation

Enzymes active in the untreated bacterial cultures and the effect of P CO₂ treatment on the activities of these enzymes was detected with the help of API ZYM strips (BioMérieux, Basingstoke, UK). These strips enabled the detection of nineteen enzymes. The strips consisted of twenty small cupules each of which was filled with 65 μ l of untreated or treated bacterial culture and incubated for 4 Hrs at 37°C. After incubation, one drop of ZYM A and ZYM B (provided with the strips) were each added to all the cupules and the change in colour of each cupule was noted. The colour change was judged as being positive or negative from the table provided with the product by the manufacturer

3.2.1.11 Detection of UV absorbing substances

UV absorbing substances (proteins and nucleic acids) expected to be released from the bacterial cells as a result of P CO₂ treatment were determined according to the method modified from 'Manual of methods for general bacteriology' (Pham et al., 2010b).

A culture of FIB was grown and after sub-culturing regrown to the desired cell density using OD with confirmation by serial dilution and spread plating on selective agar. At the same time, 2x10 ml culture samples were centrifuged at 10000g, 4°C for 30 min. The supernatant was discarded and cell pellet was suspended in equal volume of 0.85% NaCl solution. One sample was pressurised with CO₂ to the desired pressure for a specific treatment time. The other sample was kept at the ambient temperature and pressure. At the end of treatment time, both samples were centrifuged again under the same conditions as

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previously carried out. The supernatant of both samples were then observed in a UV visible spectrophotometer at the wavelengths of 260 and 280 nm using a quartz cuvette. NaCl solution (0.85%) was used as a blank.

3.2.2 Anaerobic digestion

The analytical methods used to assess the performance of anaerobic digesters were divided in three main categories.

3.2.2.1 Gravimetric analysis

a. **Total and Volatile solids (TS and VS)**

TS and VS were measured using standard method 2540 G (APHA, 2005) according to the following procedure.

The porcelain crucibles were dried at 105°C in a drying oven (Vulcan Hart-USA) followed by cooling in a desiccator with active silica gel desiccant for 15 min. Empty crucibles were weighed with an accuracy of ± 0.0001 g using a balance (Sartorius, AG, Gottingen Germany). The weight was recorded (W1). The crucibles were filled to about 2/3 of their capacity with well mixed sample and the weight was recorded (W2). The crucibles were then placed overnight in a drying oven (Vulcan, USA) at $105 \pm 5^\circ\text{C}$. After 24 Hrs, the crucibles were cooled in a desiccator for 40 min followed by weighing using the same balance (W3). Samples were then placed in a muffle furnace (Carbolite, UK) and heated to $550 \pm 10^\circ\text{C}$ for 2 Hrs. After this, the samples were again cooled in a desiccator for 1 hour and weighed again (W4). The determination was carried out in triplicate and the average value was taken.

TS and VS were calculated according to equation 3.12, 3.13 and 3.14 depending on the mode of expression

$$\%TS = \frac{W_3 - W_1}{W_2 - W_1} \times 100 \quad \text{Equation 3.12}$$

$$\%VS(\text{based on total weight}) = \frac{W_3 - W_4}{W_2 - W_1} \times 100 \quad \text{Equation 3.13}$$

$$\%VS(based \cdot on \cdot total \cdot solids) = \frac{W_3 - W_4}{W_3 - W_1} \times 100 \quad \text{Equation 3.14}$$

Where,

W_1 was the weight of empty crucible

W_2 was the weight of crucible with wet sample

W_3 was the weight of crucible and sample after drying at 105°C

W_4 was the weight of crucible and sample after drying at 550°C

Same method was used for measuring the TS and VS of both feedstock and digestate.

3.2.2.2 Chemical analysis

a. pH

pH was measured potentiometrically according to the standard method 4500-H+ (APHA, 2005) using a pH probe (Fisher Scientific) connected to a Jenway 3310 pH meter (Jenway, UK) with a sensitivity of ± 0.01 and accuracy of 0.01 ± 0.005 pH units. The pH probe was calibrated daily using buffer solutions of pH 4.0, 7.0 and 9.2 prepared from buffer tablets (Fisher Scientific). Fresh buffer solutions were prepared weekly and stored in screw capped containers. The pH probe was thoroughly rinsed with DI water between subsequent measurements to avoid cross contamination. The pH of digestate samples was recorded immediately after sampling to avoid the alteration of pH due to the evaporation of volatiles or escape of CO₂.

b. Alkalinity

Three different measurements of alkalinity; Partial (pH 5.7), Intermediate (pH 4.3) and Total (pH 4.0) (PA, IA and TA respectively) were taken as described by Ripley et al., (1986). PA is a measurement of bicarbonate buffering while IA is attributed to the buffering capacity of Volatile Fatty Acids (VFA). Alkalinity was measured according to the following procedure.

About 5 g of homogenised sample was made up to 40 ml with DI water and titrated with 0.25 N Sulphuric acid (H₂SO₄) to pH 5.7, 4.3 and 4.0 for PA, IA and TA respectively using an automatic digital titration burette system (Schott Titroline, Gerhardt UK Ltd) with pH probe and constant stirring using a magnetic stirrer to ensure thorough mixing. The pH probe was calibrated before titration as described before and washed with DI

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water between subsequent samples to avoid cross contamination. Alkalinity was calculated according to equations 3.15, 3.16 and 3.17

$$TA = \frac{(V_{4.0} + V_{4.3} + V_{5.7}) \times N \times 50000}{V} \quad \text{Equation 3.15}$$

$$PA = \frac{V_{5.75} \times N \times 50000}{V} \quad \text{Equation 3.16}$$

$$IA = \frac{V_{pH4.3} \times N \times 50000}{V} \quad \text{Equation 3.17}$$

Where,

TA was the total alkalinity (mg CaCO₃ l⁻¹)

PA was the partial alkalinity or bicarbonate alkalinity (mg CaCO₃ l⁻¹)

IA was the intermediate alkalinity or volatile fatty acid alkalinity (mg CaCO₃ l⁻¹)

N was the normality of H₂SO₄

V was the volume of sample (ml)

c. Total Ammonia Nitrogen (TAN)

TAN was measured on the basis of standard method 4500-NH₃ B and C (APHA, 2005) for digestate samples. About 3-5 g of sample was taken in a digestion tube and made up to 50 ml with DI water. The sample was then distilled in a Foss Tecator Kjeltec system 1002 distillation unit (Foss Tecator A-B, Hoganas, Sweden) with the addition of a few drops of 10 M sodium hydroxide (NaOH) in order to raise the pH above 9.5 for quick volatilisation of ammonia. Distillate (150 ml) was collected in a conical flask containing 25 ml of indicator Boric acid solution which turned from lavender to green following distillation. The distillate was then titrated with 0.25 N H₂SO₄ using an automatic digital titration system (Schott Titroline, Gerhardt UK Ltd.) until the green colour changed back to lavender. Before running the samples, a standard solution (1000mg l⁻¹) of ammonium chloride (NH₄Cl) was distilled with the addition of few drops of NaOH and the distillate was collected in the same way described above followed by titration with 0.25 N H₂SO₄. The distillation unit was cleaned before and after use by the distillation of simple DI water

containing a few drops of NaOH and then with simple DI water to avoid cross contamination.

The percentage of nitrogen in sample was calculated by using equation 3.18.

$$NH_4N \text{ mg l}^{-1} = \frac{(A - B) \times 14.0 \times 0.25 \times 100}{V_{sample}} \quad \text{Equation 3.18}$$

Where,

A was the volume (ml) of 0.25 N H₂SO₄ used to titrate the sample

B was the volume (ml) of 0.25 N H₂SO₄ used to titrate the blank

V_{sample} was the volume of sample (ml)

3.2.2.3 Gas Analysis

a. Biogas volume

Biogas was collected from CSTR bioreactors in 10 l gas collection Tedlar bags; these were connected to the gas vent of gas counter immediately after feeding the reactors. Before feeding the next day the gas bag was disconnected from the bioreactor and a 10 ml sample was collected in a syringe for analysis of gas composition. The volume of biogas collected in the Tedlar bag was then measured by a water displacement gasometer and corrected to Standard Temperature and Pressure (STP) of 0°C and 101.325 kPa, as described by Walker et al.,(2009).

b. Biogas composition

Gas samples were analysed using a Varian CP 3400 gas chromatograph with a gas sampling loop and thermal conductivity detector with Helium as a carrier gas at a flow rate of 50 ml min⁻¹ and 20 bar pressure. The GC was fitted with a Haysep C column with a molecular sieve 13 x (80-100 mesh). The temperature of column and injector was 50°C; oven was 110°C while that of the detector was 200°C. The gas chromatograph was calibrated with a standard biogas (65% CH₄ and 35% CO₂, BOC Guilford, UK). The retention time and peak areas of CH₄ and CO₂ in the calibration curve were used to determine the amount (volume %) of each gas (which was then corrected to amount of CO₂ and CH₄ in 100%) in the sample. Each sample was analysed in triplicate.

Materials and Methods

3.3 Summary

The chapter gives a complete description of the methods used in the research. Included are the source of chemicals, equipment, feedstock, inoculum, compressed gases and microorganisms used. The design and operation of CO₂ pressurisation equipment was described in detail. The design and operation of CSTR reactors and the BMP apparatus was explained along with the description of parameters used to assess the continuous and batch fed AD trials. Details of analytical and microbiological methods are provided along with reference to the original method where appropriate. It is believed that the level of detail provided in this chapter should be sufficient to allow the methods to be repeated by others.

4 Results and Discussion

4.1 Isolation of *E. coli* from CSS: its identification, confirmation and maintenance of a pure culture

Objective

To isolate and identify *E. coli* from CSS, confirm that the isolated strain is *E. coli* and preserve the isolated strain for using in future experiments.

Theory

It was decided to initially optimise process parameters for the P CO₂ treatment system (pressure, temperature, treatment time and agitation) with respect to antimicrobial action using bacterial cells cultured in broth rather than CSS. This was because of the relative ease with which this could be done and as a pointer to the likely future experimental conditions that would be required. For this purpose *E. coli* was isolated from CSS and maintained as a pure culture for die-off experiments.

Procedure

The isolation, identification, confirmation and preservation of *E. coli* were carried out as described in 3.2.1.2 and 3.2.1.4 respectively.

Results

By following the procedure described a pure culture of *E. coli* was obtained and was subsequently maintained on NA slopes. The results of the MPN, Indole and MCA tests are illustrated in Figure 4.1.

Results and Discussion



Figure 4.1 Isolation, identification, and confirmation of *E. coli*

4.2 Determination of the growth rate of Faecal Indicator Bacteria (FIB) using optical density and spread plate techniques

Objective

To determine the growth rate of FIB in broth culture by measuring the increase in OD and correlating this to viable cell numbers using the spread plate count method. Using the results to establish a growth curve makes an accurate prediction of cell numbers possible and allows detail timing of each experiment to avoid out of hours working.

Theory

OD as measured by the absorbance of light through a culture at known wavelength is proportional to the cell concentration (Heo et al., 2003) and has been used as an effective tool to replace the conventional plate count technique.

Procedure

Overnight cultures of *E. coli* and *Salmonella* were grown in TW and BPW respectively according to the procedure described in 3.2.1.5. 0.1 ml of the OC was inoculated into 100 ml of each respective growth medium and incubated in a shaking water bath at 37°C operating at 120 RPM. Increase in OD was recorded (at 400 nm) at regular intervals of 1 hour and 1 ml sample was serially diluted in MRD for *E. coli* and $\frac{1}{4}$ strength RS for *Salmonella*. *E. coli* dilutions were spread plated on MCA whereas those of *Salmonella* on XLD (for detail see 3.2.1.6 b) and the petri plates were incubated at 37°C for 24 Hrs. This procedure was repeated every hour until no significant increase in OD was observed which indicated that the culture had reached the stationary phase. The spread plate counts were then plotted against their respective OD values to give growth curves from which the generation time of FIB could be estimated. For each culture the experiment was repeated three times to check the consistency of results.

Results

The results in Figure 4.2 showed that there was a linear relationship between OD, log CFU and time and log CFU and OD which indicated that there was a consistency in the rate of growth of *E. coli* in the three runs. *E. coli* was found to be a vigorous grower with a growth rate 0.61 and a doubling time of 1.14 Hrs. It was found that when the OD was about 0.45 the culture had a cell density of about 10^7 CFU ml⁻¹ and it took around 2 Hrs for a freshly inoculated 100 ml culture in TW to reach this cell density.

Results and Discussion

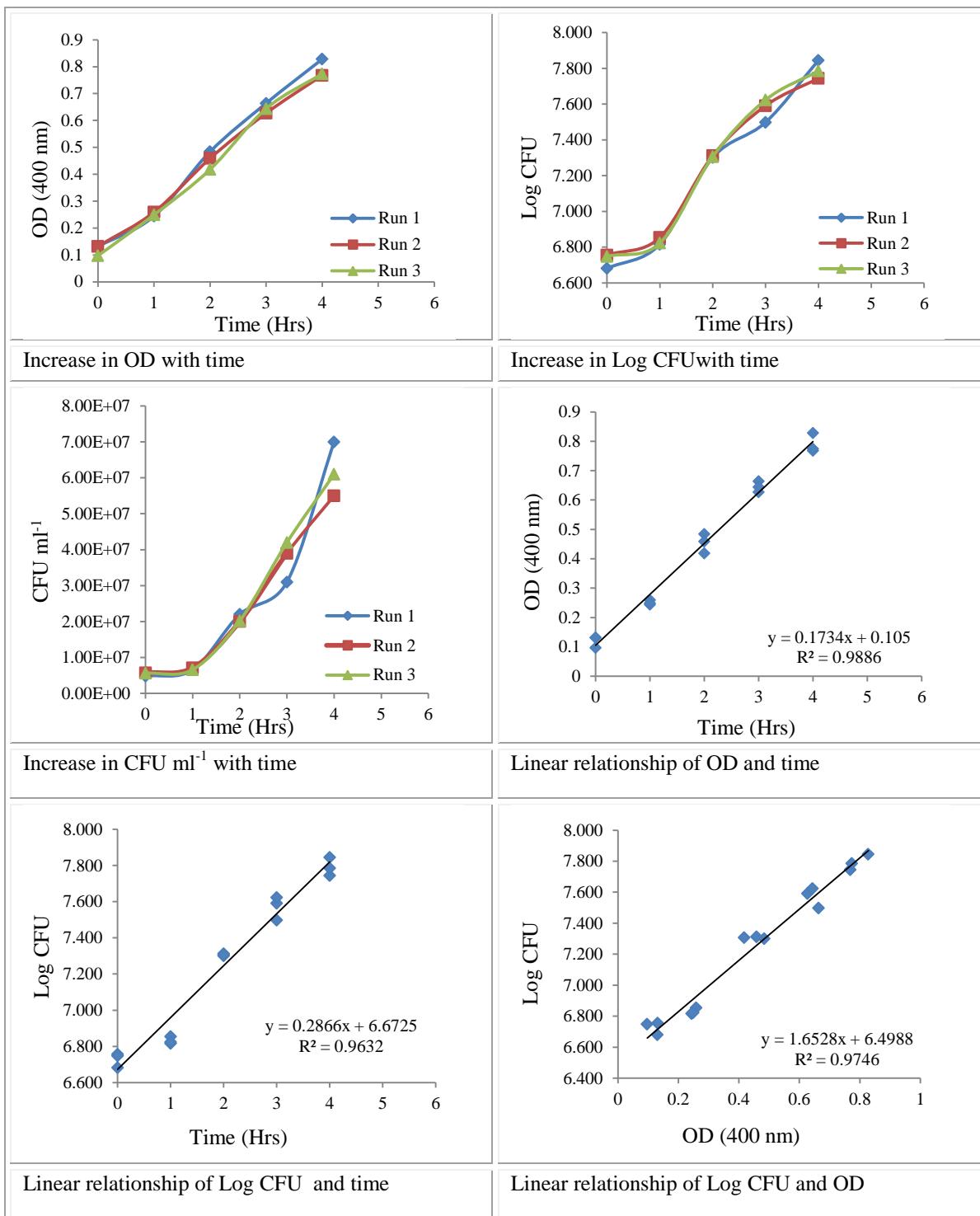


Figure 4.2 Growth curve of *E. coli* with regard to the increase in OD, log CFU, CFU ml⁻¹ and the linear relationship between these parameters

S. enterica was found to grow more rapidly than *E. coli* with a growth rate of 1.1 and doubling time of just 0.62 Hrs (Figure 4.3). When the culture had an OD of around 0.07 the cell density was 10⁷ CFU ml⁻¹ and it took about 2 Hrs to reach this value. A linear

relationship was found among log CFU and OD with time and between log CFU and OD over the first 4 hours. In the forthcoming optimisation experiments, *S. enterica* culture was grown to the OD of 0.04 in order to achieve a density of 10^6 CFU ml⁻¹.

Results and Discussion

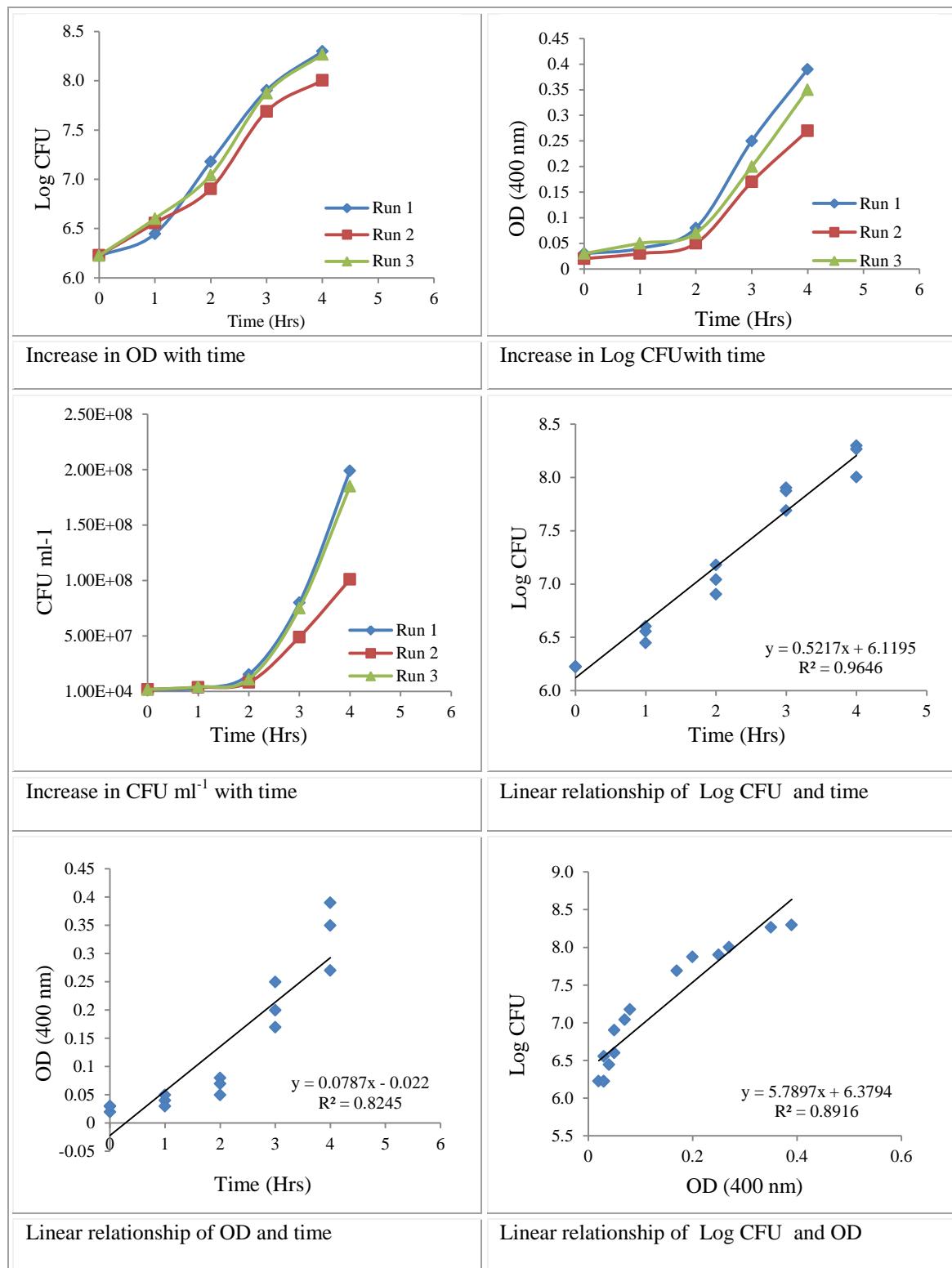


Figure 4.3 Growth curve of *S. enterica* with regard to the increase in OD, log CFU, CFU ml⁻¹ and the linear relationship between these parameters

This experiment provided a quick and easy method for growing a culture to the desired cell density based on OD as the first step towards optimisation experiments and experiments designed to study the mechanism of bactericidal action of P CO₂.

4.3 Optimisation of pressure and treatment time using P CO₂ for the inactivation of FIB

Objective

To determine the effect of pressure and treatment time on the survival of FIB in broth culture.

Theory

A number of factors influence the antimicrobial potential of P CO₂ among which, pressure and treatment time are the most important. A broth culture of FIB with a cell density of 10⁶ CFU ml⁻¹ was used a log reduction of this magnitude is required for the removal of *E. coli* in the safe sludge matrix. It is also a requirement to have an absence of *Salmonella* in 2 g of dry solids (ADAS, 2001), this starting concentration would thus be challenging. The pressures selected were: 1500, 2000 and 2800 kPa and the longest treatment 24 Hrs based on what might be possible in practice although much shorter retention times would be desirable.

Procedure

A culture of FIB was prepared (see 3.2.1.5) which was then sub cultured in 100 ml GM and grown to obtain the required cell density of 10⁶ CFU ml⁻¹ based on OD measurement. The cell suspensions were serially diluted and spread plated on their respective selective agar petri plates (3.2.1.6 b) to confirm the initial cell numbers. About 2-3 ml of culture was then treated with P CO₂ (3.2.1.7) while the rest of it was kept at ambient temperature and pressure. At the end of each selected treatment time, both the treated and control samples were serially diluted and spread plated. The selected range of pressure and treatment times used were: 1500, 2000 and 2800 kPa and 24, 18, 12, 9, 6, 3, 1.5, 1 and 0.75 Hrs respectively. The rate of inactivation was calculated as described in 3.2.1.8. Each trial was repeated at least three times to get statistically significant and repeatable results.

Results

The results indicated that inactivation increased with increase in pressure and treatment time (Table 4.1 and Figure 4.4). Complete inactivation (8 log reduction) was achieved at the longest treatment time (24 Hrs) at all selected pressures. The pH of reaction medium

Results and Discussion

dropped from 7.00 to 5.00 after P CO₂ treatment at all selected pressures and treatment times. Figure 4.4 suggests that log reduction increased linearly with duration at each pressure, with values of 0.325 ($R^2 = 0.959$), 0.616 ($R^2 = 0.925$) and 2.994 ($R^2 = 0.621$) log reduction for 1500, 2000 and 2800 kPa respectively. The high R^2 values at 1500 and 2000 kPa indicate a strong dependency on duration, while the lower R^2 value at 2800 might indicate the requirement for more trials at intermediate durations. It may also be caused by other factors, however. For example a certain amount of time is needed to fill and empty the pressure vessel, and this represents a higher proportion of the total experimental time at short test durations than at longer durations; therefore there may be practical difficulties in differentiating between intervals of less than about 15 min. The relationship between the rate of increase in log reduction with pressure does not seem to be linear, i.e. if the pressure is doubled, the rate of increase of log reduction more than doubles.

