Doctoral Dissertation

STUDY ON NOVEL DISINFECTION METHOD OF WATER AND TREATED WASTEWATER BY USING PRESSURIZED CARBON DIOXIDE

(加圧二酸化炭素を用いた水および廃水の新規消毒方法の開発)

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博士論文
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A dissertation submitted to the Division of Environmental Science and Engineering of Yamaguchi University in partial fulfillment of the requirements for the degree of Doctor of Engineering (Dr. Eng.)

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This thesis is dedicated
to my beloved parents,
Võ Thành Mai and Nguyễn Thị Kiều Nhự

This thesis is dedicated
to my beloved wife and daughter,
Hồ Thanh Trúc and Võ Hồ Tố Quyên

"What really makes science grow is new ideas,

including false ideas. "

(Sir Karl Popper)

"A theory is something nobody believes, except the person

who made it. An experiment is something

everybody believes, except the person who made it."

(Albert Einstein)

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CONTENT

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SYMBOLS & ACRONYMS

Ca^{2+}:	Calcium ion
$CaCl_2$:	Calcium chloride
CO_2:	Carbon dioxide
CO_3^{2-}:	Carbonate ion
H_2CO_3:	Cacbonic acid
HCO_3^-:	Bicarbonate ion
H^+:	Hydrogen ion
K^+:	Potassium ion
KCl:	Potassium chloride
Mg^{2+}:	magnesium ion
Na^+:	Sodium ion
NaCl:	Sodium Chloride
$NaHCO_3$:	Sodium bicarbonate
N_2O:	Nitrous oxide
N_2:	Nitrogen
HCl:	Hydrochloric acid
HH^+-ATPase:	ATP phosphohydrolase
OH*:	hydroxyl radicals
ATCC:	American Type Culture Collection
AWWA:	American Water Works Association
B. cereus:	*Bacillus cereus*
B. subtilis:	*Bacillus subtilis*
BOD:	Biochemical oxygen demand
CFU:	Colony-forming units
COD:	Chemical oxygen demand

DBPs:	Disinfection by-products
DNA:	Deoxyribonucleic acid
dsDNA:	double-stranded DNA
DW:	Distilled water
E. coli:	*Escherichia coli*
E. faecalis:	*Enterococcus faecalis*
EPA:	Environmental Protection Agency
EtOH:	Ethanol
HAAs:	Haloacetic acids
HPCD:	High Pressure Carbon Dioxide
GM:	Growth medium
K^{T}_{H}:	Proportionality constant
K^{0}_{H}:	Henry's law constant
LB:	Luria-Bertani
Log:	logarithm
Log (N/N_0):	Log reduction of microorganisms
MPa:	megapascal, 1MPa = 10^6 Pa
MS2:	Bacteriophage MS2
N:	Microorganism concentration after inactivation
N_0:	Microorganism concentration before inactivation
NOM:	Natural organic matter
$OD_{260\&280}$:	Absorbance at 260 & 280 nm wavelength
P:	Phosphate
PBS:	Phosphate buffered saline
PFU:	Plaque-forming units

P_c:	Critical pressure
P_g:	Partial pressure
PS:	Physiological saline
R^2:	Correlation coefficient
RNA:	Ribonucleic acid
rpm:	Round per minute
RT:	Room temperature
Qβ:	Bacteriophae Qβ
ssRNA:	single-strained RNA
ssDNA:	single-strained DNA
SS:	suspended solids
S. aureus:	*Staphylococcus aureus*
S. cerevisiae:	*Saccharomyces cerevisiae*
SEM:	Scanning electron microscopy
T_c:	Critical temperature
t-BuOH:	Tert-Butanol
T4:	Bacteriophage T4
THMs:	Trihalomethanes
TSA:	Tryptic Soy Agar
UV:	Ultra Violet
WHO:	World Health Organization
wt/vol:	Weight per volume
ΦX174:	Bacteriophage ΦX174