Table 4.1 Log reduction of *E. coli* at selected pressures and treatment times

| Time (Hrs) | Pressure (kPa) | | |
|------------|----------------|------|------|
| | 1500 | 2000 | 2800 |
| 24 | 8.2 | 8.3 | 8.2 |
| 18 | 4.6 | 8.3 | 8.2 |
| 12 | 3.9 | 8.1 | 8.2 |
| 9 | 3.2 | 4.9 | 8.2 |
| 6 | 2.4 | 2.9 | 8.1 |
| 3 | 0.5 | 2.1 | 7.9 |
| 1.5 | 0.3 | 1.5 | 7.7 |
| 1 | 0.2 | 1.3 | 2.3 |
| 0.75 | 0.2 | 0.3 | 0.4 |

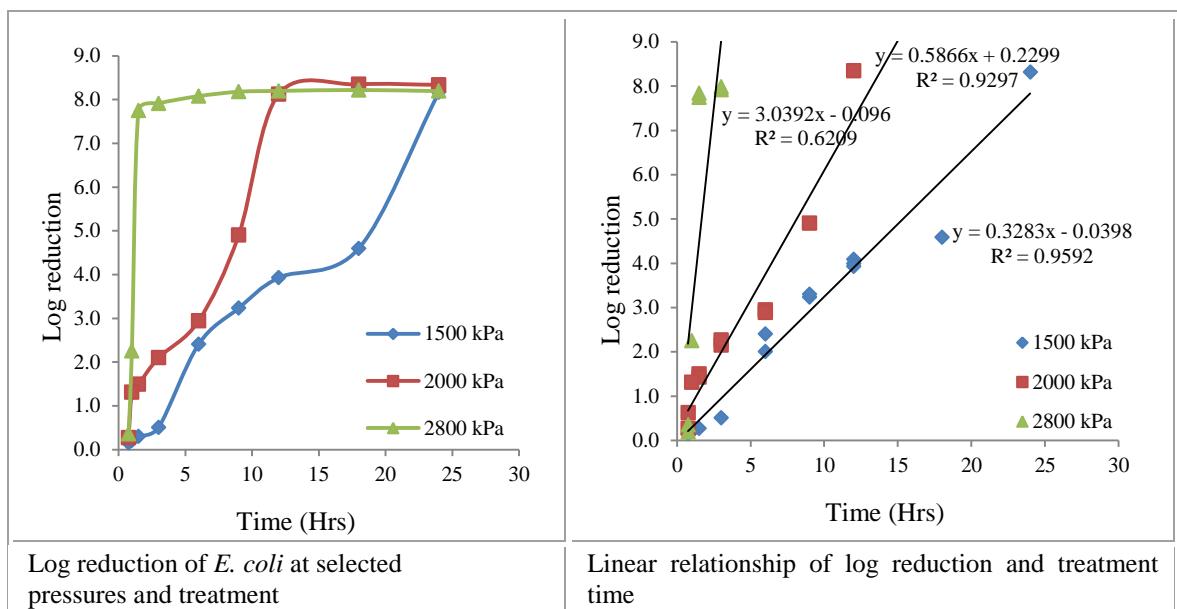


Figure 4.4 Optimisation of pressure and treatment time for inactivation of *E. coli* by P CO₂

Trials for *S. enterica* were conducted at 2800 kPa as this had proved to be the best pressure for maximum inactivation in the case of *E. coli*. The time range used (24, 12, 6 and 3 Hrs) was also narrower. Complete inactivation (8 log reduction) was accomplished at the longest exposure time (24 Hrs) (Figure 4.5 and Table 4.2).

Table 4.2 Log reduction of *S. enterica* at 2800 kPa and selected treatment time

| Time | Log reduction |
|------|---------------|
| 24 | 8.2 |
| 12 | 7.9 |
| 6 | 4.7 |
| 3 | 0.2 |

Results and Discussion

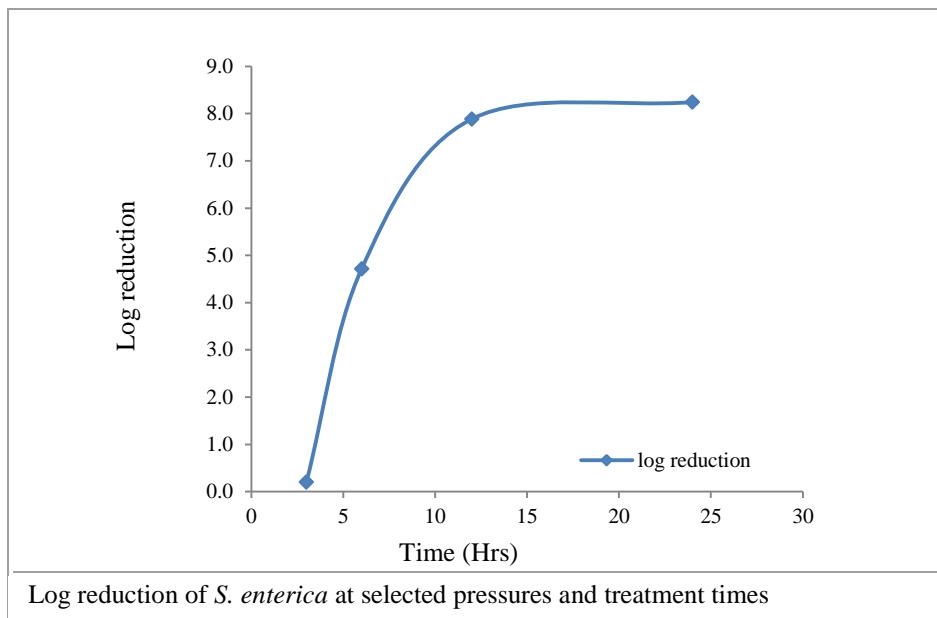


Figure 4.5 Inactivation of *S. enterica* at 2800 kPa and selected treatment times

The results for inactivation of FIB using P CO₂ were in agreement with previous studies (Sirisee et al., 1998, Erkmen, 2001, Mazzoni et al., 2001, Liao et al., 2007) which reported enhanced inactivation rate at higher pressure and longer treatment time. Since pressure controls the solubility of CO₂ in the aqueous phase of the reaction medium, dissolution of CO₂ at higher pressure is more significant which then forms carbonic acid that lowers the pH of the reaction medium (Garcia-Gonzalez et al., 2007). This acidification is supposed to play a vital role in microbial inactivation and this assumption is supported by the observed reduction in pH of reaction medium. In addition, CO₂ possesses a higher solvating power at elevated pressure as a result of which it could extract vital intracellular components (Kamihira et al., 1987, Lin et al., 1992 a, Lin et al., 1993) and enhance microbial inactivation.

4.4 Comparability of cell counts from spread plate, membrane filtration and MPN

Objective

This experiment was designed to compare the three techniques; MPN, spread plate and membrane filtration used in enumeration of the FIB.

Theory

MPN was used to determine the cell count in CSS while spread plate and membrane filtration were used to determine the cell count in pure culture; it was therefore very

important to make sure that these techniques gave the same results in order to compare the cell counts in CSS and pure culture. The three different agar media; MacConkey (MCA), Nutrient (NA) and Membrane Lactose Glucoronide Agar (MLGA) were also checked for the consistency of cell count in spread plate and membrane filtration techniques.

Procedure

A cell suspension of *E. coli* was prepared (see 3.2.1.1) with a nominal cell density of 10^7 CFU ml⁻¹ as estimated using OD, this was then enumerated by the three techniques. Serial dilutions 10^1 - 10^5 were performed (see 3.2.1.6 b) followed by the spread plating of 0.1 ml of the 10^5 dilution on duplicate petri plates of MCA, NA and MLGA followed by incubation at 37°C for 24 ± 2 Hrs. Colonies were counted at the end of incubation period.

In the case of membrane filtration, serial dilutions (10^1 - 10^6) were performed as described in 3.2.1.6 c. Last dilution (10^6) was again diluted in 90 ml of MRD in order to get the countable colony range (30 - 300) in 10 ml. Rest of the procedure was carried out as described in 3.2.1.6 c. MPN was performed as described in 3.2.1.6 a.

The results from all three techniques were expressed in CFU ml⁻¹

Results

Spread plate, membrane filtration and MPN were found to be comparable (Table 4.3). Hence it was demonstrated that the results obtained by either of these techniques were interchangeable.

Table 4.3 Enumeration of *E. coli* from pure culture by selected techniques

| Spread plate | | Membrane filtration | | MPN ml ⁻¹ |
|--------------|-----------------------------|---------------------|-----------------------------|----------------------|
| Medium | Cell count ml ⁻¹ | Medium | Cell count ml ⁻¹ | |
| NA | 3.8×10^7 | NA | 3.9×10^7 | 5.4×10^7 |
| MCA | 3.8×10^7 | MCA | 3.9×10^7 | |
| MLGA | 4.3×10^7 | MLGA | 3.4×10^7 | |

4.5 Resuscitation of *E. coli* following treatment with P CO₂

Objective

This experiment was designed to determine whether *E. coli* completely loses its viability as a consequence of P CO₂ treatment or still possesses the capability to repair, re-grow and regain viability.

Results and Discussion

Theory

Many bacteria remain viable but cannot be cultured as a response to environmental stress such as high or low temperature, osmotic pressure, oxygen, nutrient depletion and variable light intensities. In this state, the bacteria normally fail to grow on selective and complex media. Although, the bacteria cannot be cultured in this state they can recover if conditions become favourable or if the stress is reduced. The triggering of metabolic activity allowing the cells to be cultured is termed as resuscitation (Woodard and Wukasch, 1994) and is a common techniques used in water bacteriology, but even with these techniques it is believed that viability might remain but would not be expressed. It is therefore crucial to test that the P CO_2 treatment completely kills FIB rather than making them difficult to culture and apparently non-viable.

Since stressed *E. coli* lose their ability to grow on selective growth media (Özkanca et al., 2009) NA was used as a non-selective medium and compared to the selective growth medium MLGA. Firstly, resuscitation was performed without stressing *E. coli* and then the rate of growth of *E. coli* under normal and stressed conditions was determined. Finally, an attempt was made to stress *E. coli* without killing in order to check for its ability to recover and re-grow. *E. coli* (stressed or un-stressed) was allowed to resuscitate on NA and then shifted to MLGA to investigate whether it was able to re-grow and reach the cell count on NA. The cell count on NA was taken as a control.

Procedure

To test resuscitation without stressing a cell suspension of *E. coli* was prepared (see 3.2.1.1) with an estimated cell density of 10^7 CFU ml^{-1} based on the OD measurement. The culture was serially diluted ($10^1 - 10^6$) and the last dilution (10^6) was again diluted in 90 ml of MRD in order to get the countable colony range (30 – 300) in 10 ml. 4 x 10 ml aliquots of 100ml of the 10^7 dilution were then passed through replicate membrane filters which were then placed on NA petri plates and incubated at 37°C for 4 Hrs. After 4 Hrs, two membrane filters were shifted from NA to MLGA and the petri plates were incubated at 44°C for 14 Hrs. At the end of incubation period, the colonies were counted and were expected to give comparable results between NA and MLGA cultures.

To test resuscitation of a stressed *E. coli* culture a 10^7 CFU ml^{-1} culture was prepared, serially diluted (10^1-10^5) and spread plated on NA and incubated at 37°C to check the recoverable cell density. The cell suspension was divided in two equal portions: one

portion was incubated in a shaking water bath at 37°C (control) while the other was incubated in a shaking water bath at 50°C (stressed). Serial dilution and spread plating (10^1 - 10^5) on NA petri plates was carried out at 20 minute intervals for a period of one hour. The petri plates were incubated at 37°C for 24 Hrs. The colonies on control and stressed petri plates were counted and compared.

In the next step one sample of *E. coli* was exposed to heat (50°C) and another to pressure (2800 kPa, 9 Hrs) to induce stress. A cell suspension with 10^7 CFU ml⁻¹ was prepared as before and a proportion placed in a water bath maintained at 50°C for 1 hour. A second portion was exposed to P CO₂ (2800 kPa, 9 Hrs). Plating for cell recovery from both stressed and unstressed cultures was carried out using serial dilution, membrane filtration and incubation. For membrane filtration six replicate membrane filters were prepared and two of these were placed directly on MLGA and incubated at 44°C. The other four filters were placed on NA petri plates and incubated at 37°C for 4 Hrs. After 4 Hrs, two of the four membrane filters were shifted from NA to MLGA and the petri plates were incubated at 44°C for 14 Hrs. Colony count from the membrane filters incubated directly on MLGA was expected to be less than those shifted from NA to MLGA as the stressed cells lose their ability to grow on selective media while non-selective media such as NA are mild and the stressed cells could easily recover on them (Özkanca et al., 2009). Therefore the recovery step (4 Hrs incubation period on NA) was expected to help the stressed bacteria recover and grow more easily on MLGA.

Results

In the experiment where no stress was applied, cell recovery was comparable both on membrane filters incubated on NA and on those shifted from NA to MLGA after 4 Hrs (4.2×10^7 and 3.6×10^7 respectively).

In the experiment where cells were subjected to heat treatment of the cell suspension for a period of one hour at 50°C the results showed that the cell count of the control culture increased, whereas that of the treated culture slowly decreased (Table 4.4, Figure 4.6) indicating that *E. coli* was stressed.

This test was repeated with the cultures spread plated on both NA and MCA to assess whether the selective agar (MCA) had any additional effects on the cell count. The results showed that the cell counts from both agars were comparable (Table 4.5 and Figure 4.7).

Results and Discussion

Table 4.4 Cell counts of control and stressed cell suspensions

| Time (Min) | Control | Stressed |
|------------|----------|----------|
| 0 | 3.00E+07 | 3.00E+07 |
| 20 | 6.00E+07 | 4.30E+07 |
| 40 | 6.50E+07 | 3.90E+07 |
| 60 | 1.15E+08 | 3.10E+07 |

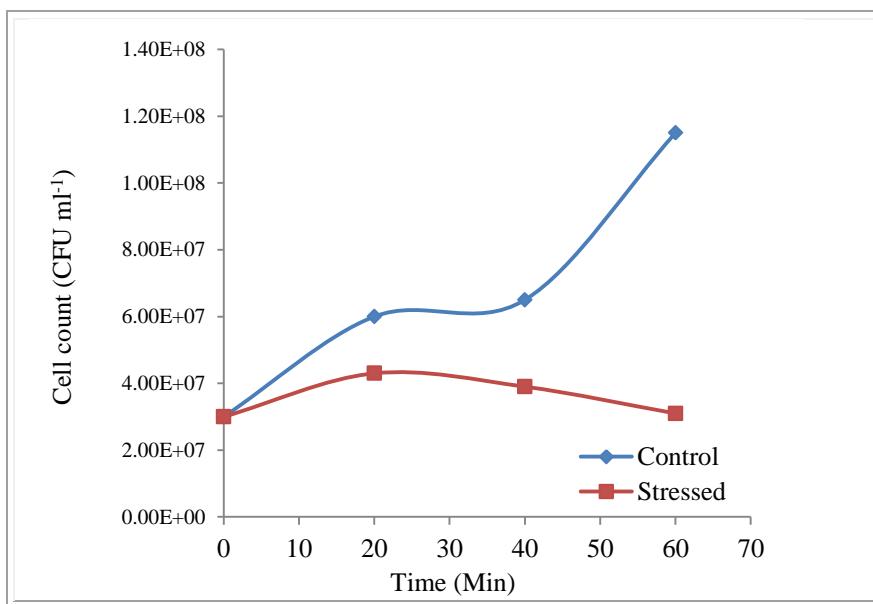


Figure 4.6 Growth rate of control and stressed cell suspensions

Table 4.5 Cell counts of control and stressed cell suspension from NA and MCA

| Time | NA | | MCA | |
|------|-----------|-----------|-----------|-----------|
| | Control | Stressed | Control | Stressed |
| 0 | 3.40E+107 | 3.40E+107 | 3.70E+107 | 3.70E+107 |
| 20 | 6.90E+107 | 3.40E+107 | 6.00E+107 | 3.35E+107 |
| 40 | 7.50E+107 | 6.40E+107 | 7.90E+107 | 5.20E+107 |
| 60 | 1.57E+108 | 4.20E+107 | 1.48E+108 | 3.20E+107 |

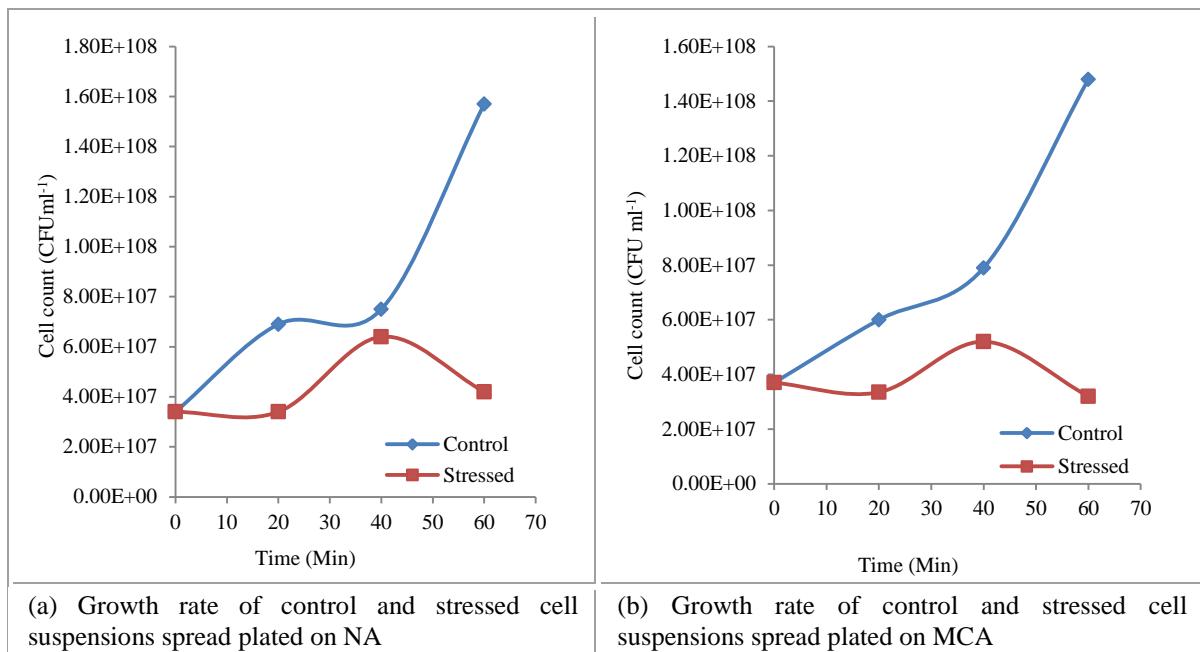


Figure 4.7 Rate of growth of control and stressed cell suspensions spread plated on NA (a) and MCA (b)

The final experiment showed that the colony count from the membrane filters incubated on NA and then shifted on to MLGA ($3.20E+07$) after heat stress was equal to the spread plate colony count from NA ($3.20E+07$) at the end of stress period (Table 4.6). This indicated that the stressed *E. coli* recovered from heat injury during the resuscitation step (4 Hrs incubation period on NA). After exposure to pressure, however, *E. coli* could not be resuscitated using these techniques. Zero cell counts were obtained from NA (spread plate). Membrane filter colony counts were zero from NA and MLGA, and also from the filter papers shifted from NA to MLGA (Table 4.6). This showed that *E. coli* treated with PCO_2 was unable to recover from pressure stress and regain viability.

Table 4.6 Colony counts (spread plate and membrane filters) from stressed and resuscitated *E. coli*

| Type of stress | Spread plate colony counts (NA) | | Membrane filter colony counts (After stress) | | |
|-----------------|---------------------------------|--------------|--|----------|------------|
| | Before stress | After stress | MLGA | NA | NA to MLGA |
| Heat stress | 2.00E+07 | 3.20E+07 | 1.90E+07 | 2.40E+07 | 3.20E+07 |
| Pressure stress | 2.00E+07 | 0.00E+0 | 0.00E+0 | 0.00E+0 | 0.00E+0 |

Results and Discussion

4.6 Mechanism of bactericidal action of PCO₂

4.6.1 Electron microscopic studies (SEM and TEM) of FIB treated with P CO₂

Objective

Electron microscope studies were used to determine the effect of P CO₂ treatment on cell structure and therefore gain insight into the mechanism of inactivation.

Theory

Electron microscopy has been employed as a powerful tool to examine the changes in cell morphology and internal structure (SEM and TEM respectively) following P CO₂ treatment (Hong and Pyun, 1999, Kim et al., 2007a, Kim et al., 2007b).

Procedure

The preparation of a cell suspension and P CO₂ treatment (2800 kPa for 24Hrs) was carried out as described in 3.2.1.5 and 3.2.1.7 respectively. The preparation, processing and observation of samples for SEM and TEM were conducted as described in 3.2.1.9.

Results

SEM images of FIB treated with P CO₂ (2800 kPa for 24 Hrs) showed clear signs of rupture and damage (Figure 4.8 b and Figure 4.9 b) as compared to the relatively smooth appearance of untreated cells (Figure 4.8 a and Figure 4.9 a). The treated cells of *S. enterica* appeared flat (Figure 4.9 b) as compared to the three dimensional appearance of normal cells. Earlier researchers (Nakamura et al., 1994, Folkes, 2004) also found that the SEMs of *S. cerevisiae* treated with P CO₂ showed holes and wrinkles and the cells were shrunken. In some studies, however, cells treated with P CO₂ were either unaffected or showed only minor changes in their morphology (Ballestra et al., 1996, Dillow et al., 1999, Kim et al., 2007a, Kim et al., 2007b, Kim et al., 2008).

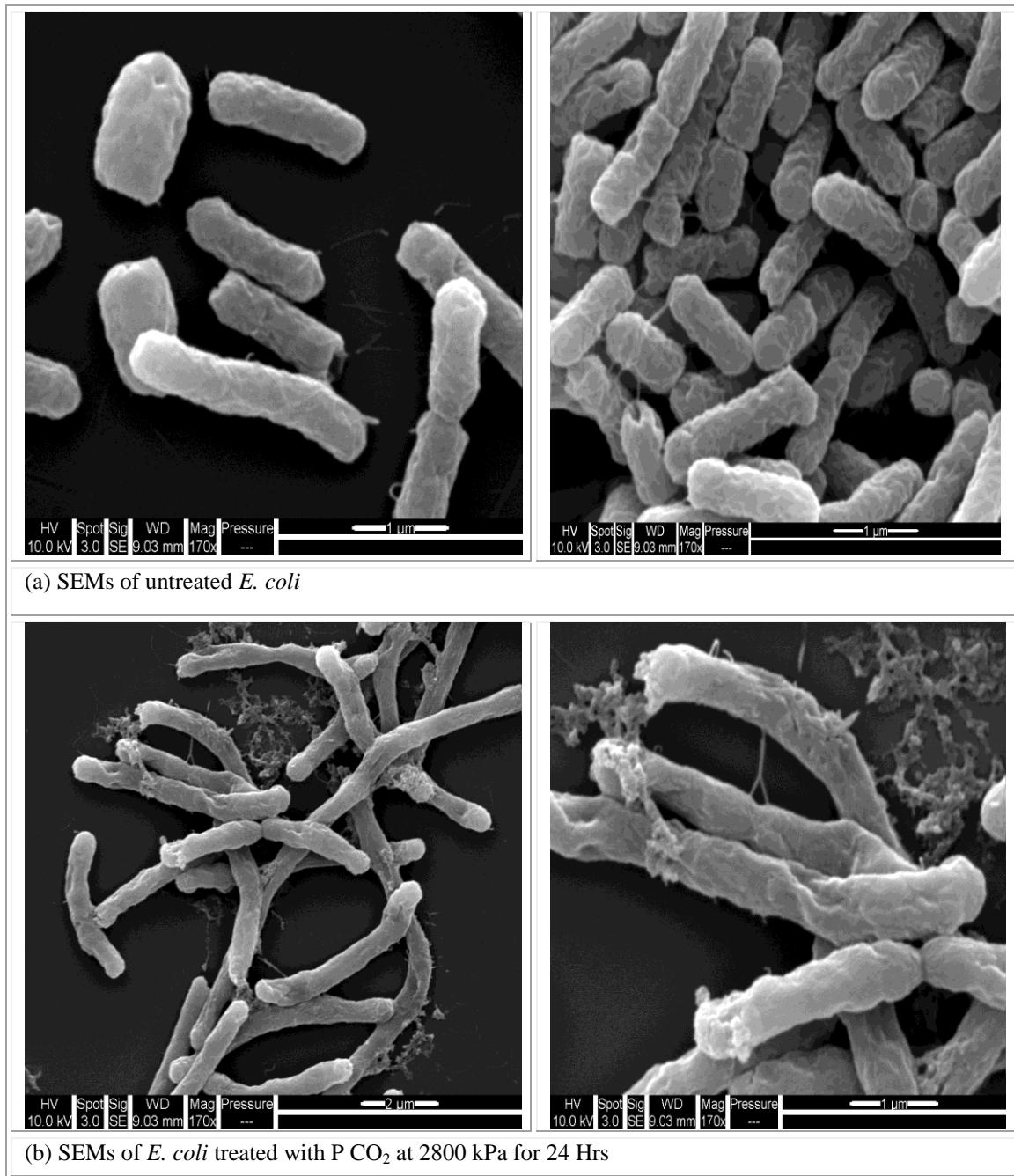
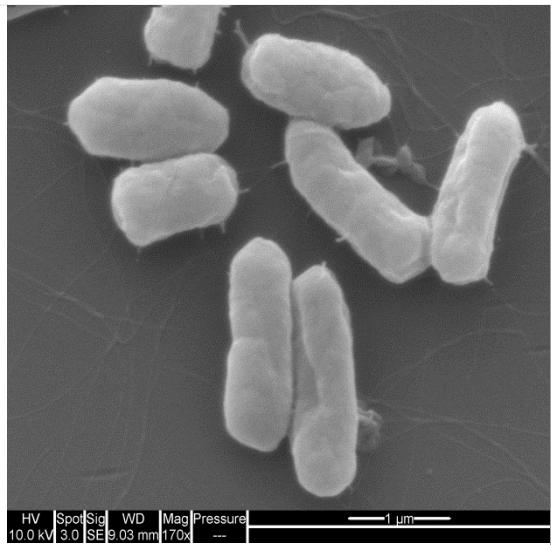
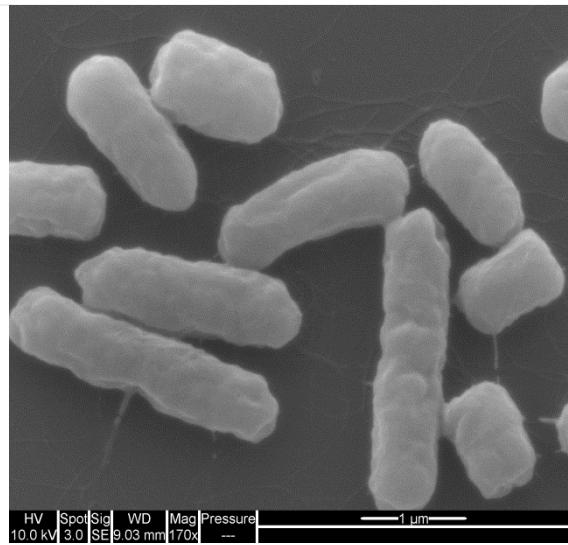
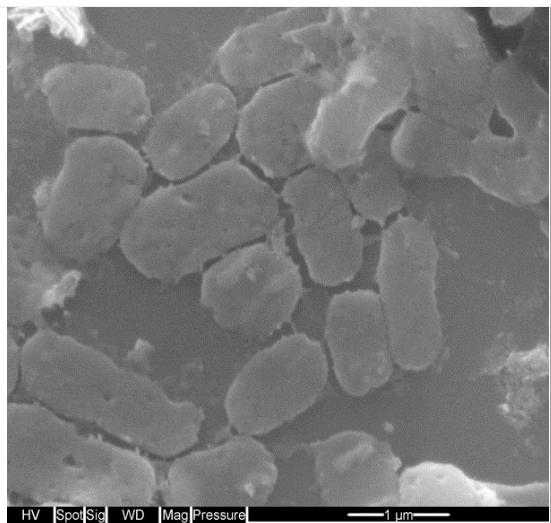
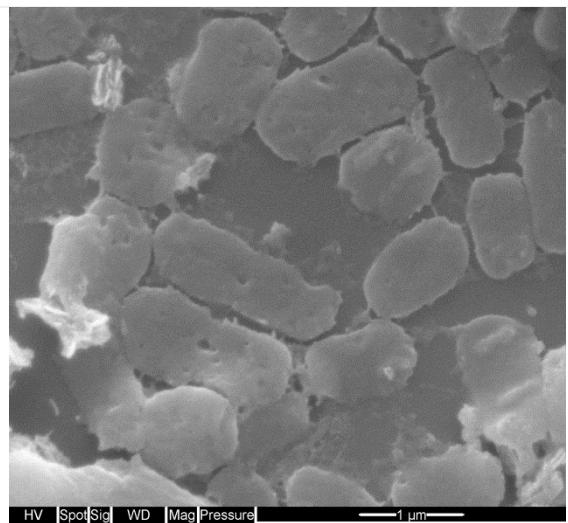


Figure 4.8 SEMs of *E. coli* (a) untreated and (b) treated with PCO_2 at 2800 kPa for 24 Hrs

Results and Discussion



(a) SEMs of untreated *S. enterica*



(b) SEMs of *S. enterica* treated with $P\text{ CO}_2$ at 2800 kPa for 24 Hrs

Figure 4.9 SEMs of *S. enterica* (a) untreated and (b) treated with $P\text{ CO}_2$ at 2800 kPa for 24 Hrs

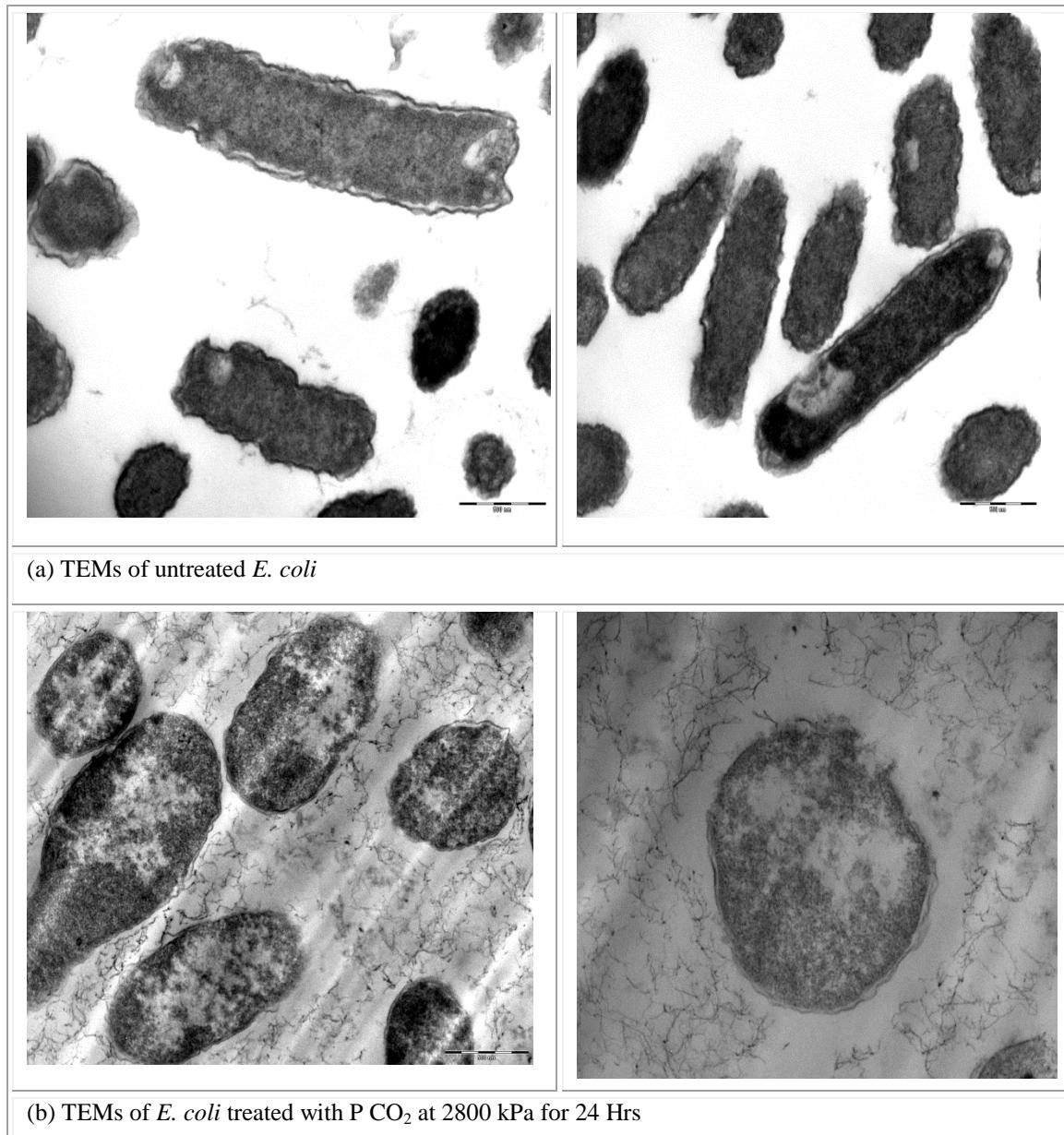


Figure 4.10 TEMs of *E. coli* (a) Untreated and (b) Treated at 2800 kPa for 9 Hrs

TEM images demonstrated that untreated FIB cells (Figure 4.10 a and Figure 4.11 a) had evenly distributed cytoplasm; whereas the treated cells (Figure 4.10 b and Figure 4.11 b) showed empty spaces in the cytoplasm, cell components were unevenly distributed and intracellular substances were agglomerated suggesting possible leakage of cell components during $P\text{ CO}_2$ treatment. Moreover, some cells were completely ruptured and their contents were lost (Figure 4.11b).

Results and Discussion

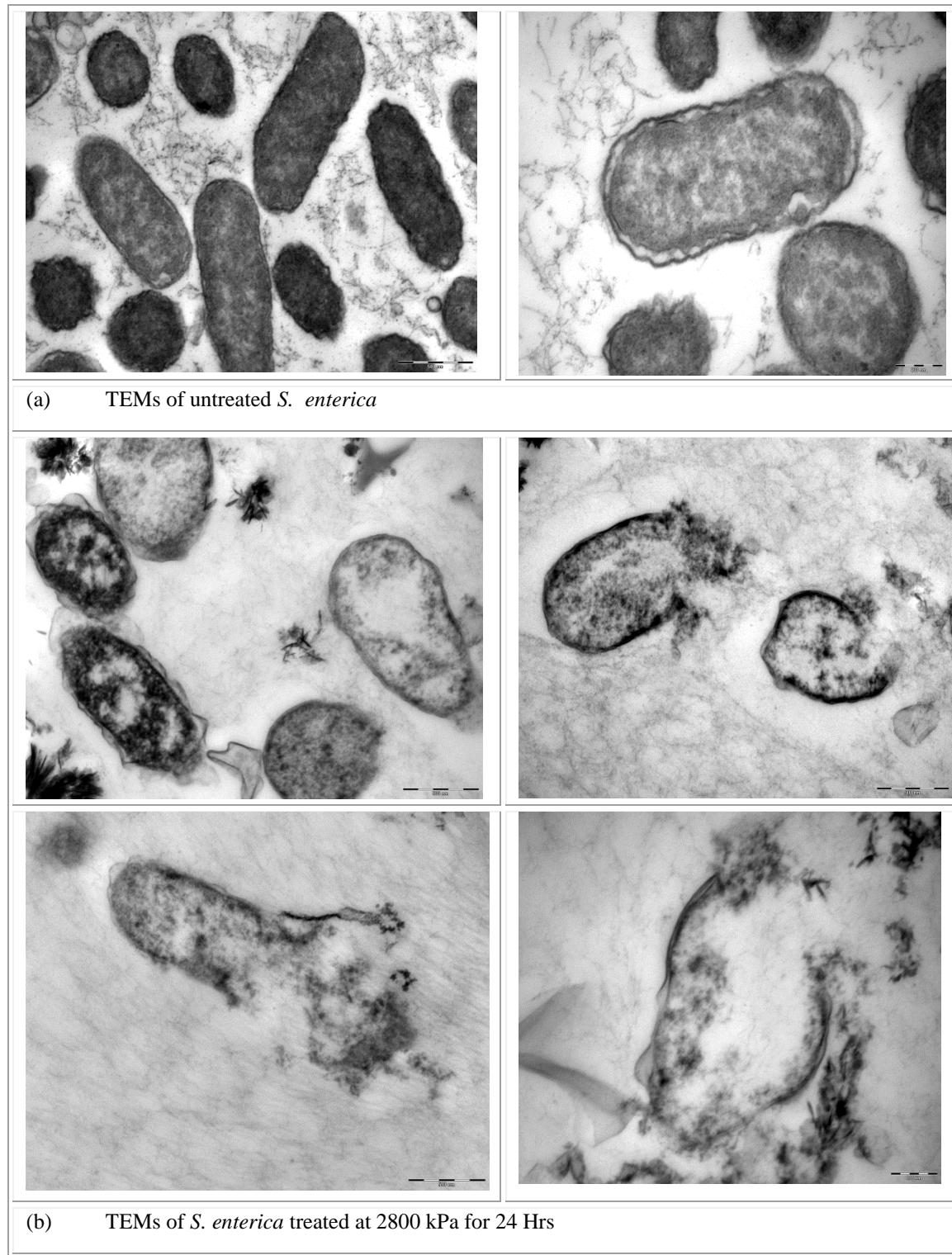


Figure 4.11 TEMs of *S. enterica* (a) untreated and (b) treated at 2800 kPa for 24 Hrs

These results agreed with those from TEM studies of *E. coli* O157:H7 and *S. typhimurium* treated with P CO₂ (10000 kPa, 35°C for 30 min) conducted by Kim et al. (2007a, 2007b). The TEMs of treated cells showed empty spaces in the cytoplasm, segregation of cell membrane from the cytoplasm and agglomeration of cytosol. Similar results were reported

by Hong and Pyun (1999) whereby TEMs of *Lactobacillus plantarum* treated with P CO₂ (6865 kPa, 30°C for 1 hour) showed enlarged periplasmic space between cell wall and cytoplasmic membrane. Blank areas were evident in the cytoplasm pointing towards the extraction of intracellular substances as a result of the process of P CO₂ treatment.

On the basis of electron micrographs, it was evident that the cells were ruptured or mechanically damaged during the process of P CO₂ treatment. If the cell wall was ruptured this would also affect the activity of enzymes (Garcia-Gonzalez et al., 2007). Further investigation was carried out to verify this assumption.

4.6.2 Effect of P CO₂ treatment on the enzyme activities of FIB

Objective

To determine the effect of P CO₂ treatment on the enzyme activities of FIB treated with P CO₂ in order to gain insight into the inactivation mechanism.

Theory

Loss of enzyme activities has been used as a strong explanation of antimicrobial action of P CO₂ treatment. Enzymes are a vital component of cytoplasm whose efficiency is maximum at the optimal pH. Drop of intracellular pH as a consequence of the dissolution of CO₂ in the aqueous phase of cytoplasm can lead to the inhibition and /or inactivation of key enzymes and therefore could contribute to the loss of cell viability (Hutkins and Nannen, 1993).

Procedure

Enzyme activities of FIB (untreated and treated with P CO₂ at 2800 kPa for 24 Hrs) were determined with the help of API ZYM strips (BioMérieux, Basingstoke, UK). Details of the procedure used are described in 3.2.1.10.

Results

Enzyme activity studies showed that the enzymes active in pure culture of *E. coli* were: alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-B1-phosphohydrolase and β glucosidase. While, alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, acid phosphatase, naphthol-AS-B1- phosphohydrolase and α glucosidase were the enzymes active in untreated culture of *Salmonella*. It was, however, shown that none of the enzymes active in the untreated cell culture were active in the cultures treated with P CO₂ at 2800 kPa for 24 Hrs. Inactivation of cellular enzymes as a result of P CO₂ treatment has been reported by earlier researchers.

Results and Discussion

Ishikawa et al. (1995a) found that the enzymes lipase and alkaline phosphatase completely lost their activities as a result of P CO₂ treatment at 15000 kPa and 35°C. P CO₂ treatment of *Lactobacillus plantarum* resulted in the loss of 5 out of 13 enzymes active before treatment (Hong and Pyun, 2001). Ballestra et al. (1996) detected that the intracellular acidification as a result of dissolution of CO₂ causes the selective inhibition of enzymes with acidic isoelectric points whereas, those with basic isoelectric points remained unaffected. Effect of P CO₂ treatment over a range of pressure (8500 and 12000 kPa), temperature (30, 50 and 70°C) and a treatment time of 40 min on the activity of enzymes; alkaline phosphatase, acid phosphatase, ATPase and pectinase was studied by Bertoloni et al. (2006). Increase in pressure and treatment time enhanced the inactivation of all enzymes except ATPase. Kim et al. (2007b) found that 4 out of 9 enzymes active in untreated *Salmonella typhimurium* lost their activities as a consequence of P CO₂ treatment at 10000 kPa and 35°C. Therefore, enzyme inactivation was identified as one of the major reasons of bacterial inactivation and it was in agreement with the previous research.

4.6.3 Release of UV absorbing substances from FIB as a consequence of P CO₂ treatment

Objective

To determine whether P CO₂ treatment results in the release of UV absorbing substances from the FIB.

Theory

Since CO₂ is lipophilic in nature (Kamihira et al., 1987), it can interfere with the structure and function of proteins (Jones and Greenfield, 1982) and biological membranes by replacing the membrane lipids with CO₂ molecules (Sears and Eisenberg, 1961, Dixon and Kell, 1989). Hence the intracellular materials (majority of which are proteins) are released when the applied CO₂ pressure is reduced (Lin et al., 1992, Lin et al., 1993). Proteins absorb maximum light at 280nm due to the presence of aromatic amino acids tyrosine and tryptophan which are present in almost all proteins in variable proportions (Pham et al., 2010b). Some researchers have used this absorbance as a tool for detecting the presence of proteins in the supernatant of bacterial culture treated with P CO₂ (Hong and Pyun, 2001, Kim et al., 2007a, Kim et al., 2007b).

Procedure

Detection of UV absorbing substances at 260 and 280 nm in the supernatant of FIB as a result of P CO₂ treatment (2800 kPa for 24 Hrs) was carried out according to the procedure described in detail in 3.2.1.11.

Results

These studies showed that UV absorbance in the centrifuged supernatant from treated cells was higher than for untreated cells (Table 4.7) pointing towards the loss of proteins and other UV absorbing substances as a consequence of P CO₂ treatment.

Table 4.7 UV absorbance by untreated and treated supernatant of FIB

| Wavelength | <i>E. coli</i> | | <i>S. enterica</i> | |
|------------|----------------|---------|--------------------|---------|
| | Control | Treated | Control | Treated |
| 260 nm | 0.06 | 1.23 | 0.49 | 0.73 |
| 280 nm | 0.05 | 0.90 | 0.23 | 0.45 |

An increase in the UV absorption by the supernatant of cell suspension treated with P CO₂ in a continuous flow system was noted by Shimoda et al. (1998) although no data was shown. Enhanced UV absorbance (260 and 280 nm) by the supernatant of cell suspension treated by P CO₂ has also been reported by Hong and Pyun (2001), Kim et al. (2007a) and (2007b) and has been linked to the extraction of intracellular substances such as proteins and nucleic acids from the microbial cells during treatment.

Taking into account the results from all three experiments conducted to determine the mechanism of action of P CO₂ for the inactivation of FIB, it can be concluded on the basis of SEMs that the treated cells were mechanically injured. The TEMs supported this and showed empty spaces in the cytoplasm suggesting possible leakage of intracellular substances, which was further supported by enhanced UV absorbance in the supernatant from treated cell suspensions. Even when the cells seemed intact, their cytoplasm had either leaked out or aggregated. Some images, however, clearly showed the ruptured cells and the cell walls with lost cytoplasm: this was particularly evident for *S. enterica* where the organisms appeared completely flattened in the third dimension. Enzyme activity was found to be completely lost following P CO₂ treatment, further supporting the loss of viability. These results support the concept of complex interrelated steps involved in microbial inactivation as a result of P CO₂ treatment (Garcia-Gonzalez et al., 2007).

Results and Discussion

4.7 Assessment of the initial concentration and natural die away of *E. coli* in CSS

Objective

This experiment was conducted to determine the initial concentration of *E. coli* in freshly collected CSS as well as to monitor the drop in the initial concentration of *E. coli* over a period of 16 days. The basic objective was to determine the time intervals between collections of CSS during which there was likely to be no significant drop in the initial concentration of *E. coli*.

Theory

It was necessary to make sure that the anaerobic digesters receive a constant input of *E. coli* since the reduction of initial concentration of *E. coli* in mesophilic anaerobic digestion would be monitored in the subsequent experiments.

Procedure

After collection, the CSS was stored in a refrigerator in a loosely closed screw capped bottle to avoid any pressurisation of the container. The MPN technique was used to determine the initial concentration of *E. coli* (3.2.1.6 a) on the day of collection and then on the 4th, 8th, 12th and 16th day after collection. Cell count of *E. coli* was expressed as MPN g⁻¹.

Results

Initial concentration of *E. coli* in CSS was found to be about 10⁵ MPN g⁻¹ of wet weight (WW) which declined to about 10³ MPN g⁻¹ WW by day 16 of storage (Figure 4.12).

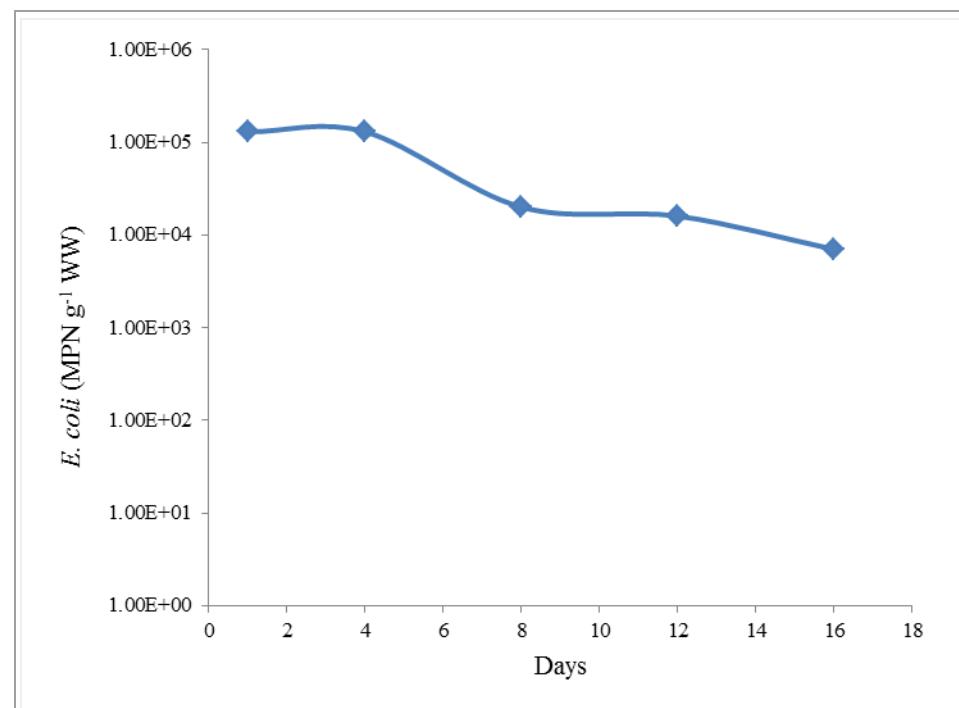


Figure 4.12 Natural die away of *E. coli* in CSS

On the basis of these results it was decided that the sludge would be collected every fortnight for semi-continuous CSTR AD trials in order to maintain a relatively consistent feedstock while avoiding excessive natural die off of *E. coli*.