GLOSSARY

1. **Bactericidal**: chemical that can kill or inactivate bacteria. Such chemicals may be called variously depending on the spectrum of activity, such as bactericidal, virucidal, fungicidal, microbicidal, sporicidal, tuberculocidal or germicidal.
2. **Bactericidal effect:** An antimicrobial that kills a microorganism (or, more specifically, a bacterium) is said to be bactericidal.
3. **Bacteriophage:** a virus whose host is a bacterium, commonly called phage. Following is listed common bacteria and their viral parasites: *E. coli* ATCC 11303/ phage T4, *E. coli* ATCC 23631/ phage Qβ, *E. coli* ATCC 15597/ phage MS2, *E. coli* ATCC 13706/ phage ΦX174.
4. **Disinfectant:** A disinfectant is a chemical or physical agent that is applied to inanimate objects to kill microbes.
5. **Disinfection**: Disinfection means reducing the number of viable microorganisms present in a sample. Not all disinfectants are capable of sterilizing, but, of course, all disinfectants are employed with the hope of disinfecting.
6. **Disinfection by-products, DBPs**: disinfectants, such as chlorine, react with a number of chemicals present in the water or wastewater. Some of these by-products are dangerous to health, while others are disinfectants.
7. **Inactivation**: the destruction of biological activity, as of a bacteria or a virus, by the action of pressurized CO_2 bubble or disinfectants.
8. **Microbubble**: an extremely bubble, usually only a few hundred micrometers in diameter.
9. **Pressurized**: in a pressure device that the pressure inside different from the pressure outside.
10. **Pressurized CO_2**: CO_2 gas is dissolved in water in a pressurized condition
11. **Sterilization**: Sterilization is the killing of all microorganisms in a source of water, a media, a material or on the surface of an object.
12. **Virucidal**: having the capacity to or tending to destroy or inactivate viruses.

ABSTRACT, 抽象的な, TÓM TẮT, ABSTRAK, 摘要 and บทคัดย่อ

ABSTRACT

There is increasing concern that conventional disinfection methods are being disadvantaged with hazardous by-products (chlorine, ozone), high cost, complicated setup and high maintenance (UV, membrane, advanced oxidation process)… Latest technologies of water disinfection must develop from exploiting of advantages of conventional methods and eradicating their handicaps. For this, our study relies on transferring the antiseptic of carbon dioxide effectively used in food preservation to wastewater and water disinfection as a novel finding. Accordingly, the inactivation effects of pressurized CO_2 microbubbles on disinfection efficiency against microorganisms (including bacteria and viruses) and other related aspects of the pH role by dissolved CO_2 in inactivation mechanism, temperature, pressure and environmental water samples were investigated.

These present results confirm previous findings in the field of food preservation and contribute additional evidence that suggests pressurized CO_2 may be applied in water treatment. For example, under identical treatment conditions at 0.7 MPa and room temperature, a greater than 5.0-log reduction in *E. coli* was achieved by CO_2, while a nearly 4.0-log reduction for phage T4, over 3.3-log reduction for phage Qβ and approximately 3-log for phage MS2 and phage ΦX174 were observed. The decrease of pH in water and high diffusivity of dissolved gas induced by treatment with CO_2 is considered to be the most effective factor leading to its microbicidal effects. In addition, intracellular release of proteins and nucleic acids and cell damage under SEM observation supported clearly to microorganism deaths. Increasing pressure and temperature leads to the adjustment of CO_2 state and have a strongly effect on the microbicidal efficiency. However, the suitable operating conditions found in this study are the pressure of 0.7 MPa and a temperature range from

20 °C to 25 °C. Finally, a little difference of inactivation effect between the real wastewater and laboratory wastewater (distilled water and artificial wastewater) revealed that this method has the potential application for water treatment. A secondary disinfectant such as chlorine, chloramines or chlorine dioxide may be used with pressurized CO_2 for a complete disinfection system.

These findings were originally inherited from the discoveries of using high pressure CO_2 to inactivate pathogens in food industry. Carbon dioxide on the other hand is safe to handle (it becomes active only when dissolved in water, no special alloy or plastic distribution piping is required for CO_2 system, CO_2 leaks dissipate safely into atmosphere) easy to apply, efficient, relatively low toxicity and naturally abundant. Whilst the present disinfecting methods are facing to the problems with disinfection by-products, use of pressurized CO_2 for the target inactivation of pathogens does partially substantiate no forming the residual toxicity. The current research was not specifically designed to evaluate factor related to intracellular pH of inactivated cells as well the continuous system. The issue of successful inactivation by CO_2 treatment in this study is an intriguing one which could be usefully explored in further research.