4.8 Treatment of CSS with P CO₂

Objective

The experiment was designed to determine if the highest pressure (2800 kPa) and longest treatment time (24 Hrs) were sufficient to inactivate a significant proportion of FIB in the CSS and to promote biogas production.

Theory

Spooner et al.,(2007) have reported about 30-40% increase in biogas production by pre-treating secondary sludge with compressed biogas (400-600 kPa) prior to AD. It was assumed that the dissolution of CO₂ in the aqueous phase of sludge, penetration in the bacterial cells and expansion upon decompression resulted in the cell rupture and release of cell contents. Hence the biomass was readily available to methanogenic bacteria resulting in the increase in biogas production. Based on the results from pure culture experiments with FIB it was anticipated that these conditions would give sufficient inactivation of FIB to meet the requirements of the safe sludge matrix (ADAS, 2001).

Results and Discussion

Procedure

P CO₂ treatment of CSS was carried out at 2800 kPa for 23 Hrs (23 Hrs treatment time was selected to allow for 1 hour time for degassing of sample before feeding the treated CSS to the anaerobic digesters). Initial concentration of FIB in the CSS was determined using the MPN technique as described in 3.2.1.6 (a). About 600g of CSS was added to the pressure vessel, the same sample weight was treated alongside in a plastic bottle. After 23 Hrs, the vessel was depressurised and both samples tested for FIB using the MPN technique.

Results

Surprisingly, it was found that only 0.3 log reduction was achieved for *Salmonella* and 0.2 log reduction for *E. coli* in CSS treated at 2800 kPa for 23 Hrs (Table 4.8).

Table 4.8 Log reduction of FIB in CSS treated at 2800 kPa for 23 Hrs

| Bacterium | Time (Hrs) | Control 1 | Control 2 | Treated | Log reduction |
|---|------------|-----------|-----------|----------|---------------|
| <i>Salmonella</i> (MPN g ⁻¹ WW) | 24 | 91 | 24 | 13 | 0.3 |
| <i>E. coli</i> (MPN g ⁻¹ WW) | 24 | 2.80E+06 | 5.40E+05 | 3.10E+05 | 0.2 |

This result could be due to the influence of the solids content of the suspending medium on the inactivation of microorganisms. There is a consensus that the water content of material to be sterilised by P CO₂ plays a positive role towards the inactivation of microorganisms (Kamihira et al., 1987, Debs-Louka et al., 1999, Dillow et al., 1999, Schmidt et al., 2005). CO₂ dissolves in the water to produce H₂CO₃ which dissociates to produce H⁺ ions, and lowers the pH of substrate leading to the intracellular penetration of dissolved CO₂ due to the increase in cell wall permeability. Intracellular pH drop then leads to a series of interrelated events eventually causing the loss of viability (Garcia-Gonzalez et al., 2007). In the present study, significant inactivation in the case of broth culture as compared to very low log reduction in the case of CSS could be attributable to the higher water content of broth culture, although at only about 3-5% VS the water content of the CSS is still very high. In the case of presence of oil or fat, however, the dissolution of CO₂ is inhibited and bacterial cells are protected from inactivation by the formation of an extra protective coating around them (Kim et al., 2008). This might provide an alternative protective mechanism as these materials are present in CSS.

Another possibility is that the larger sample size with a smaller surface area used in the CSS experiments might cause a reduction in rate of inactivation. Further experiments were conducted to verify this assumption.

4.9 Comparison of two pressure vessels for the inactivation of FIB in CSS and broth culture

Objective

To examine the difference in extent of inactivation of FIB when different and same volumes of broth culture and CSS were treated in two different pressure vessels at the same pressure and treatment time. It was also designed to assess the influence of sample size, matrix, size of pressure vessel and sample volume on the rate of inactivation.

Theory

The experiments using broth cultures of FIB and CSS were carried out using pressure vessels of different size and capacity. There were differences in the density of broth culture and of CSS. As the water content of broth culture was much higher than CSS the dissolution of CO_2 in the former might be higher and as dissolution of CO_2 has been identified as the cause of inactivation (Isenschmid et al., 1995, Hong et al., 1999) this might have a large impact. It might also be the case that CO_2 might dissolve and would be released faster where the sample size is smaller and the surface area to volume ratio is higher. The effect of sample density, volume and size of pressure vessel on the rate of inactivation of FIB were compared in these experiments.

Procedure

Two experiments each were carried out with CSS and broth culture of *E. coli*. In the first experiment, same volume (100 g) of either substrate was treated in each pressure vessel. In the second experiment, different volumes (600 g and 3 g) of either substrate were treated in the large and small vessels respectively (corresponding to the volumes used in optimisation of broth culture and P CO_2 treatment of CSS).

Since the natural concentration of *Salmonella* in CSS was less than 10^6 CFU ml^{-1} , therefore 950 g of sludge was spiked with 50 ml of a pure culture of *Salmonella* with a cell density of 10^9 CFU ml^{-1} in order to achieve the desired 10^6 CFU ml^{-1} of *Salmonella* in CSS. The culture of *Salmonella* was prepared according to the procedure described in 3.2.1.1 and the initial numbers of *E. coli* and *Salmonella* confirmed using the MPN technique 3.2.1.6 a. In the first experiment with CSS, 3g of sludge was added to the small vessel and 600g to the

Results and Discussion

large vessel and each vessel pressurised to 2800 kPa for 23 Hrs. A sample was kept at ambient temperature and pressure as control. At the end of treatment time, number of FIB in treated and control samples was determined using MPN technique. In the second experiment 100g of sludge was added to both the large and small pressure vessels and each vessel pressurised to 2800 kPa for 23 Hrs. A sample was kept at ambient temperature and pressure as control. At the end of treatment time, number of FIB in treated and control samples was determined using MPN technique (3.2.1.6 a).

E. coli culture with a cell density of 10^6 CFU ml⁻¹ was prepared as described in 3.2.1.1 and the initial number of cells enumerated using MPN technique (3.2.1.6 a). Both experiments (same and different volume) were conducted at 2800 kPa for 23 Hrs. Control samples were kept alongside the pressure vessels in both experiments. Number of *E. coli* in treated and control samples was determined using MPN technique (3.2.1.6 a).

Results

In the tests using 100 g of CSS, there was very little difference in log reductions for both *E. coli* (0.3 and 0.4) and *Salmonella* (1.4 and 1.3) in the small and large pressure vessels respectively (Table 4.9). In the test using 3 g of CSS in the small vessel and 600 g in the larger vessel, the small vessel showed 3.0 log reduction in *E. coli*, compared to only 0.2 log reduction in the large vessel. The result for *Salmonella* was less marked with log reductions of 2.6 and 1.6 for CSS in the small and large vessels respectively (Table 4.9).

Table 4.9 Comparison of two pressure vessels for the inactivation of FIB in CSS

| Microorganism | Small pressure vessel | | Large pressure vessel | |
|----------------------|-----------------------|-------------------|-----------------------|-------------------|
| | <i>E. coli</i> | <i>Salmonella</i> | <i>E. coli</i> | <i>Salmonella</i> |
| Same volume CSS | 0.3 | 1.4 | 0.4 | 1.3 |
| Different volume CSS | 3.0 | 2.6 | 0.2 | 1.6 |

Treating the same amount (100 g) of broth culture of *E. coli* in the small and large vessels resulted in an 8 log reduction equivalent to complete inactivation. There was however a fall in log reduction to 5.4 in the large vessel when the amount treated was increased to 600 g (Table 4.10). This result strongly suggested that sample size affects the rate of inactivation of microorganisms. This was supported by the results for CSS but the difference was smaller and thus sample composition clearly played a major role.

Table 4.10 Comparison of two pressure vessels for the inactivation of *E. coli* in broth culture

| | Small pressure vessel | Large pressure vessel |
|--------------------------------|-----------------------|-----------------------|
| | Log reduction | |
| Same volume broth culture | 7.9 | 7.9 |
| Different volume broth culture | 7.5 | 5.4 |

4.10 Biochemical Methane Potential tests on raw and P CO₂ pre-treated CSS and WAS***Objective***

To determine the effect of P CO₂ pre-treatment on the biochemical methane potential of CSS.

Theory

The BMP test shows the amount of substrate carbon that can be converted in to methane under favourable incubation conditions with a healthy methanogenic inoculum and an adequate supply of nutrients. It may also provide an insight into the performance of CSTR digesters fed with that substrate (Chiu et al., 1997). A BMP test of untreated and P CO₂ pre-treated CSS should be able to show if the P CO₂ treatment can make more of the substrate carbon bioavailable for conversion to methane to study the influence of this pre-treatment on the biogas potential and VS destruction. The BMP test included samples of Co - Settled Sewage Sludge (CSS) and Waste Activated Sludge (WAS) both components of the combined settled sludge used in previous experiments.

Procedure

Two BMP tests were carried out, the first for treated and untreated CSS only and the second for treated and untreated CSS and WAS.

In the first test the inoculum to substrate (I/S) ratio was approximately 5:1 on a VS basis, and appropriate amounts of inoculum and substrate were weighed into each digester individually. A positive control of cellulose was also included.

In the second test, samples of two substrates and inoculum were collected from Millbrook WWTW, Southampton on the same day. A sub-sample of CSS was immediately pressurised and left for 23 Hrs while another sub-sample was maintained at room temperate and ambient pressure nearby. The WAS was allowed to settle for 24 Hrs then the supernatant was decanted and subsamples were respectively pressurised and

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maintained under local ambient conditions. The inoculum was stored in a water bath at 37°C before use. The BMP test on the treated and untreated CSS samples thus began one day earlier than the treated and untreated WAS, but used the same inoculum. Since the procedure adopted of individually weighing the inoculum and substrate in digesters in 1st BMP test was found to have some accuracy problems therefore this method was modified in 2nd test. Modified method involved the mixing of inoculum and substrate in the desired ratio and then the mixture was filled in reactors. The number of reactors was also increased from 3 to 5 which were denoted as 1-5 (see table 4.1). For the CSS, 2550 g of inoculum was mixed with 450 g of treated or untreated CSS to give a 5:1 I/S ratio on a VS basis. In the case of WAS the ratio was 3:1 which equated to 1650 g of inoculum mixed with 550 g of treated or untreated WAS. This lower ratio was determined by the quantity of thickened WAS available. For each test replicate bottles were made up each containing 400 g of the respective mixtures; inoculum only controls were run in parallel. The initial conditions for the second BMP tests are summarised in Table 4.11.

Table 4.11 Initial conditions of second BMP test

| Sample | Material | Inoculum | | | Substrate | | | I:S |
|------------|--------------------------------|----------|--------------------------|------|-----------|--------------------------|------|------|
| | | g WW | g VS kg ⁻¹ WW | g VS | g WW | g VS kg ⁻¹ WW | g VS | |
| C (1-5) | Inoculum | 400 | 23.1 | 9.3 | 0 | 0 | 0 | |
| SS (1-5) | Raw Sewage Sludge | 340 | 23.1 | 7.86 | 60 | 52.1 | 3.13 | 2.51 |
| TSS (1-5) | Treated Sewage Sludge | 340 | 23.1 | 7.86 | 60 | 52.1 | 3.13 | 2.51 |
| WAS (1-5) | Waste Activated Sludge | 300 | 23.1 | 6.94 | 100 | 14.7 | 1.47 | 4.72 |
| TWAS (1-5) | Treated Waste Activated Sludge | 300 | 23.1 | 6.94 | 100 | 14.7 | 1.47 | 4.72 |

The procedure for carrying out the BMP is described in detail in 3.1.6.2.

Results

First BMP test

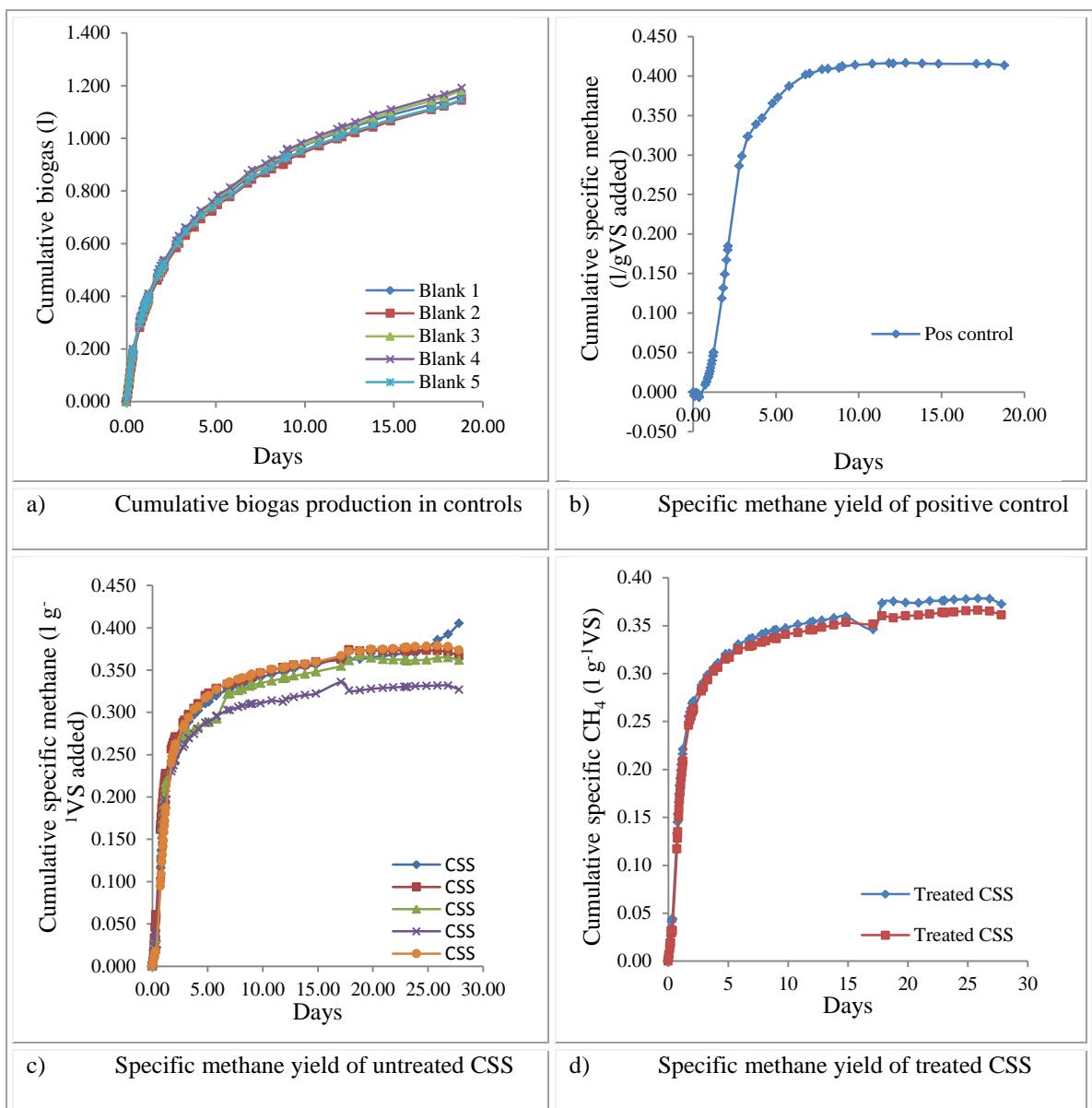


Figure 4.13 Biogas and methane yield in first BMP test

Figure 4.13 shows the results from the first BMP test. The cumulative biogas production of the controls showed good reproducibility and the positive control gave a net specific methane yield of cellulose of around $0.425 \text{ l CH}_4 \text{ g}^{-1} \text{ VS added}$, close to the theoretical value, indicating a healthy inoculum. The results for specific methane yield from both treated and untreated CSS were similar at around $0.37 \text{ l CH}_4 \text{ g}^{-1} \text{ VS added}$ but showed too much variability to draw a firm conclusion on any effect of the treatment. This may have been due to the method adopted of filling the bottles individually, which could lead to small variations in the weight and consistency of both sample and inoculum. The method used in the second test was therefore modified to avoid this problem.

Results and Discussion

Second BMP test

Quality control:

Figure 4.14 shows the cumulative biogas and methane yields from control and test samples. It can be seen that the values for the three control samples in each test run are in good agreement with each other (Figure 4.13 a and Figure 4.14 a, b), and the gas production indicates a healthy and active inoculum. The difference between the two sets of controls reflects the fact that the test on treated and untreated WAS started one day later, and the biogas generated by the second set of controls during this time was not included. The cumulative gas production curves for the two sets of test substrates also show good agreement, with no sudden discontinuities. The only exception to this is for sample SS5 which appeared to have a small leak on the digester: the results for this replicate were therefore ignored in further calculations. There was no sign of early stage acidification in the BMP curves indicating that the inoculum substrate ratios used were satisfactory

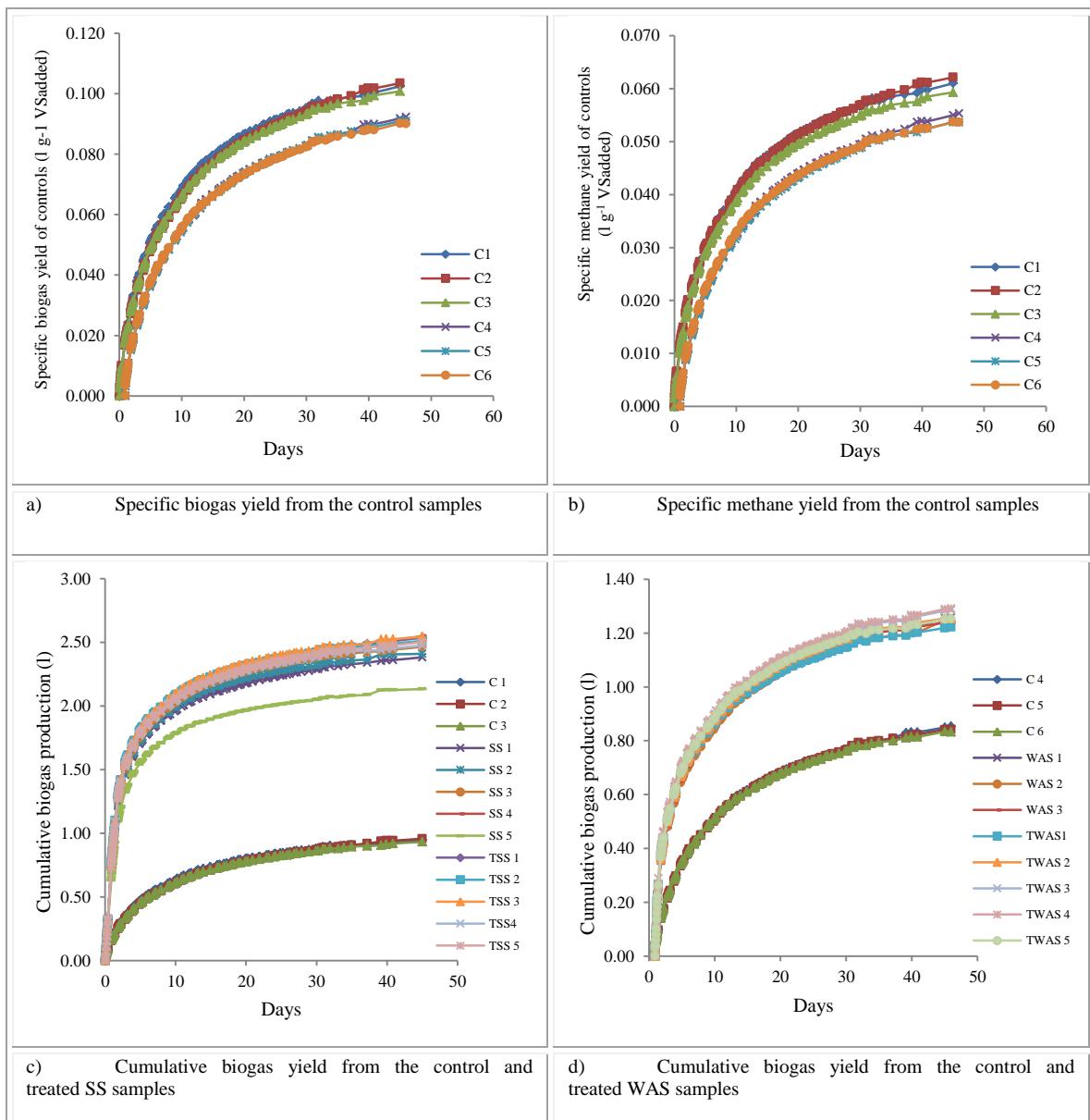
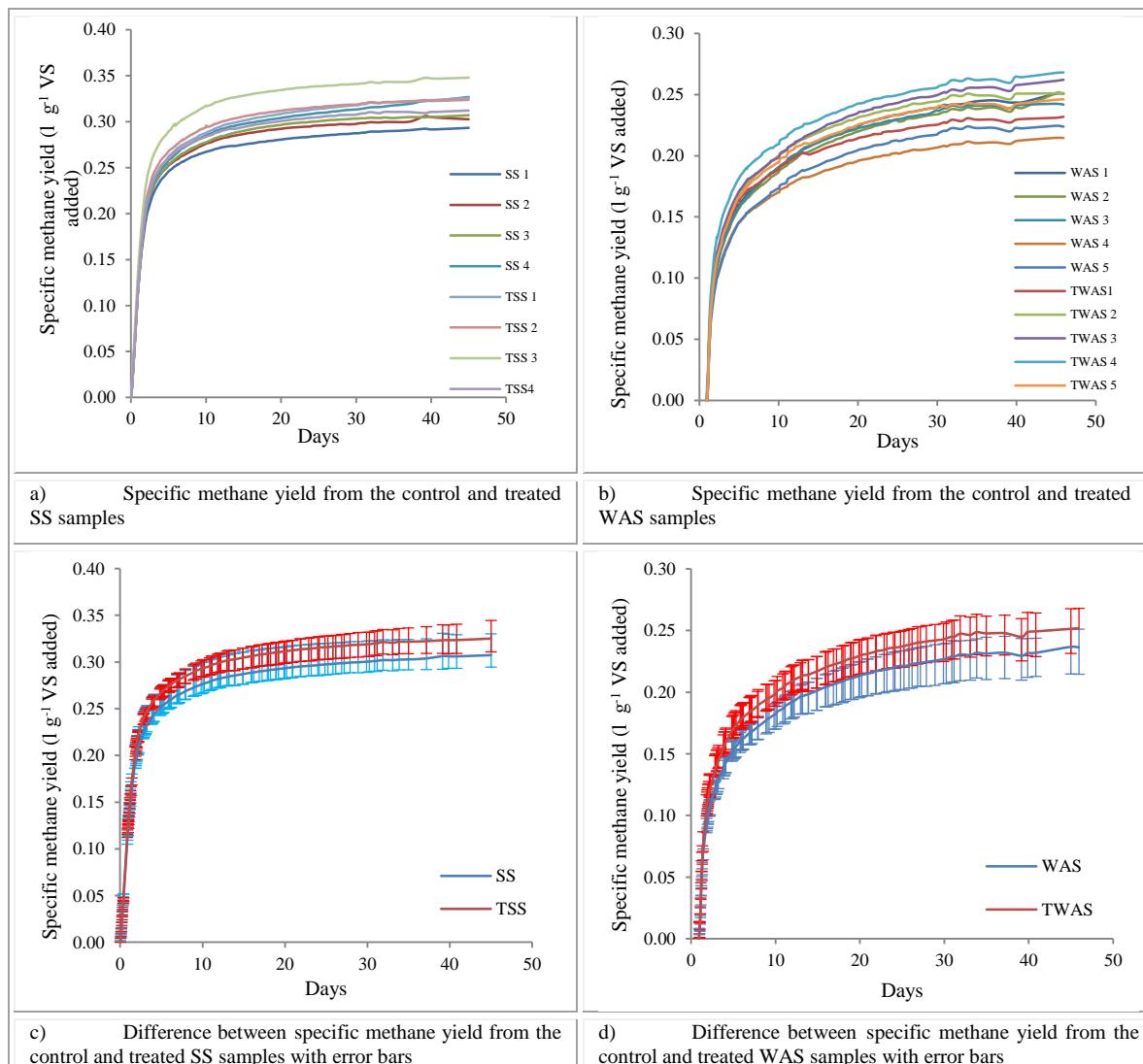


Figure 4.14 Specific biogas and methane yield from the control samples

Specific methane yields from the untreated and treated CSS and WAS samples are shown in Table 4.12 and Figure 4.15 a and b. There was a small amount of variability between samples: this was slightly greater for the WAS, probably reflecting the very small quantity of VS added in the test due to the low solids content of this material. As expected the methane yield of the CSS was higher than for the WAS. There appeared to be small differences between the average specific methane yields for treated and untreated CSS and also for treated and untreated WAS. Even with quintuplicate replicates and the modified method, however, the difference was too small in relation to the natural variability in the test to be confirmed as significant, as indicated by the overlapping error bars in Figure 4.15 c and d.

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**Figure 4.15 Specific methane yield from the untreated and treated SS and WAS samples
(Error bars represent range)**

Table 4.12 Specific biogas and methane yield (BMP test value)

| Samples | Biogas ($1 \text{ kg}^{-1} \text{ VS added}$) | Methane ($1 \text{ kg}^{-1} \text{ VS added}$) |
|---------------------------------------|--|---|
| Control (C) | 0.894 | 0.532 |
| Sewage Sludge (SS) | 2.387 | 1.403 |
| Treated Sewage Sludge (TSS) | 2.513 | 1.495 |
| Waste Activated Sludge (WAS) | 1.221 | 0.724 |
| Treated Waste Activated Sludge (TWAS) | 1.261 | 0.747 |

Kinetic modelling

Kinetic modelling of BMP test results is generally undertaken to help estimate the performance of the AD process and provide insight into the potential behaviour of the feedstock in full-scale applications (Rincón et al., 2010). In the current case, modelling was undertaken to see whether any difference could be identified between the treated and

untreated samples for each of the two substrates. To determine the kinetic constants, the specific methane production was modelled using a pseudo-parallel first-order model (Equation 4.1). This approach assumes that the gas production curve represents the rapid breakdown of readily degradable components, followed by slower degradation of the more recalcitrant proportion (Chynoweth et al., 1993, Rao et al., 2000). The methane production is therefore governed by two rate constants k_1 and k_2 :

$$Y = Y_{\max} (1 - Pe^{-k_1 t} - (1-P) e^{-k_2 t}) \quad (\text{Equation 4.1})$$

Where

Y is the cumulative methane yield at time t

Y_{\max} is the ultimate methane yield

k_1 is the first order rate constant for the proportion of readily degradable material

k_2 is the first order rate constant for the proportion of less readily degradable material

P is the proportion of readily degradable material

Table 4.13 Kinetic constants from modelling SS and TSS methane production

| Parameter values | SS1 | SS2 | SS3 | SS4 | SS5 | TSS1 | TSS2 | TSS3 | TSS4 | TSS5 |
|------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Y_m | 0.292 | 0.304 | 0.305 | 0.323 | 0.251 | 0.324 | 0.323 | 0.347 | 0.311 | 0.314 |
| P | 0.76 | 0.76 | 0.75 | 0.73 | 0.78 | 0.74 | 0.75 | 0.76 | 0.78 | 0.75 |
| k_1 | 0.85 | 0.84 | 0.86 | 0.84 | 0.87 | 0.84 | 0.83 | 0.85 | 0.81 | 0.84 |
| k_2 | 0.12 | 0.10 | 0.11 | 0.10 | 0.17 | 0.10 | 0.11 | 0.12 | 0.10 | 0.11 |
| R_2 | 0.9993 | 0.9989 | 0.9990 | 0.9990 | 0.9989 | 0.9991 | 0.9990 | 0.9992 | 0.9990 | 0.9990 |

Table 4.14 Kinetic constants from modelling WAS and TWAS methane production

| Parameter values | WAS1 | WAS2 | WAS3 | WAS4 | WAS5 | TWA S1 | TWA S2 | TWA S3 | TWA S4 | TWA S5 |
|------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Y_m | 0.251 | 0.250 | 0.250 | 0.215 | 0.225 | 0.232 | 0.251 | 0.262 | 0.268 | 0.246 |
| P | 0.46 | 0.46 | 0.45 | 0.49 | 0.45 | 0.53 | 0.46 | 0.46 | 0.5 | 0.47 |
| k_1 | 1.68 | 1.74 | 1.68 | 1.68 | 1.68 | 1.76 | 1.67 | 1.68 | 1.72 | 1.68 |
| k_2 | 0.10 | 0.10 | 0.10 | 0.11 | 0.10 | 0.11 | 0.11 | 0.10 | 0.10 | 0.12 |
| R_2 | 0.9993 | 0.9991 | 0.9994 | 0.9993 | 0.9992 | 0.9991 | 0.9991 | 0.9990 | 0.9991 | 0.9990 |

Table 4.15 Kinetic constant values for the average of all replicates of both substrates

| Parameter values | SS | TSS | WAS | TWAS |
|------------------|-------|-------|-------|-------|
| Y_m | 0.307 | 0.325 | 0.237 | 0.251 |
| P | 0.71 | 0.75 | 0.50 | 0.47 |
| k_1 | 0.81 | 0.84 | 1.72 | 1.68 |

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| Parameter values | SS | TSS | WAS | TWAS |
|------------------|--------|--------|--------|--------|
| k2 | 0.10 | 0.11 | 0.10 | 0.12 |
| R2 | 0.9961 | 0.9991 | 0.9993 | 0.9992 |

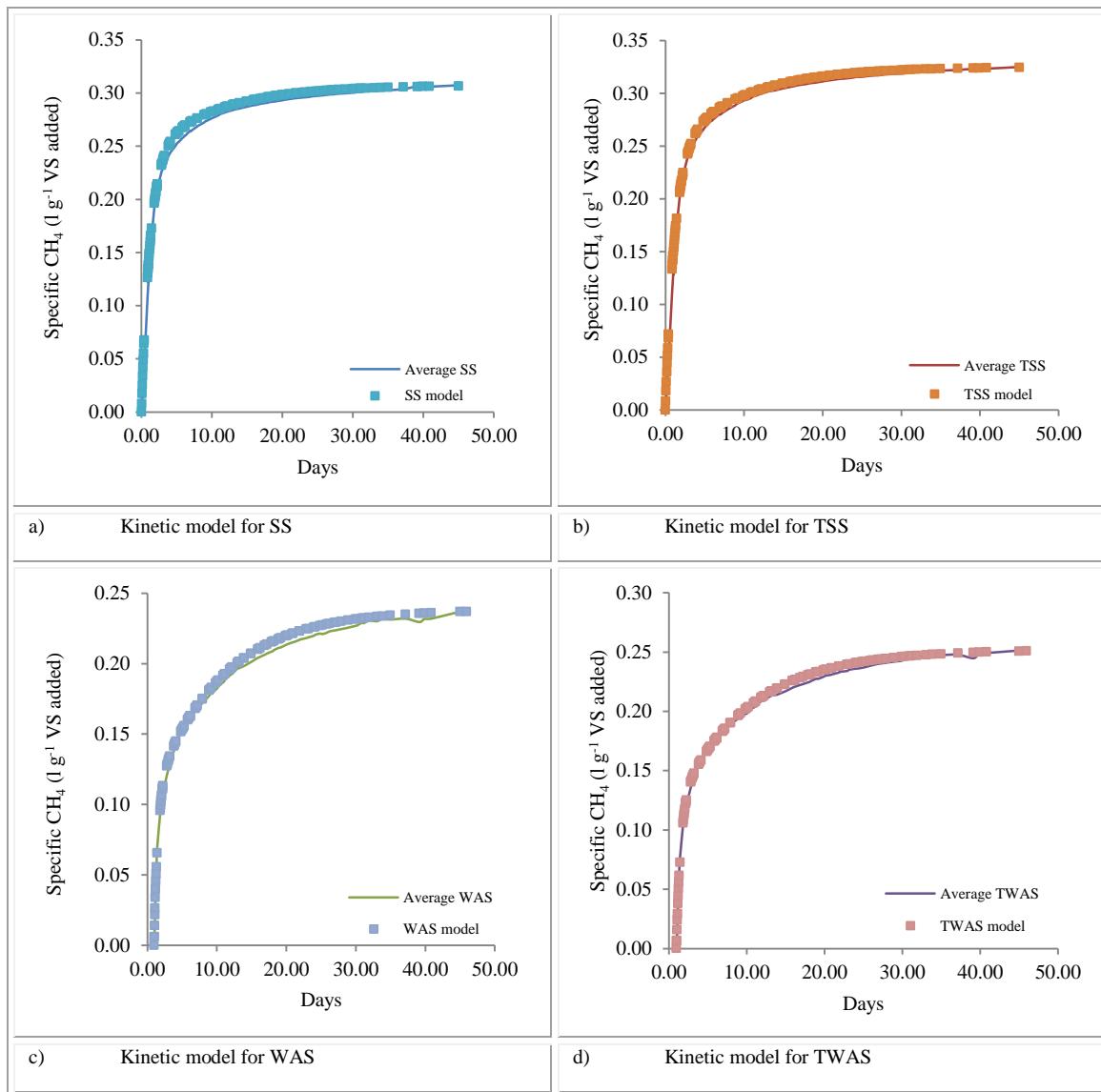


Figure 4.16 Kinetic models for untreated and treated SS and WAS

Table 4.13, Table 4.14 and Table 4.15 show the kinetic constant values for individual test replicates and for the average of two samples, respectively. Plotting the real data against the modelled values showed that the models fitted well (Figure 4.16), and this was confirmed by the correlation coefficients shown in the tables. For both untreated and treated CSS 90% of methane was released in the first 10 days confirming that a semi-continuous AD system operating at a short retention time of about 15 days might be expected to yield a high proportion of the methane potential. Methane release from the

WAS took slightly longer and appeared to reach a maximum value after 15 days indicating that a slightly longer retention time would be preferable in a semi continuous system. It can be seen, however, that there is no significant difference between the kinetic coefficients for the treated and untreated samples in either substrate. This confirmed the view that, under the treatment conditions used, no significant improvement in the specific methane yield of either substrate is likely to occur in semi-continuous digestion.

4.11 Semi continuous AD of CSS

Objective

To acclimate mesophilic anaerobic digesters to CSS and to quantify the effect of P CO₂ pre-treatment on biogas potential and concentration of FIB in the digestate.

Theory

The experiment was designed to assess the effect of P CO₂ pre-treatment of CSS on the biogas kinetics and pathogen inactivation. Two digestion trials were carried out. The first assessed the natural variability between digesters treated in the same way and receiving CSS as the feedstock: this trial aimed at sorting out any issues regarding the running of the digesters or limitations of the monitoring systems or analytical procedures. The second trial aimed to ascertain the effect of P CO₂ pre-treatment on the anaerobic digestibility of CSS, and to monitor the survival of FIB during the process of mesophilic anaerobic digestion.

Procedure

Digestion trial 1

Four semi-continuous CSTR anaerobic digesters (R1-R4) were set up as described in 3.1.6.1. The digesters had a planned OLR of 3 g VS l⁻¹ D⁻¹ and a HRT of 15 days. The digesters were initially loaded with 127 g day⁻¹ of CSS which was increased to 267 g by day 31. By day 52 a control strategy was adopted such that the digesters were fed on 267 g WW of CSS in which the VS content was adjusted to 44.9 g VS l⁻¹ by the addition of tap water. 250g of digestate was removed each day to maintain a constant digester volume, with the 17 g difference between feed and effluent quantities accounted for by the removal of biogas and losses of digestate during sampling. The pH of digestate and the gas counter reading for each digester was monitored daily. Analyses for ammonia nitrogen and VS destruction in the digestate were conducted once a week. Gas composition was determined

Results and Discussion

and the gas counter calibration checked twice a week. The trial was carried out for 170 days (about 11 HRT).

Digestion trial 2

This trial used two controls (C1 and C2) and two test (T1 and T2) digesters. The OLR was 2.5 g VS l⁻¹ day⁻¹ and the HRT was 15 days using a feedstock with the VS adjusted to 37.5g VS l⁻¹ by the addition of tap water. Each digester was fed with 267 g sludge and 250 g digestate was removed daily. Initially all four digesters were operated with untreated sludge for an acclimation period of 190 days (12 HRT). From day 191 onwards, the concentration of *E. coli* in digestate (MPN ml⁻¹) of all digesters was monitored twice a week for 3 HRT. From day 263 onwards, the feed to the two test digesters (T1 and T2) was switched to P CO₂ pre-treated (2800 kPa, 23 Hrs) sludge while the two control digesters (C1 and C2) continued to receive the raw sludge. The concentration of *E. coli* (MPN ml⁻¹) in the digestate of all digesters was again monitored for a period of 3 HRT to determine the effect of pre-treatment on the number of *E. coli*. The digesters were monitored for performance (specific biogas and methane and average daily biogas and methane production) and stability parameters (pH, alkalinity and ammonia) as before.

Results

Digestion trial 1

The characteristics of the CSS feedstock (% age of TS and VS) used during this trial were found to vary considerably (Figure 4.17). TS values ranged from 5.2-7.2% and VS from 3.9-5.7%. The variability in TS is apparent in Figure 4.17 but it can be seen that the proportion of volatile matter remained relatively similar at around 78% TS.

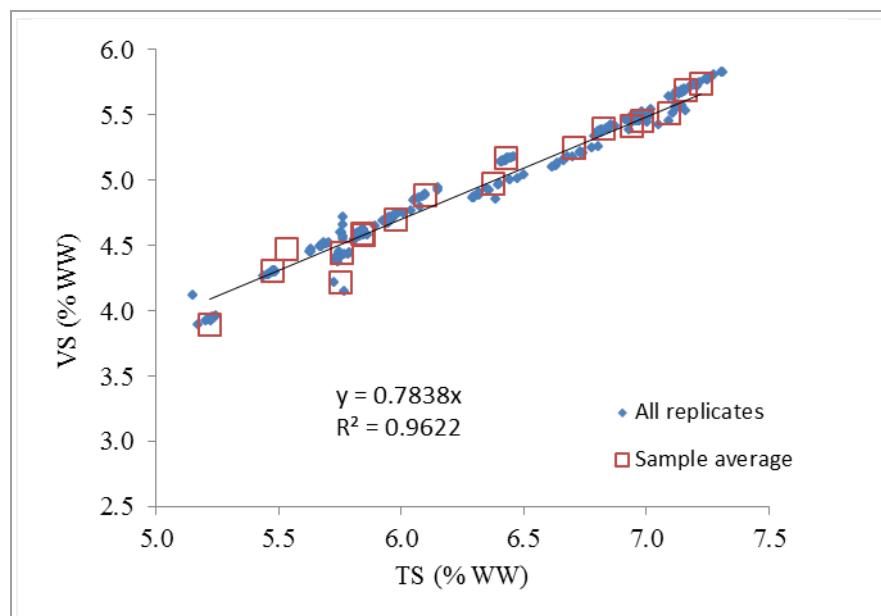


Figure 4.17 TS and VS of feed stock

The pH in the digesters fell slightly over the first ~60 days, which may be attributable to the gradual increase in loading. To maintain stable conditions the VS concentration of the feedstock was kept constant from day 52 onwards. From day 60 onwards the pH stabilised in all digesters at values between 7.2 and 7.3 (Figure 4.18 a). The total ammonia nitrogen (TAN) concentration in the digestate initially increased and there were slight differences in values between the digesters. From day 100 onwards however the TAN concentration in the digestate was stable at around from 1.14-1.69 g l⁻¹ and in close agreement in all four digesters (Figure 4.18 b). This value is typical of a sewage sludge digester and well below concentrations that may become inhibitory to methanogenesis (Koster and Lettinga, 1988). Alkalinity, which is an indicator of buffering capacity, had stabilised by day 100 at around 700 mg l⁻¹ CaCO₃. IA and PA are the indicators of volatile fatty acid (VFA) alkalinity and bicarbonate alkalinity respectively, and their ratio (IA/PA) is considered to be a useful parameter of digester stability. The ratio of IA/PA during this trial did not exceed 0.6 (Figure 4.18 d) indicating stable operation without any VFA accumulation (Ripley et al., 1986).

Results and Discussion

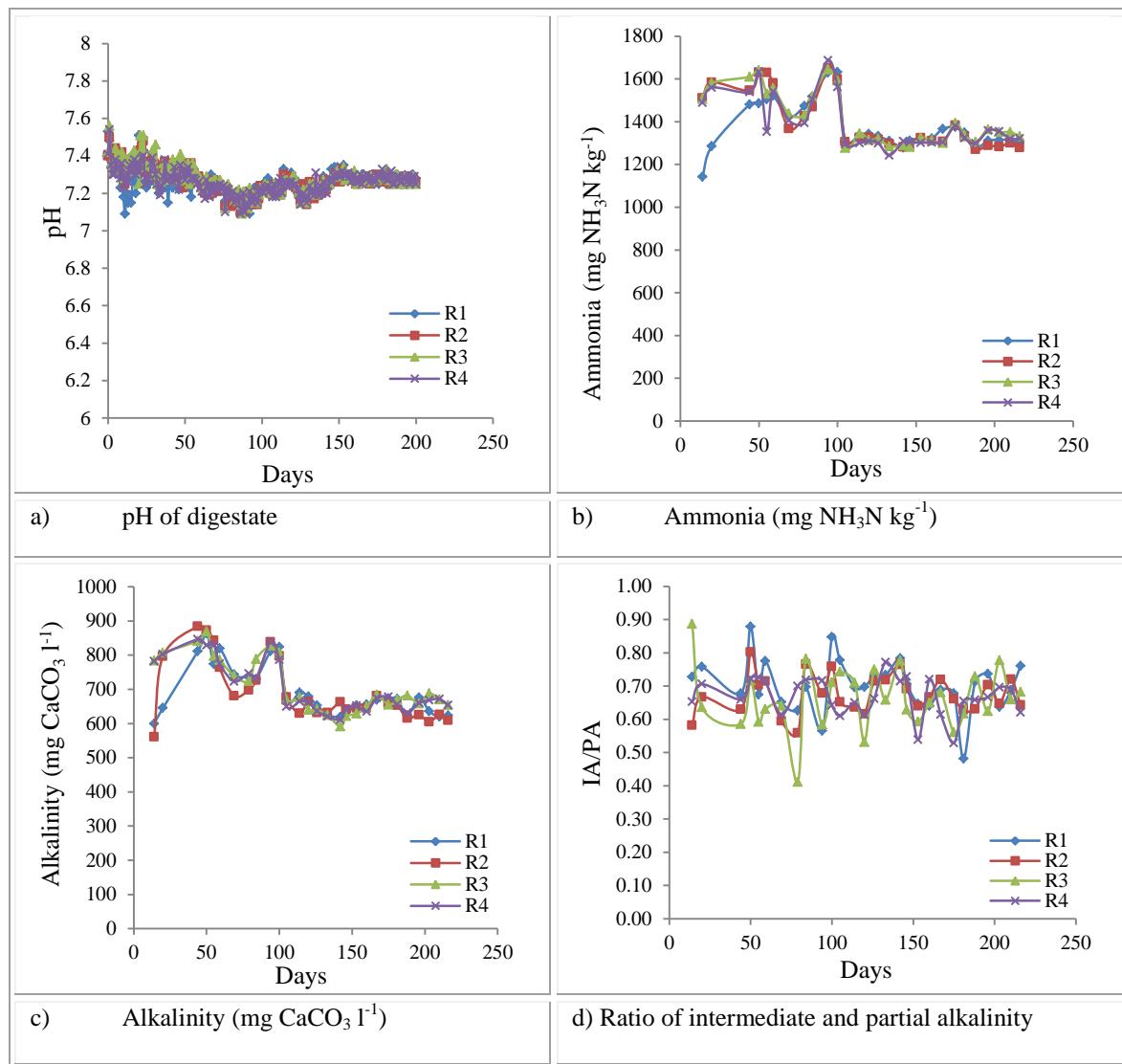


Figure 4.18 Stability parameters of digesters (Digestion trial 1)

The performance of digesters was initially slightly inconsistent but gradually stabilised from day 100 onwards (Figure 4.19). Average biogas production was about 6 l day⁻¹ and average specific biogas yield was 0.50 l g⁻¹ VS added. Average CH₄ production was about 3 l day⁻¹ and specific CH₄ yield was 0.32 l g⁻¹ VS added. Average CH₄ content of biogas and VS destruction were 65% and 48% respectively (Table 4.16). For the final 100 days of the run the agreement between gas production values for the 4 digesters was extremely good (Figure 4.19), and indicated that this mode of operation would be adequate for detecting differences in specific biogas and methane yields for treated and untreated feed stocks on the order of 5%. A baseline was therefore established for the upcoming trial in which two digesters would be fed with CSS pre-treated with P CO₂.