抽象的な

従来、水の消毒には主に塩素による方法が用いられてきた。しかしながら、塩素消毒による弊害（発ガン性物質であるトリハロメタン類の生成や生態系への悪影響）や塩素消毒が効かない微生物の出現（クリプトスポリディウム原虫やウイルス類など）によって、塩素消毒に代わるより安全・安心な消毒技術が必要とされている。これまで、紫外線、膜分離、オゾン等による代替塩素消毒技術も開発されてきたが、その複雑な運転操作や初期コスト・運転コストの高額さなどから未だ普及には至っていない。そこで本研究では、これまで食品の殺菌などに用いられてきた二酸化炭素による方法を水の代替塩素消毒技術として発展させ、薬品を用いない新しい消毒技術、すなわち加圧二酸化炭素を用いた水及び排水の新規消毒方法の開発を行った。消毒対象として、大腸菌及びウイルスを選定し、様々に圧力や温度等の条件を変化させて、最適な運転条件の検討を行った。また、消毒のメカニズムを検討するために適用するガスの種類を変化させた実験やpHに着目した実験を行った。実験結果から、これまで食品の殺菌に用いられてきた二酸化炭素による消毒は水処理にも適用できることが確認された。最適な運転条件（0.7MPa の圧力条件＋室温＋運転時間 25 分）下で、大腸菌の場合に 5log 以上、バクテリオファージ T4 の場合にほぼ 4log、バクテリオファージQβの場合に 3.3log 以上、バクテリオファージ MS2 の場合にほぼ 3log の消毒が可能であった。また、消毒効果としては使用するガスの溶解度のみでなくpHの低下も重要な要素であることが示された。さらに、タンパク分析による細胞内物質の漏出の確認、SEM による細胞破壊の確認により、微生物の確たる消毒（殺菌）が示された。重要な運転条件である圧力、水温が上昇するほど消毒効果は高くなったが、適切な運転条件は、0.7MPa の圧力条件＋20～25℃の温度範囲と確認された。最後に、浮遊性物質（SS）を含む実環境水を用いて消毒実験を行ったところ、SSの存在は消毒効果を阻害するため、SSを除去して消毒処理を行う必要性のあることが示された。本法の後処理として残留性のある塩素消毒やクロラミン消毒による処理を行うことでより完全な消毒システムを構築できると考えられる。二酸化炭素は基本的に無害であり、配水時の管内部をプラスチックによるライニングにする等の工夫により、より安全な消毒システムを構築できる。さらに二酸化炭素による消毒では副生物は生成せず、従来の塩素消毒に優る。

以上から、本法は薬品を用いない水及び排水の新規消毒方法としての十分な可能性を有することが示されたと考えられる。

TÓM TẮT

Các phương pháp khử trùng truyền thống hiện đang gặp một số khó khăn về các sản phẩm phụ nguy hại (như phương pháp dùng clo, ozone), đòi hỏi chi phí cao, lắp đặt phức tạp và bão dưỡng cao (phương pháp tia cực tím, oxi hóa nâng cao, lọc màng)….Những công nghệ khử trùng mới nhất phải dựa trên khai thác những ưu điểm của khử trùng truyền thống và hạn chế những nhược điểm của chúng. Vì những lí do này, nghiên cứu chúng tôi hướng đến sử dụng chất tiệt trùng khí cacbonic mà đã được sử dụng hiệu quả trong lĩnh vực bảo tồn thực phẩm áp dụng cho lĩnh vực khử trùng nước và nước thải như là một chất khử trùng mới. Theo đó, hiệu quả bật hoạt của các vi bong bóng CO_2 áp lực cao vào hiệu quả khử trùng chống lại các vi sinh vật (bao gồm vi khuẩn lẫn vi rút) và những khía cạnh khác như vai trò pH gây ra bởi CO_2 hòa tan cao trong cơ chế bất hoạt, nhiệt độ, áp suất và các mẫu nước môi trường đã được đầu tư.