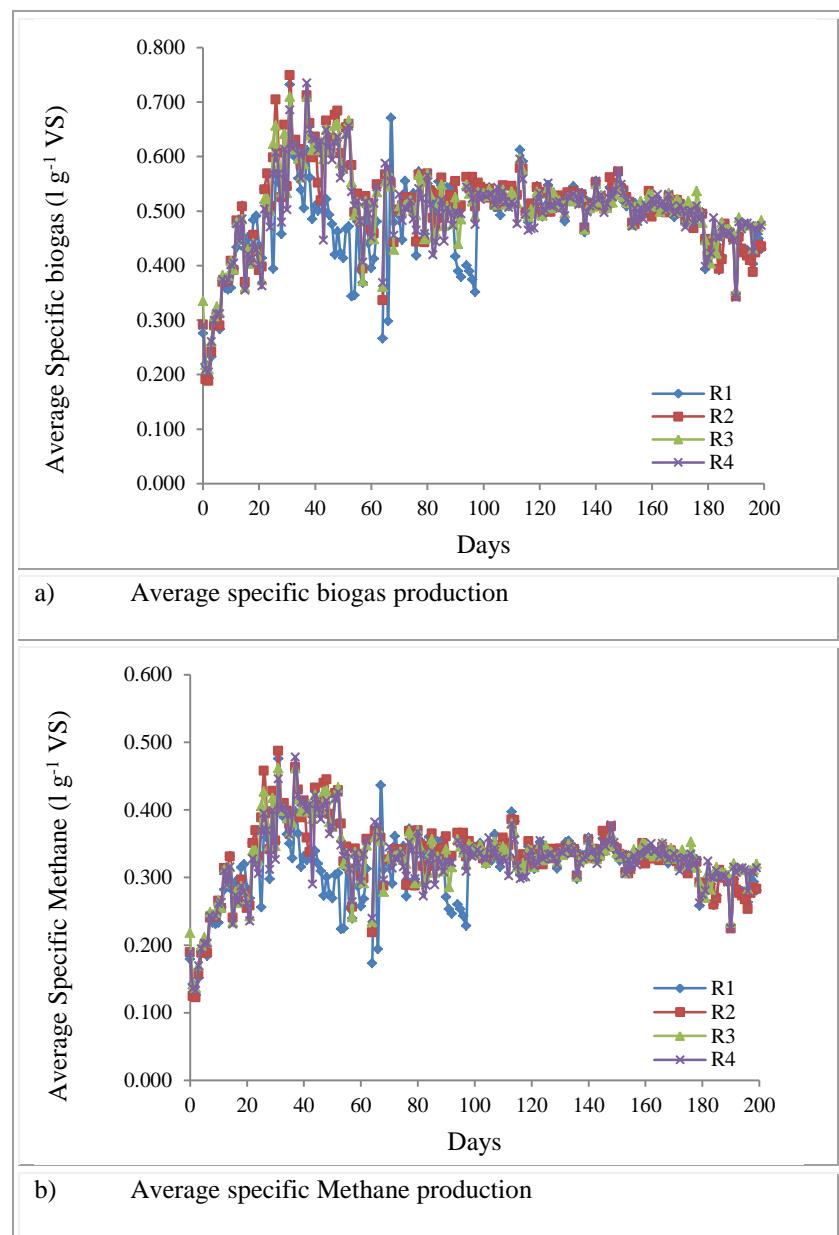


Figure 4.19 Performance parameters of digesters (Digestion trial 1)

Table 4.16 Performance parameters of anaerobic digesters (Digestion trial 1)
(average values for day 100-200)

| Digesters | Specific biogas yield (l g ⁻¹ VS) | Methane content in biogas (%) | Specific CH ₄ yield (l g ⁻¹ VS) | Volatile Solid destruction (%) |
|-----------|---|-------------------------------|--|--------------------------------|
| R 1 | 0.499 | 63.2 | 0.324 | 49 |
| R 2 | 0.499 | 63.2 | 0.324 | 48 |
| R 3 | 0.504 | 63.4 | 0.328 | 48 |
| R 4 | 0.500 | 63.2 | 0.325 | 49 |

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Digestion trial 2

In the second digestion trial the digesters were operated for 338 days (22 HRT). During the first 190 days (12 HRT) the digesters were acclimated and monitored to ensure stable operation. The concentration of *E. coli* (MPN ml⁻¹) was measured in the digestate of all four digesters for 3 HRT between days 190 - 263. From day 263 onwards, digesters R3 and R4 were fed with CSS pre-treated with P CO₂ (2800 kPa, 23 Hrs and 100 RPM). The concentration of *E. coli* (MPN ml⁻¹) in the digestate of all digesters was again monitored for 3 HRT.

The feedstock showed some variation over the course of the trial, with the TS ranging from 3.99 to 6.6%, and an average VS of 4.2%. The variability in TS is evident in Figure 4.20 but the proportion of volatile matter remained very similar throughout and close to the value in the previous trial of around 78 %TS. The method of ensuring a constant loading and HRT in the digesters by dilution of the collected sludge to 3.75 %VS was possible for most of the study period but there was a period towards the end of the trial where the VS of the sampled material dropped to around 3.4%; during this period the HRT was kept constant at 15 days and the OLR was allowed to vary.

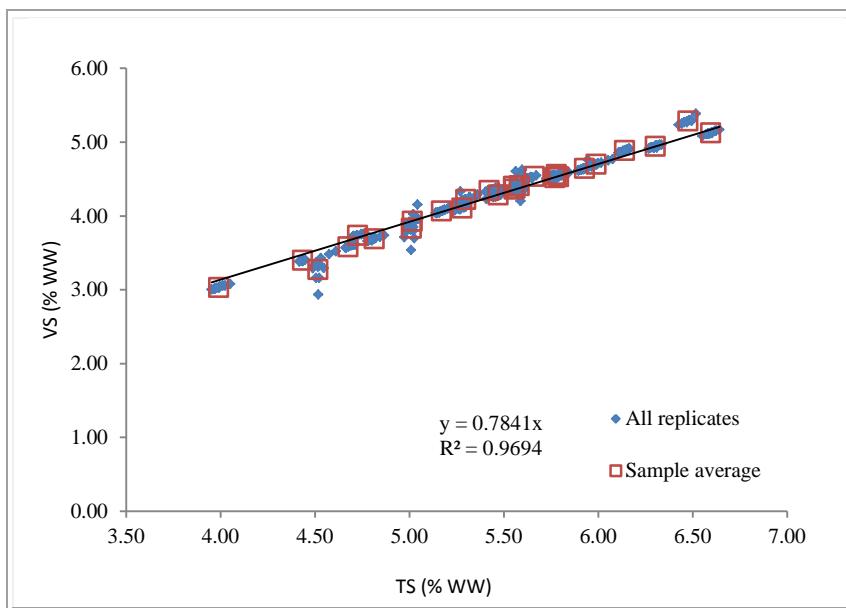


Figure 4.20 TS and VS of feed stock

The pH of the digesters initially showed some variation but from day 50 onwards stabilised in the range 7.2 to 7.3 (Figure 4.21 a), with no apparent difference due to P CO₂ pre-treatment. Total ammonia nitrogen in the digestate showed a very gradual increase in concentration over the trial period (Figure 4.21 b) as for the previous trial. There was no

difference in ammonia concentration between the control and the test digesters. Some difference might have been expected if the pressure treatment was leading to extensive cell disruption and improved hydrolysis: if this had occurred then more nitrogen would have been released from cell protein and reduced to ammonia as the carbon components were degraded. Total, partial and intermediate alkalinity were measured: the ratio of IA/PA (Figure 4.21 d) did not exceed the value of 0.6. The digesters can therefore be regarded as operating stably (Ripley et al., 1986), and again there was no apparent difference between the test and control digesters.

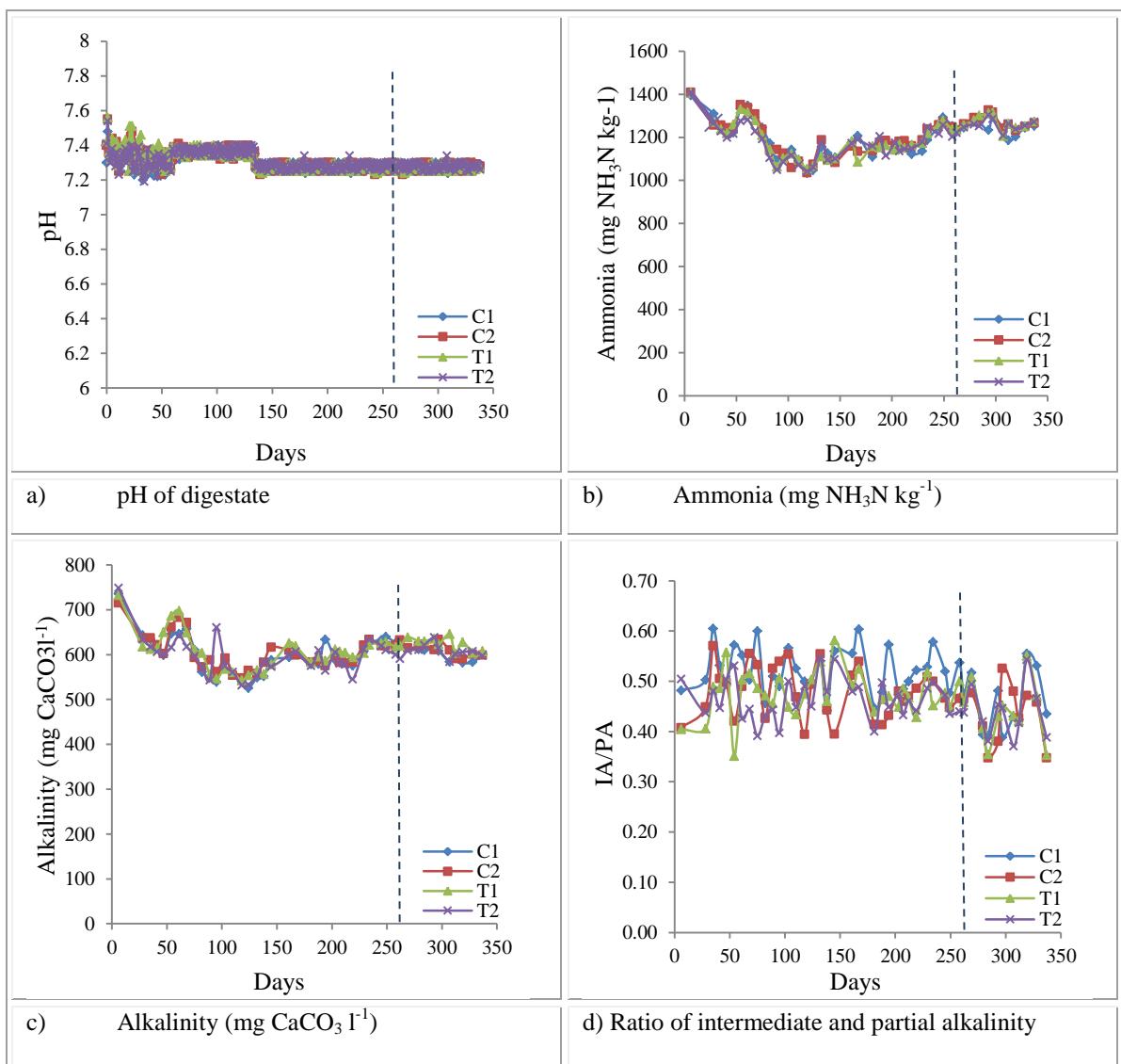


Figure 4.21 Stability parameters of digesters (Digestion trial 2)

(Vertical line indicates the start of feeding with P CO_2 pre-treated feedstock)

Average specific biogas production was $0.501 \text{ l g}^{-1} \text{ VS added}$, very similar to the value in the first trial. In the pre-trial period from day 0-262 it showed small variations which

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tended to follow the same pattern in all the digesters (Figure 4.22 a). This type of natural variation can be expected when running an experiment of this type and may be caused by small differences in daily feed due to slight inhomogeneity of the feedstock material. A similar pattern can be seen after the start of CO₂ pressurisation trial (day 263 onwards) and throughout that duration, but in this case there is also a slight downward trend in all digesters. The reason for this is unknown but is evidently related to the properties of the sludge itself, which appeared to be more dilute during the summer period as shown by the low %TS and VS obtained in the later part of the trial. It may be that in warmer weather some of the more putrescible components in the sludge were degraded at the treatment works in both sedimentation and storage tanks before being sampled.

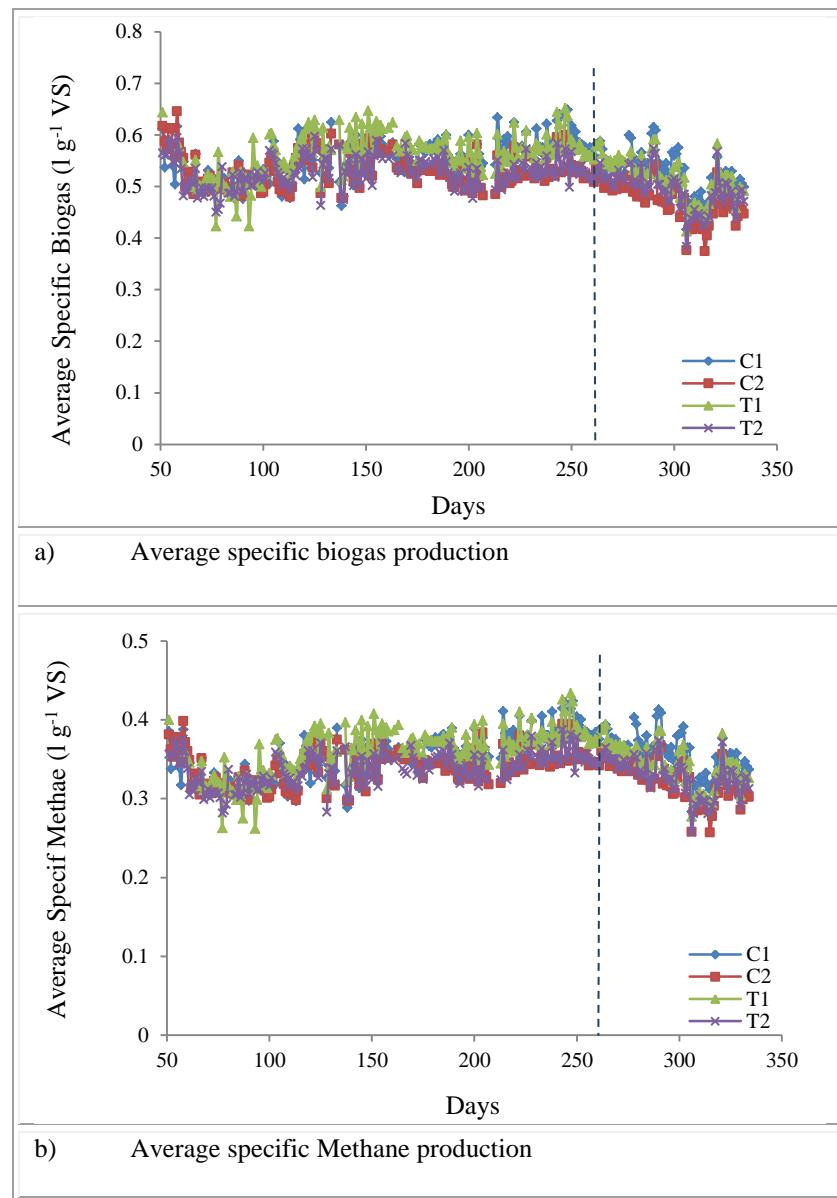


Figure 4.22 Performance parameters of digesters (Digestion trial 2)

(Vertical line indicates the start of feeding with P CO₂ pre-treated feedstock)

As expected the methane production (Figure 4.22 b) showed a similar trend to that for biogas: the average specific methane yield was 0.32 L g⁻¹VS added. VS destruction in control and test digesters was very similar with the two test digesters showing 48.4 and 48.0% destruction and the two controls 48.1 and 47.0 % destruction (Table 4.17). Performance was therefore typical of a single stage mesophilic digestion process for sewage sludge (Tchobanoglous et al., 2004). There appeared to be no benefit in terms of increased biogas yield as a result of the CO₂ pressurisation pre-treatment.

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The conditions applied in the pre-treatment used were much more stringent than those described by Spooner et al. (2007), where pre-treatment of waste activated sludge at 600 kPa for a duration of up to 60 minutes gave an apparent increase in gas production. This conflicts with the results of the current study but direct comparison is not be possible as the type and size of equipment used was different as well as the difference in feedstock.

Table 4.17 Performance parameters of anaerobic digesters (Digestion trial 2)

| Digesters | Specific biogas yield ($1 \text{ g}^{-1} \text{ VS}$) | Methane content in biogas (%) | Specific CH_4 yield ($1 \text{ g}^{-1} \text{ VS}$) | Volatile Solid destruction (%) |
|-----------|--|-------------------------------|---|--------------------------------|
| C 1 | 5.0 | 64.6 | 3.2 | 48 |
| C 2 | 4.9 | 64.7 | 3.1 | 47 |
| T 1 | 5.0 | 64.4 | 3.2 | 48 |
| T 2 | 4.7 | 64.5 | 3.0 | 48 |

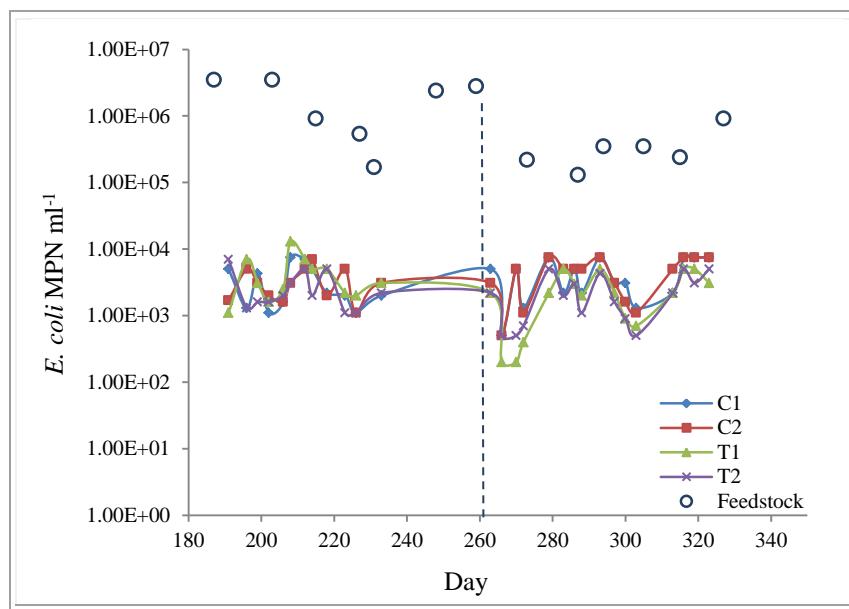


Figure 4.23 Concentration of *E. coli* (MPN g⁻¹ WW) in the feedstock and in the digestate of digesters (Vertical line indicates the start of feeding with P CO₂ pre-treated feedstock)

The concentration of *E. coli* in the feedstock on the day of collection ranged from 1.30×10^5 to 2.80×10^6 MPN g⁻¹ WW (Figure 4.23) and decreased on the storage of material under refrigerated conditions (see section 4.7). It is thus not possible to show directly a reduction in numbers of *E. coli* between the feed material and the digestate removed from the digester that can be attributed to the digestion process alone. The results are therefore presented as a comparison between the test and control digesters over the test period, compared to the differences observed in the pre-trial comparison of the digesters running under identical conditions.

The concentration of *E. coli* in the digesters over the study period ranged from 10^2 to 10^4 MPN ml⁻¹ which was on average about two log reductions below the concentration in the feedstock (10^5 to 10^7 MPN ml⁻¹). This was typical of the performance of a mesophilic digester fed on co-settled sewage sludge (Godfree and Farrell, 2005). There was no apparent difference in *E. coli* destruction before and after the start of CO₂ pressurisation treatment. The general pattern of removal in all of the digesters was similar, suggesting that the differences on a sample to sample basis probably reflected the *E. coli* load entering the digester, which can also be seen to vary in the feed sludge.

Digester operation was stable for 338 days over which the trial was carried out. During this time the performance was typical of a full-scale sewage sludge digester treating co-

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settled sludge, in terms of both the biogas production and *E. coli* destruction. The test digesters fed with CO₂ pressurised sludge showed no enhancement in either biogas production or *E. coli* destruction under the conditions used.

5 Conclusions and Future Work

5.1 Conclusions

- The research showed that pressurised CO₂ could be an effective means of reducing the numbers of faecal indicator bacteria present in pure culture even when used at relatively low pressures (1500-2800 kPa) and at ambient temperature. This provides new information, as previous studies have concentrated on much higher pressures where the CO₂ is in a supercritical state.
- The effect of different conditions of pressure and exposure time was clearly demonstrated in a series of experiments covering a range of conditions (1500 to 2800 kPa and 0.75 - 24 hours). The results showed that at higher pressure shorter exposure periods were sufficient to achieve a given degree of inactivation compared to the time required at lower pressures. The highest pressure was able to achieve 8 log reduction even at lower exposure times (18, 12, 9, 6, 3 and 1.5 hours). At a given pressure the relationship between log reduction and exposure time was linear.
- To achieve a 6-fold log reduction in a realistic timespan of less than 6 hours a pressure of 2800 kPa would be required, which is the highest cylinder pressure that can be achieved for CO₂ as a compressed gas. It must however be recognised that these times and pressures are dependent on the culture volume and the configuration of the apparatus, as was clearly shown in later experiments.
- The time of exposure required to reduce bacterial numbers was relatively long, however, in comparison with other recognised techniques for sanitisation.
- There appeared to be some difference in the sensitivity of different organisms, as shown by the shorter retention times and pressures required to achieve the same log reduction in numbers of *Salmonella enterica* compared to that for a strain of *E. coli* isolate from sewage sludge.
- The research developed a test methodology using simple equipment and a selection of standard microbiological techniques (MPN, spread plating and membrane filtration), which were found to be comparable in their ability to recover pure cultures of the test organisms.
- When tests were performed after stressing the test organisms by exposure to heat, use of a resuscitation technique was found to be beneficial in recovering viable cells. Resuscitation techniques were ineffective in recovering test organisms after

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treatment with pressurised CO₂, although this technique was only applied in situations where the organisms had been exposed to the highest pressure and duration of treatment.

- It is clear that pressurised CO₂ treatment is far less effective in inactivating faecal indicator bacteria that may be present as part of a community in wastewater sludges, than in pure culture. Even under the harshest conditions and using the more sensitive *S. enterica* as a test organism spiked into the sample there was only a small reduction in numbers compared to that occurring in pure culture.
- Although a number of theories have been proposed as to why pressurised CO₂ can cause a loss of viability in a wide range of microorganisms, the mechanism involved has not been clearly demonstrated. In the current study it was clear that inactivation was as a result of disruption of the cell wall and membrane. This was confirmed by both transmission and scanning electron microscopy, which showed physical damage to the cell structure, including evidence of the loss of cell contents through ruptured cell wall.
- Further evidence that the cells were completely inactivated was obtained by assaying the enzyme complement and those enzymes found to be active in healthy and viable cells were lost in cells which had been treated. This is again indicative of catastrophic failure, most likely associated with result of damage to the cell membranes and organelles.
- Yet further evidence for the loss of integrity of the cell wall and membranes was the presence of proteins in the supernatant liquor of treated cells after centrifugation. This has been clearly linked by previous studies to cell lysis and loss of viability.

In summary the results showed conclusively that under the test conditions used pressurisation and rapid depressurisation with CO₂ caused irreversible cell damage resulting in the loss of viability of both *S. enterica* and a cultured strain of *E. coli*. The effect was reproducible and a time-pressure relationship was established for the apparatus used. It is likely however that the sanitising effect will be influenced by culture volume, probably because the ratio of surface area to volume affects the dissolution of CO₂ and its penetration into the cell. This finding may limit the practical application of the approach, and further work is needed to establish the parameters and to develop reactor systems that

could overcome restrictions of this type. It must be appreciated however that even under the very favourable conditions used in these experiments the exposure times required for 6 log reduction were long enough to make commercial application problematic without significant improvements in performance.

- Reported increases in methane production after pressurised CO₂ treatment were investigated in batch and semi-continuous culture. The results from batch Biochemical Methane Potential testing were unable to show a statistically significant difference in methane yield or gas production kinetics between the treated and untreated material. There was however an apparent small difference in methane yield which could be of interest in long-term continuous operation of a digester treating co-settled sludge.
- Operation of semi-continuous digesters at a constant organic loading rate and hydraulic retention time confirmed that it was possible to identify differences in gas yield of 5 % and this technique would therefore be applicable to assessing differences of this order between treated and untreated samples. On running duplicate pairs of digesters with and without pressurised CO₂ treatment of the feedstock over a steady-state operating period of more than 100 days, no difference could be found in the biogas production or specific methane yield.
- The concentration of *E. coli* in the sewage sludge feedstock was found to drop from 10⁵ MPN ml⁻¹ to 10³ MPN ml⁻¹ (wet weight) over a 16-day storage period from the day of collection. This natural reduction meant that the only reliable method of ascertaining the effect of treatment was to compare the numbers present in digesters fed untreated material or material treated after storage.
- During this continuous trial the numbers of *E. coli* in the digestate from digesters receiving treated and untreated feedstocks showed no significant difference. It can be concluded that the pressurised CO₂ treatment used at a maximum cylinder pressure of 2800 kPa and an exposure time of 23 hours with a sludge volume of 600 ml in a 2-litre cylindrical pressure vessel agitated on an orbital shaker was ineffective at either increasing the biogas or methane yield from co-settled sewage sludge, or at reducing the number of *E. coli* found in the digestate.
- Experiments were conducted out to look at the inactivation of *E. coli* naturally present in co-settled sewage sludge and also of *S. enterica* spiked into a sewage sludge matrix. These experiments were carried out in a larger pressurisation

Conclusions and Future work

system than that used for the initial pure culture inactivation trials and the loss of viability was insignificant in *E. coli* although some reduction in numbers of spiked *S. enterica* was noted.

In summary, pressurised CO₂ treatment was not found to be effective in the reduction of numbers of *E. coli* found in sewage sludge or of *S. enterica* when added to a sewage sludge matrix. Although the size and headspace of the pressurisation vessel was shown to affect the degree of log reduction this could not fully explain the lack of performance in comparison with pure culture. It was concluded that the sludge matrix itself must play a role in protecting the microbial consortia from the effects of pressurised CO₂. The exact reasons for this were not discovered but may be due to the effect of dissolved compounds present in the sludge and/or the structure and nature of the sludge flocs themselves. The long-term digestion simulation experiments showed that even treatment conditions which had shown 8 log reduction in *E. coli* in pure culture were ineffective in reducing the number of indigenous *E. coli* present in the sludge. The experiments also showed conclusively that pressurisation and depressurisation under the conditions used did not improve either the biogas productivity or the specific methane yield. Optimisation of the design of the pressurisation vessel may improve this and should be a focus of any future investigations.

5.2 Future Work

Where pressurised CO₂ treatment has been used in an intermediate range in which the loss of viability is low, further testing should be carried out to determine the effectiveness of resuscitation techniques. This would help to identify whether there is an intermediate state between total cell destruction where cell membranes have been shown to be ruptured, and those where less severe conditions may have caused changes in cytoplasmic pH leading to localised inhibition of enzyme systems that could be recovered in favourable conditions for resuscitation and restoration of viability. This may be particularly important in establishing the effectiveness of this type of treatment in natural populations where the cells may be protected by being part of a floc or due to the presence of dissolved compounds in the medium. One key direction for future work is to attempt to elucidate the protective mechanisms operating in the sludge matrix with a view to reducing or eliminating their effectiveness and increasing the effectiveness of pressurisation.

It is essential that future work should fully evaluate the effects of sample volume, surface area and headspace volume on log reduction. Although moderate mixing was induced when using the larger pressure vessel by placing it on an orbital shaker, this may not have been effective at increasing the surface area and gas transfer rate. Other methods of agitation should be investigated including rolling and tumbling, and even the use of non-coupled stirring devices although this may be more difficult to achieve in the case of a heavyweight pressure vessel.

The aim of future work in this area must be to couple experimental findings with the design of potential larger-scale treatment systems. Future work should also consider the benefit of this type of technology in increasing the methane yield or reducing the number of faecal indicator bacteria in relation to alternative approaches and technologies that could be used for the same purpose. Even the use of pressurised CO₂ at cylinder pressure of 2800 kPa is at a higher pressure than can be used in industrial-scale reactors without special certification. Future work should therefore look at enhancing the effect of CO₂ pressurisation-depressurisation cycles at lower operating pressures, preferably less than 1000 kPa. The work should therefore focus on the engineering design necessary to maximise gas transfer of CO₂ from the headspace into the bulk sludge.

If an effective engineering solution can be found that allows improvement in performance then a full energy balance and life cycle assessment should be undertaken to allow proper comparison with competing new and established technologies.

6 Appendices

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 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 (1500, 2000 and 2800 kPa) and treatment time (from 0.75 - 24 hours). 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.

Keywords: 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 (Water UK, 2007, Luduice, 2001). 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 and 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 *E. 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.

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 7380 kPa) (Kamihira et al., 1987, Ishikawa et al., 1995b, Kim et al., 2007a, Kim et al., 2007b) 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

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unsuitable for a large-volume, low-value product such as sewage sludge. 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 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 a). Recently, an attempt has been made to use P CO₂ in subcritical conditions for enhancement of biogas production by treatment of sewage sludge prior to anaerobic digestion. 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 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 (Spooner 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 pre-treatment 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).

E. coli was enriched from freshly collected PSS according to the procedure in SCA (2003). *E. 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 hours. 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 hours then stored at 4 °C. These slants were used to inoculate TW to grow a pure culture in each subsequent experiment. *E. coli* was sub-cultured on fresh NA slants every fortnight to retain viability.

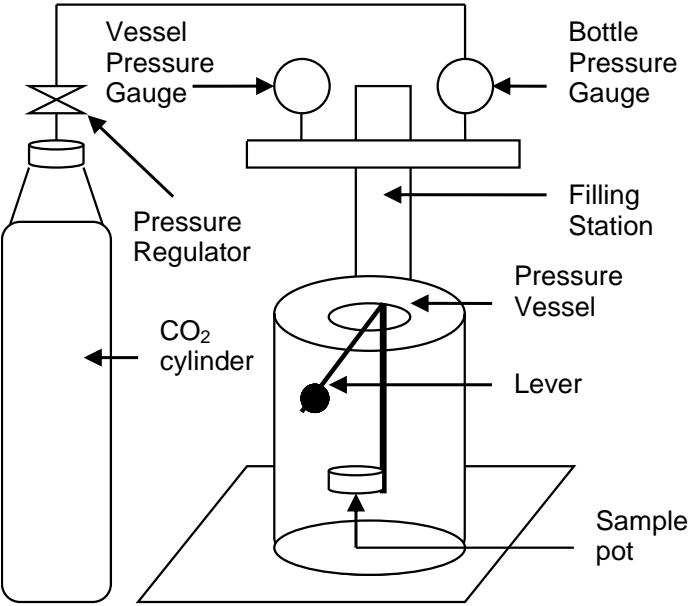
A culture of *E. coli* was prepared by inoculating 100 ml TW in an Erlenmeyer flask from NA slants and incubating in a shaking incubator at 37 ± 2 °C for ~12 hours. 0.1 ml of this was aseptically transferred to 100 ml TW and placed on an orbital incubator at 37±2 °C. At hourly intervals a sample was aseptically removed, a portion of which was placed into a cuvette for OD determination. 1 ml was transferred into 9 ml of Maximum Recovery Diluent (MRD). This 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 hours 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:

$$\text{Growth rate} = \left(\log_{10} \left(\frac{N}{N_0} \right) \cdot \log_e(10) \right) / (T - T_0) \quad [1]$$

$$\text{Doubling time} = \log_e(2) / \text{Growth rate} \quad [2]$$

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 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 (1500, 2000, 2800 kPa) and treatment times (24, 18, 12, 9, 6, 3, 1.5, 1 and 0.75 hours) 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 hours. 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 serially 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.

Appendices

| | |
|---|--|
|  |  |
| a) Schematic of P CO ₂ treatment unit | b) Pressurisation vessel |
| Figure 1. Carbon dioxide pressurisation equipment | |

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:

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

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 P CO₂, Scanning (SEM), and Transmission (TEM) Electron Microscopy studies were conducted with the untreated and treated (2800 kPa, 9 hours) *E. coli* cultures. Samples for microscopic study were centrifuged at 10000 x 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 M 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, 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 *E. coli* measured by OD and by \log_{10} CFU are shown in Figures 2a and b. OD versus \log_{10} CFU for the exponential growth phase of approximately 4 hours is shown in Figure 2c.

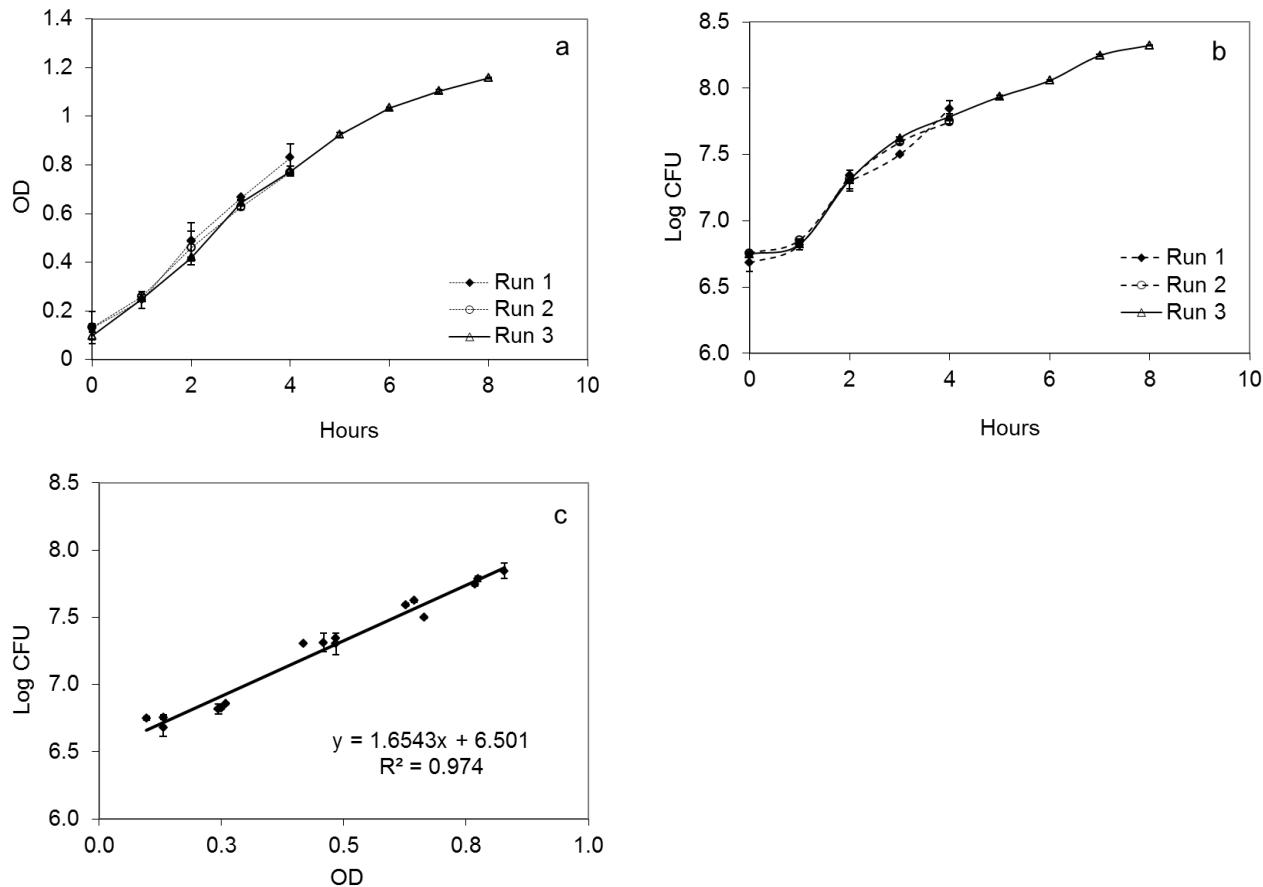


Figure 2. Growth curves and relationship between OD and log CFU

In the test runs the culture was grown for 3 - 4 hours 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 - 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 *E. coli* was around 0.6 hour^{-1} corresponding to a doubling time for the culture of 1.0-1.2 hours.

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 was expected, since the higher pressure increases the solubility of CO_2 in the aqueous phase of the reaction medium, which then forms carbonic acid that lowers the pH (Garcia-Gonzalez et al., 2007). 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).

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,

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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 2800 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 could be an important factor, but higher pressures or exposure periods may be required to produce satisfactory inactivation. At the highest pressure of 2800 kPa a 7.8 \log_{10} reduction was achieved in 1.5 hours, which would satisfy the requirements of the SSM and may also represent a feasible duration for practical application in wastewater treatment.

Table 6.1 Log₁₀ reduction in CFU at selected pressures and exposure times

| Hours | Pressure (kPa) | | |
|----------------|----------------|-------|-------|
| | 1500 | 2000 | 2800 |
| 24 | 8.2 | 8.3 | 8.2 |
| 18 | 4.6 | 8.1 | 8.2 |
| 12 | 4.0 | 8.2 | 8.2 |
| 9 | 3.3 | 5.0 | 8.2 |
| 6 | 2.5 | 2.9 | 8.1 |
| 3 | 0.5 | 2.2 | 7.9 |
| 1.5 | 0.3 | 1.5 | 7.8 |
| 1 | 0.2 | 1.3 | 2.3 |
| 0.75 | 0.2 | 0.4 | 0.27 |
| Slope* | 0.32 | 0.60 | 10.18 |
| R ² | 0.960 | 0.949 | 0.994 |

* Log₁₀ reduction in CFU per hour of treatment

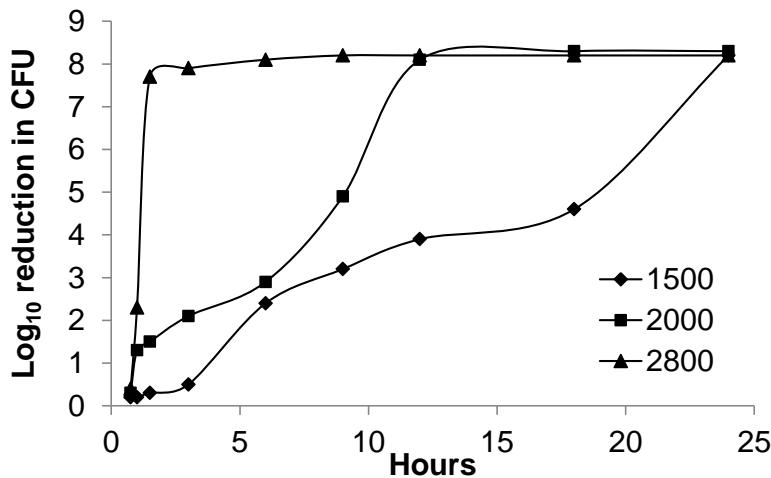


Figure 3. Log₁₀ reduction in CFU at selected pressures and treatment times

Scanning Electron Micrographs of *E. coli* treated with P CO₂ (2800 kPa for 9 hours) showed clear signs of rupture, holes and damage to the cells (Figure 4a) as compared to the smooth appearance of untreated cells (Figure 4b). This 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 was not seen on the micrographs of untreated cells.

Transmission Electron Micrographs showed that the cytoplasm of treated cells (Figure 4d) (2800 kPa, 9 hr) was unevenly distributed and had empty spaces, again indicating the possible loss of cell components, whereas that of untreated cells was evenly distributed (Figure 4c).

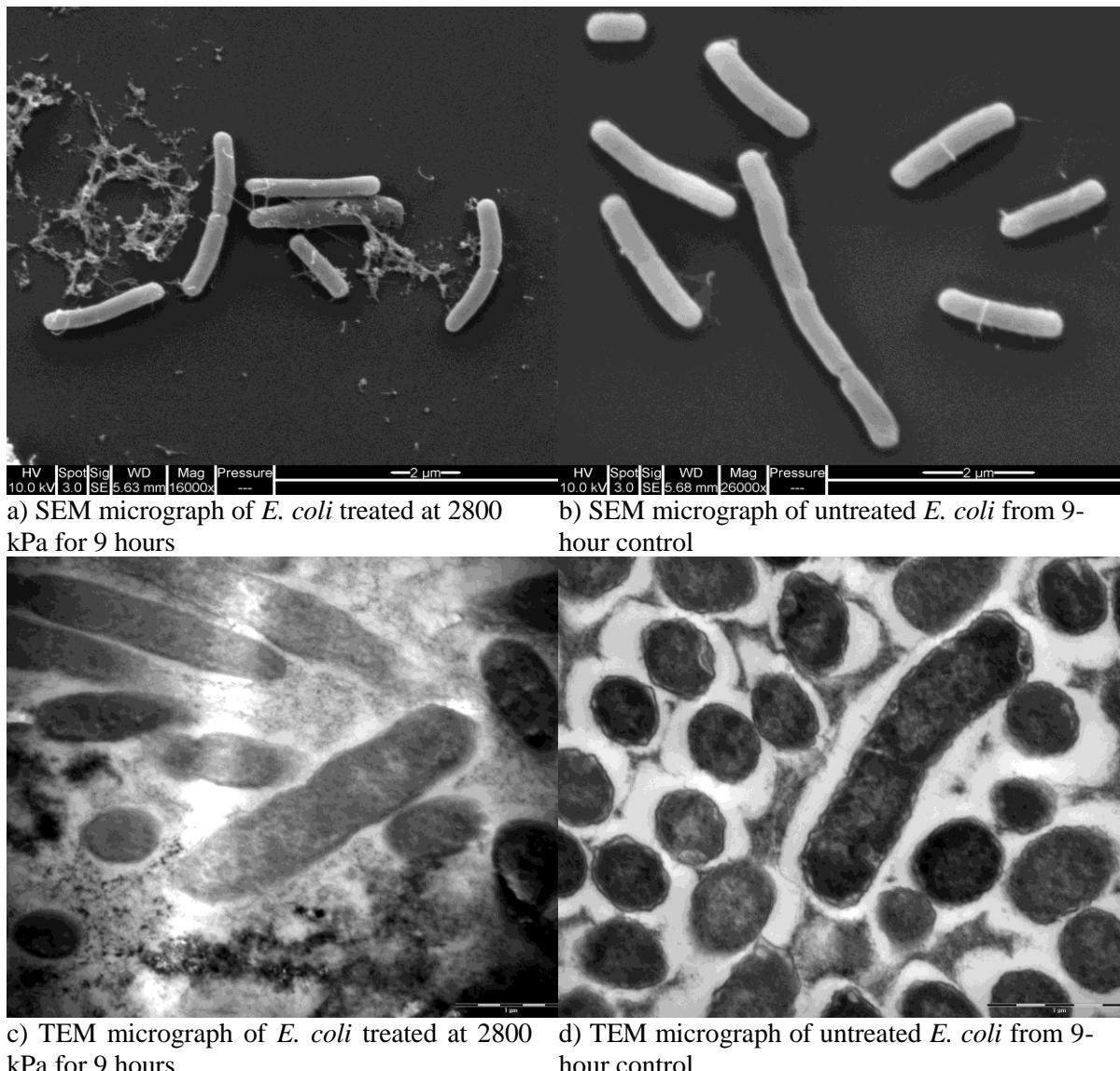


Figure 4. Electron micrographs of treated and untreated samples

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 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

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(Kamihira et al., 1987, Lin et al., 1992 a, Lin et al., 1993), and thus contribute to disruption as seen in Figure 4c.

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 hours). 7.8 log₁₀ reduction was achieved in 1.5 hours at the highest pressure (2800 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 biodegradability of SS in AD. Further studies on the anaerobic digestion 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.

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Appendices

Evaluation of pressurised carbon dioxide pre-treatment aimed at improving the sanitisation and anaerobic digestibility of co-settled sewage sludg

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Key words

Pathogen, biogas, cell ruptor, faecal indicator organism

Abstract

Pressurised carbon dioxide (P CO₂) is a useful technique for non-thermal food sterilisation and has also been reported to enhance biogas production in anaerobic digestion. In the current work, co-settled sewage sludge was pressurised with CO₂ at 2800 kPa for 23 hours before daily feeding to a pair of semi-continuous mesophilic digesters over a period of 70 days. The results were compared to those obtained from an identical pair of control digesters fed on the same material but without the pressure treatment. Both cases were compared to the results taken over a 90-day period where all 4 digesters were run on the same feed without any pre-treatment, to establish a baseline for performance and stability. As test parameters the digesters were monitored for specific biogas and methane production, volatile solids destruction and destruction of *Escherichia coli*. The results showed that the pre-treatment had no enhancement effect on any of these parameters.

Introduction

Anaerobic digestion (AD) is one of the most favoured technologies for retrieving renewable energy from organic wastes (DEFRA, 2007). Sewage sludge (SS) is a huge bio-resource whose production has been consistently increasing in the UK and elsewhere. Average annual production of SS in the UK during 2006-07 was 1.73 million tonnes (dry weight) (Water UK, 2008), 82.6% of which was either recycled to agricultural land or used for land reclamation (Water UK, 2007). AD is the method most frequently used by wastewater treatment plants for the stabilisation of SS (Pérez-Elvira et al., 2006) while also generating renewable energy (biogas) and organic soil conditioner (digestate) (Dohanyos and Zabranska, 2001). However, SS contains a significant proportion of pathogenic microorganisms (Strauch, 1991) that can survive the digestion process (Sahlström, 2006) and therefore potentially pose serious threats to human and animal health when digestate is applied to arable land (Sahlström, 2003). Increased public awareness due to the outbreak of food-borne diseases has intensified the demand for improved biosecurity and food safety. This became the driving factor for an agreement between the British Retail Consortium and UK Water in February 1998, known as 'The Safe Sludge Matrix' (SSM), which determines the criteria for safe land recycling of SS. As a result of this agreement, agricultural utilisation of untreated SS has been banned in the UK since 31 December 1999. Moreover, proper sanitisation of SS for the removal of pathogens in line with the quality criteria described in SSM is mandatory for making it safe for land recycling (ADAS, 2001).

There are various techniques, such as thermal, chemical, thermo-chemical, sonication, biological, enzymatic and mechanical methods, for pre-treatment of SS prior to AD (Appels et al., 2008). These are aimed at making the organic matter easily accessible to anaerobic bacteria, and thereby enhancing the digestibility and biogas production as well as decreasing the quantity of sludge for final disposal (Pérez-Elvira et al., 2006). Additional benefits could potentially include improved sludge dewatering, reduction in foaming and enhanced pathogen kill (Pérez-Elvira et al., 2006); but to be considered an ideal method the pre-treatment must also be economic both to install and to operate (Tyagi and Lo, 2011).

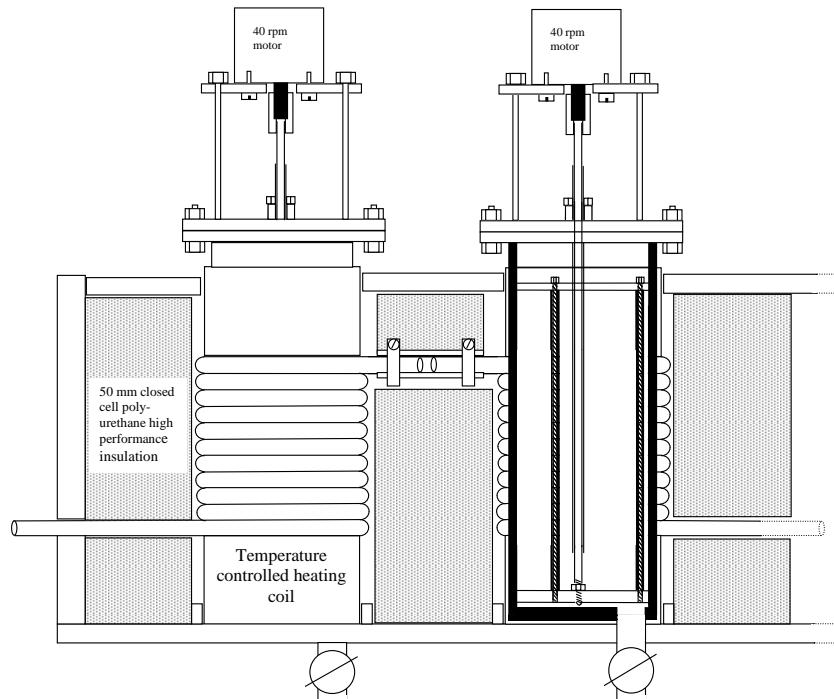
The detrimental effect of carbon dioxide (CO₂) on bacterial growth was first reported by (Valley and Rettger, 1927), but it is only over the last two decades that P CO₂ has become established as a technique for non thermal sterilisation (Garcia-Gonzalez et al., 2007). Its application has, however, been confined to the food industry until recently, when a novel process called 'Cellruptor' (initially Bug Buster) was developed by Eco-Solids International (Spooner et al., 2007) and was reported to enhance biogas production by around 30-40 % by using pressurised biogas (35-45% CO₂) to pre-treat secondary sludge biosolids before anaerobic digestion. The principle of pressurised CO₂ treatment as applied elsewhere is that CO₂ dissolves in the aqueous phase of a

liquid to form carbonic acid (H_2CO_3) which diffuses into any microbial cells present. This causes a drop in intracellular pH (Daniels et al., 1985) which affects the enzymatic activities (Jones and Greenfield, 1982) and triggers a series of complex interrelated processes leading to the loss of cell viability (Garcia-Gonzalez et al., 2007). Upon decompression, CO_2 dissolved in the cytoplasm expands causing the microbial cells to rupture and release their contents (Nakamura et al., 1994). It has been assumed in the case of anaerobic digestion that this cell rupturing process will make the biomass more readily available to anaerobic bacteria, thereby helping to overcome the rate-limiting hydrolysis step of AD (Spooner et al., 2007). The process could therefore boost biogas production and an additional benefit would be the enhanced sanitation of digestate as pathogenic bacteria might also be expected to be inactivated. The present study was carried out to test whether $P\ CO_2$ pre-treatment has these benefits when applied to co-settled sewage sludge before mesophilic digestion, by testing for improved biogas production and enhanced removal of *Escherichia coli* as a pathogen indicator organism.

Materials and Methods

Digesters. Four 5-litre capacity digesters with a 4-litre working volume were used in the study. These were constructed from PVC tube with gas-tight top and bottom plates. The top plate was fitted with a gas outlet, a feed port sealed with a rubber bung, and a draught-tube liquid seal through which a stainless steel asymmetric bar stirrer was inserted with a 40 rpm motor mounted directly on the top plate. Temperature was maintained at $37^\circ C \pm 0.5$ by water circulating through an external heating coil. The digesters were connected to tipping-bucket gas counters with continuous data logging. Calibration of gas counters was checked weekly by collecting the gas in a Tedlar bag (SKC Ltd, Blandford Forum, UK): the volume was then measured accurately in a weight-type water displacement gasometer (Walker et al., 2009). All gas volumes reported are corrected to standard temperature and pressure of $0^\circ C$, 101.325 kPa as described by Walker et al. (2009). Semi-continuous operation was achieved by removing digestate through an outlet port in the base plate before adding feed via the feed port in the top plate (Figure).

Inoculum and feedstock. The digesters were filled to working volume with anaerobic digester sludge obtained from Millbrook Wastewater Treatment Plant (Southern Water) Southampton, UK. Co-settled Sewage Sludge (SS) from the same site was used as feedstock over the experimental period; this was collected every two weeks and stored at $4^\circ C$ before use.



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Figure 1. Cut-away diagram showing the construction of the 5-litre digesters vessels, method of mixing and temperature control

Feedstock pre-treatment. P CO₂ pre-treatment was carried out in a 2.2-litre capacity stainless steel pressure vessel (Prosep Filter Systems Ltd. West Yorkshire, UK, model No. 530110XN10B10V). The vessel was fitted with a pressure gauge to measure vessel pressure and one port was fitted with a valve through which it could be pressurised with CO₂ and through which the pressure could subsequently be released. The pressure vessel was mounted on an orbital shaker (Barloward Scientific Ltd. Staffordshire, UK) to provide agitation for the duration of the pressurisation period. The vessel was pressurised to 2800 kPa by connection to a cylinder of CO₂ (BOC Ltd, UK)

1 litre of PPS feedstock was added to the vessel by unscrewing the body of the vessel from the inlet manifold and then re-assembling the two parts. After adding the sludge the vessel was pressurised for 23 hours whilst being shaken at 100 rpm. The pressure was then released rapidly, keeping the end of tube in a plastic bottle to minimise aerosol escape. The pressure vessel was then opened and the treated sludge was removed and allowed to de-gas for one hour before being used. This operation was performed daily to provide the feedstock for the digesters.

Digester operation. The digesters were operated with daily addition of feed to maintain an organic loading rate (OLR) of 2.5 g volatile solids (VS) /l/day and a Hydraulic Retention Time (HRT) of 15 days. To facilitate the control of both OLR and HRT the solids content of the co-settled sludge sampled every two weeks was measured and the concentration was then adjusted to give a VS of 37.5 g VS/l. The wet weight of feedstock added to each digester was always equal every day throughout the experimental period (267 g). The weight of digestate removed each day was 250 g (17 g less than the amount of feed added) so as to maintain a constant volume in the digester after taking into account biogas production and minor losses in sampling.



Figure 2. P CO₂ treatment vessel

Figure 3. Set of 5 l digesters in temperature controlled housing

All four digesters were initially operated with identical untreated sludge and allowed to stabilise before recording data for the purposes of comparison. The current study then used a data period equivalent to 6 x HRT (90 days) to provide baseline values to compare the operational stability and other digestion parameters for all 4 digesters running under identical conditions on the same feed. Two digesters were then switched to being fed with the pressure-treated sludge while the other two continued to receive untreated material, and the trial continued for another 71 days (4.7 HRT). When the 4 digesters were run as duplicates of test and control the feedstock PSS was taken from the same batch at the same time and treated equally with the exception of the pressure treatment. All 4 digesters were monitored in the same way and for the same parameters as used from the start of the trial. These were: pH, alkalinity, ammonia, biogas, biogas composition,

volatile solids (VS) destruction and concentration of *E. coli* in the digestate. Gas composition was measured on samples collected in a gas sampling bag over a 24 hour period.

Analytical methods. TS and VS were measured according to Standard Methods 2540 G (APHA, 2005). pH was determined using a Jenway 3010 meter (Bibby Scientific Ltd, UK) with a combination glass electrode calibrated in buffers at pH 4, 7 and 9.2 (Fisher Scientific, UK). Alkalinity was measured by titration with 0.25N H₂SO₄ to endpoints of pH 5.7 and 4.3, allowing calculation of total (TA), partial (PA) and intermediate alkalinity (IA) (Ripley et al., 1986). Ammonia concentration was determined using a Kjeltech steam distillation unit according to the manufacturer's instructions (Foss Ltd, Warrington, UK). Biogas composition (CH₄ and CO₂) was determined using a Varian star 3400 CX Gas Chromatograph with a porapak column operated at 50°C and a thermal conductivity detector. The GC was calibrated with a standard gas mix of 65% (v/v) CH₄ and 35% (v/v) CO₂ (BOC, Guildford, UK).

Concentration of *E. coli* (Most Probable Number per millilitre MPN/ml) determined by the Most Probable Number (MPN) technique as described in method 2 of the 'Methods for the isolation and enumeration of *Escherichia coli* including verocytotoxigenic *Escherichia coli*' (Standing committee of analysts, 2003). All microbiological reagents were obtained from Oxoid Ltd. (Basingstoke, UK) and all sterile media and equipment requirements met by sterilisation at 121°C for 15 min.

Results and Discussion

The co-settled sludge obtained from Millbrook STW in Southampton varied over the course of the trial with the TS ranging from 4.0 to 6.6%, and an average VS of 4.1%. The variability in TS can be seen in Figure 4 but the proportion of volatile matter remained very similar at around 78 %TS. The method of diluting the sludge to ensure a constant loading and HRT in the digesters by dilution of the collected sludge to 3.75%VS was possible for most of the study period but there was a period towards the end of the trial where the VS of the sampled material dropped to around 3.4%; during this period the HRT was kept constant at 15 days and the OLR was allowed to vary.

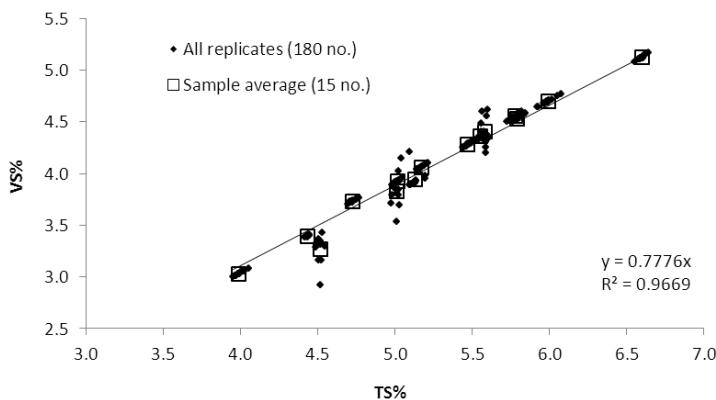


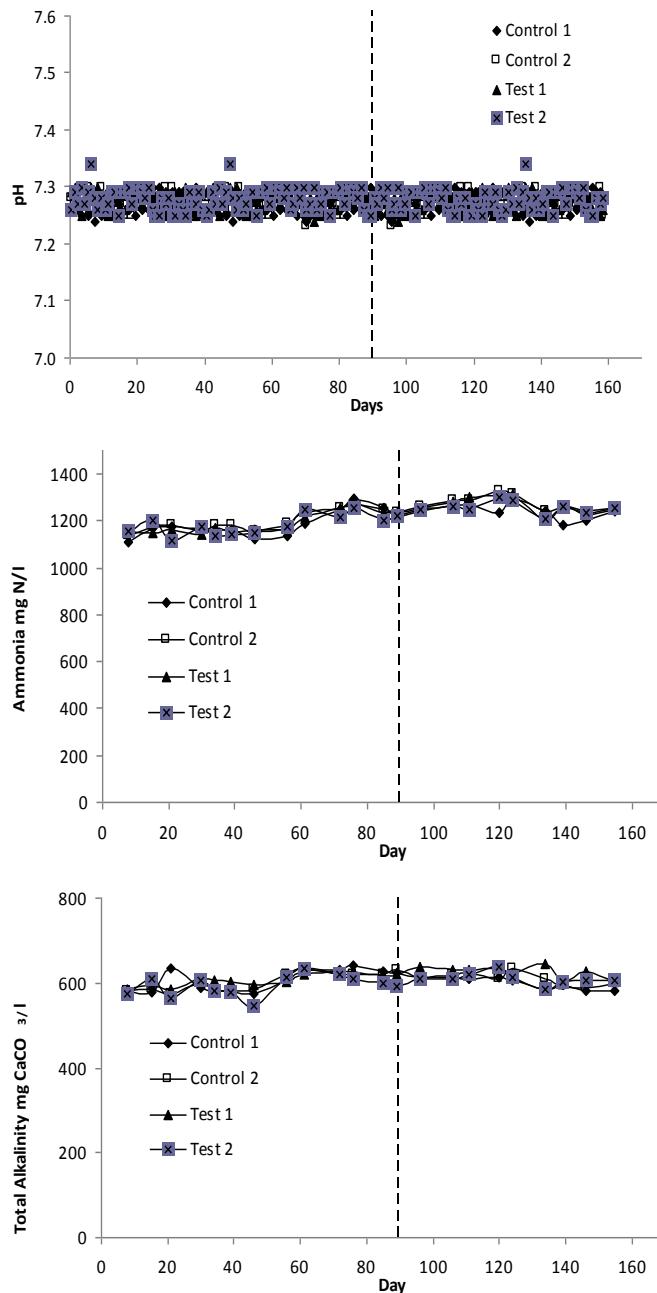
Figure 3. Feedstock TS and VS during the trial period

The pH of all the digesters remained in the range 7.2 to 7.3 (Figure 5) with no apparent difference due to the CO₂ pressure pre-treatment. The total ammonia in digestate showed a very gradual increase in concentration over the trial period (Figure 6) but remained in the range 1.1 - 1.35 g/l which is typical of that found in a sewage sludge digester, and well below the concentration where it may become inhibitory to the methanogens (Koster and Lettinga, 1988). There was no difference in ammonia concentration between the control and the test digesters (Figure 5), such as might have been expected if the pressure treatment was leading to extensive cell disruption and improved hydrolysis. If this occurred then more nitrogen would have been released from cell protein and reduced to ammonia as the carbon components were degraded. Total, partial and intermediate alkalinity were measured: the ratio of IA/PA is shown in Figure 5 and did not exceed a value of 0.6. The digesters can therefore be regarded as operating stably without any accumulation of volatile fatty acids (Ripley et al., 1986), and again no apparent difference between test and control digesters.

Average specific biogas production was 0.499 l/g VS_{added}. In the pre-trial period from day 0-90 this showed small variations that tended to follow the same pattern in each of the 4 digesters

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(Figure 6). This type of natural variation can be expected when running an experiment of this type and may be caused by small differences in daily feed due to slight inhomogeneity of the feedstock material. A similar pattern can be seen after the start of the CO₂ pressurisation trial and throughout its duration, but in this case there is also a slight downward trend in all digesters. The reason for this is unknown but is evidently related to the properties of the sludge itself, which appeared to be more dilute during the summer period as shown by the low %TS and VS obtained in the later part of the trial. It may be that in warmer weather some of the more putrescible components in the sludge were degraded at the treatment works in both sedimentation and storage tanks before being sampled. There appeared to be no benefit in terms of increased biogas yield as a result of the CO₂ pressurisation pre-treatment.



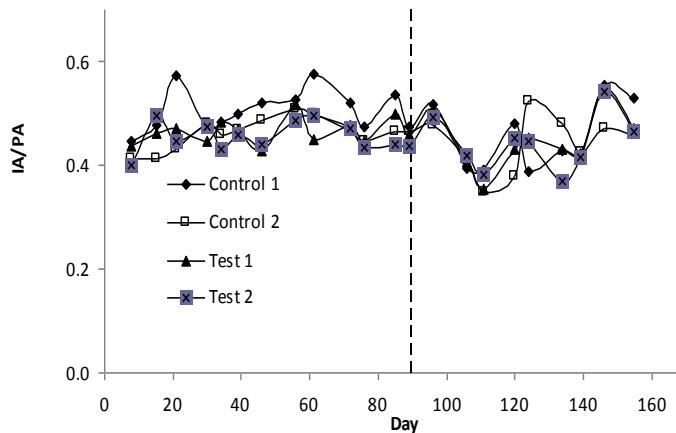
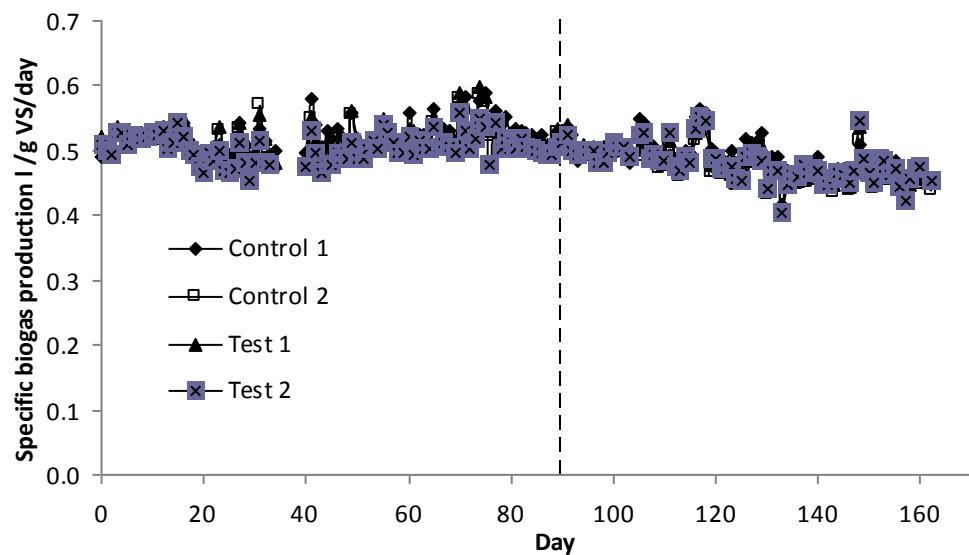


Figure 4. pH, ammonia, total alkalinity and IA/PA ratio in test and control digesters. Vertical line indicates start of feeding with CO_2 -pressurised digestate

As expected the specific methane production graph (Figure 6) showed a similar trend to that for specific biogas production: the average specific methane yield was $0.331 \text{ g VS}_{\text{added}}$ and was in the range $0.27 - 0.39 \text{ g VS}_{\text{added}}$. VS destruction was calculated both by simple difference in percentage solids entering and leaving the digester and also by a mass balance taking into account loss of mass in the biogas. In both cases the result was very similar with values for the two test digesters showing 47.7 and 49.1% destruction and the two controls 48.5 and 48.8% destruction. Performance was therefore typical of a single stage high-rate mesophilic digestion process for sewage sludge (Metcalf and Eddy, 2003).



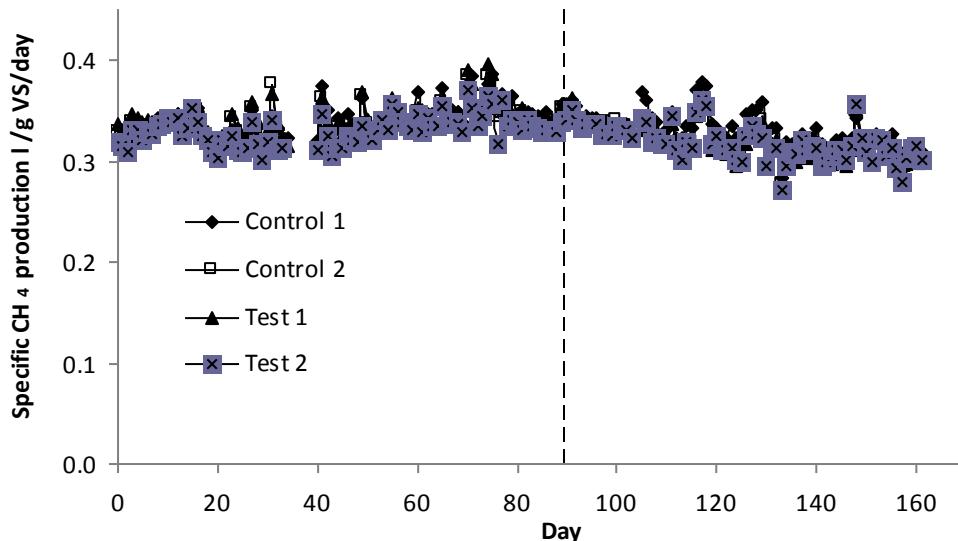


Figure 5. Specific biogas and methane potentials in test and control digesters during the experimental period. Vertical line indicates start of feeding with CO₂-pressurised digestate

The concentration of *E. coli* in the co-settled sludge on the day of collection ranged from 1.30×10^5 to 2.80×10^6 MPN/ml (Figure 7) and decreased on storage of the material under refrigerated conditions (data not shown). It is therefore not possible to show directly a reduction in numbers of *E. coli* between the feed material and the digestate removed from the digester that can be attributed to the digestion process alone. The results are thus presented as a comparison between the test and control digesters over the test period, compared to the differences observed in the pre-trial comparison of the digesters running under identical conditions.

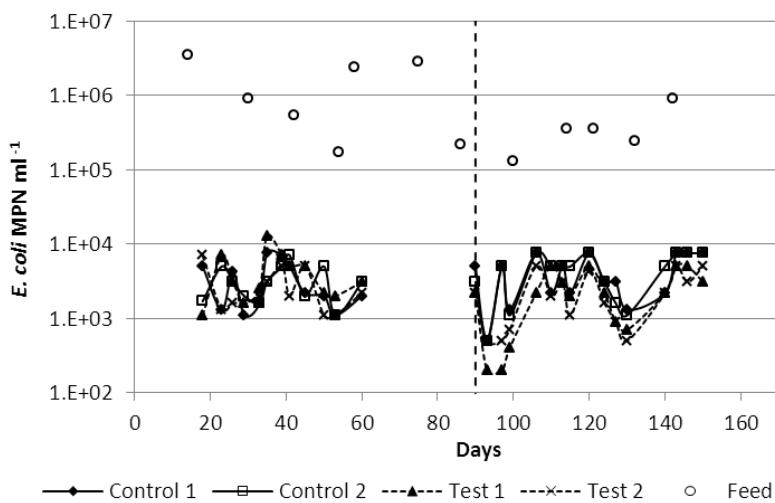


Figure 6. *E. coli* numbers in fresh feedstock before storage and in control and test digesters. Vertical line indicates start of feeding with CO₂-pressurised digestate

The concentration of *E. coli* in the digesters over the study period ranged from 10^2 to 10^4 MPN ml⁻¹ which was on average about two log reductions below the concentration in the feedstock (10^5 to 10^7 MPN/ml). This is typical of the performance of a mesophilic digester fed on co-settled sewage sludge (Godfree and Farrell, 2005). There was no apparent difference in *E. coli* destruction before and after the start of the CO₂ pressurisation treatment. The general pattern of removal in all of the digesters was similar, suggesting that the differences on sample to sample basis probably reflected the *E. coli* load entering the digester which can also be seen to vary in the feed sludge. This result was rather surprising as treatment of a pure culture of *E. coli* grown in nutrient broth and treated with CO₂ at the same pressure and exposure time had shown an 8-log reduction in numbers (Mushtaq *et al.*, 2011), although in the previous work a smaller volume of sample and of pressure

cell was used. Tests are currently under way to assess whether the size of sample may be the primary reason for the difference in performance, or whether other factors might also contribute. For example, it is possible that the presence of oils or fats in raw sludge can interfere with the dissolution of CO₂ and protect the bacterial cells by making an extra protective coating on them (Kim et al., 2008). It is also possible that a larger sample size with a smaller surface area to CO₂ exposure could cause differences; this is considered unlikely, however, as the larger sample was shaken to improve gas transfer. Indications that gas transfer into the liquid at least was successful were also given by a small drop in pressure of the vessel over the first few minutes of exposure, and by the extensive foaming and degassing observed on depressurisation.

The conditions applied in the current pre-treatment were much more stringent than those described by Spooner *et al.* (2007), where the conditions recommended for pre-treatment of waste activated sludge were 600 kPa with an exposure time of up to 60 minutes. These less stringent conditions may be better suited to materials such as waste activated sludge, but tests carried out for *E. coli* destruction in pure culture (Mushtaq *et al.*, 2011) showed a clear dependence on the time and pressure of exposure with longer times and higher pressures being the most successful.

Conclusions

Digester operation proved to be very stable for the 160 days over which the trial was carried out. During this time performance was typical of a full-scale sewage sludge digester treating co-settled sludge in terms of both the biogas production and *E. coli* destruction. The test digesters fed with CO₂ pressurised sludge showed no enhancement in either biogas production or *E. coli* destruction under the conditions used. This result was slightly unexpected, as the pressure treatment had been effective at reducing the number of broth-cultured *E. coli* in a smaller pressure vessel and with a smaller sample size. It is possible that the treatment might be better suited to other substrates or pressure-time profiles, as described in the original work

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