Các kết quả hiện tại xác nhận từ các kết quả trước trong lĩnh vựa bảo tồn thực phẩm và đóng góp thêm các bằng chứng xác thực rằng khí CO_2 áp lực có thể được ứng dụng trong xử lí nước. Ví dụ, dưới những điều kiện đã xác định ở 0.7 MPa và nhiệt độ phòng, giảm hơn 5 log vi khuẩn *E. coli* được tìm thấy, trong khi đó giảm gần 4 log cho vi rút T4, hơn 3.3 log đối với vi rút Qβ và xấp xỉ giảm 3 log cho vi rút MS2 và ΦX174 cũng được quan sát. Việc giảm pH trong nước và độ khuếch tán cao của khí hòa tan được chỉ dẫn bởi xử lí CO_2 được cân nhắc là nhân tố hiệu quả nhất dẫn đến những cái chết của vi khuẩn. Bên cạnh đó, sự phóng thích prô-tê-in và các axit nu-clê-ic và phá hủy tế bào dưới quan sát kính hiển vi điện tử SEM đã giải thích rõ ràng cho những cái chết của vi sinh vật. Việc tăng nhiệt độ và áp suất dẫn đến thay đổi trạng thái khí CO_2 và có một sự tác động mạnh mẽ vào hiệu quả diệt khuẩn. Tuy nhiên, điều kiện vận hành thích hợp đã được xác định từ nghiên cứu này là tại áp suất 0.7 MPa và khoảng nhiệt độ từ 20°C đến 25°C. Cuối cùng, có một sự khác biệt nhỏ về hiệu quả bất hoạt giữa nước thải thực tế và nước thải được tổng hợp từ phòng thí nghiệm (nước cất và nước ngầm nhân tạo) đã tiết lộ rằng phương pháp này có tiềm năng ứng dụng cho xử lí nước. Tuy nhiên một quá trình khử trùng thứ cấp như thêm clo, chlorine dioxide hay chloramine có lẽ nên được dùng kết hợp với khí CO_2 áp lực cho một hệ thống khử trùng hoàn hảo hơn.

Những kết quả tìm kiếm này được thừa hưởng những khám phá về việc dùng khí CO_2 áp lực cao để bật hoạt các mầm bệnh trong lĩnh vực thực phẩm. Mặc khác khí CO_2 lại an toàn để sử dụng (nó trở nên kích hoạt khi hòa tan vào nước, đường ống phân phối không đòi hỏi phụ gia hay hợp kim bảo vệ, nếu rò rỉ CO_2 trở nên an toàn trong khí quyển), dễ ứng dụng, hiệu quả, ít độc hại và có trử lượng dồi dào trong tự nhiên. Trong khí các phương pháp khử trùng hiện nay đang đối mặt với vấn đề các sản phẩm phụ của quá trình khử trùng thì việc dùng CO_2 áp lực cho mục đích bất hoạt các mầm bệnh cho thấy phần nào sẽ không hình thành các sản phẩm độc hại này. Nghiên cứu này cũng không được thiết kế chi tiết để đánh giá nhân tố liên quan đến pH nội bào của vi khuẩn cũng như cho các hệ thống xử lí liên tục. Sự thành công sử dụng CO_2 cho bất hoạt trong nghiên cứu này là một điều thú vị mà cần được khám phá cho các nghiên cứu tương lai.

ABSTRAK

Dalam perkembangan tahun-tahun terakhir ini, ada perhatian lebih terhadap kerugian dari metode desinfeksi konvensional. Kerugian tersebut diantaranya produk samping yang berbahaya (dari metode klorin, ozon), biaya yang mahal, persiapan yang rumit, dan perawatan yang mahal (untuk metode UV, membrane, proses oksidasi lanjutan). Teknologi terkini dari desinfeksi untuk air bersih, seyogyanya dikembangkan dari keuntungan yang telah ada pada metode desinfeksi konvensional dengan meminimalkan kerugian yang ada. Berdasarkan hal tersebut, studi ini menitikberatkan pada penemuan baru aplikasi karbondioksida antiseptic terhadap desinfeksi air bersih dan air limbah, yang sebelumnya sering digunakan untuk pengawetan makanan. Oleh karena itu efek penonaktifan mikroorganisme (termasuk bakteri dan virus) dengan gelembung mikro CO_2 bertekanan dan efek pH oleh CO_2 terlarut terhadap mekanisme penonaktifan, suhu, tekanan dan kondisi lingkungan air setempat akan diselidiki pada studi ini.

Hasil yang diperoleh di studi ini memperkuat hasil penelitian sebelumnya di bidang pengawetan makanan dan berkontribusi dalam menambah bukti yang menyarankan CO_2 bertekanan dapat diaplikasikan di pengolahan air bersih. Pada kondisi yang sama pada tekanan 0.7 MPa dan suhu ruang, hasil yang diperoleh untuk reduksi *E. Coli* oleh CO_2 adalah 5.0 log dimana reduksi phage T4 hanya mencapai hampir 4.0 log, reduksi phage $Q\beta$ mencapai lebih 3.3 log serta reduksi phage MS2 and reduksi phage ΦX174 mendekati 3-log. Penurunan pH di air dan tingginya difusiti gas terlarut yang diinduksi oleh pengolahan dengan CO_2 adalah dua faktor yang paling efektif mempengaruhi mikroba. Sebagai lanjutannya, pelepasan intraselular untuk protein, asam nukleat dan kerusakan sel menunjukkan kematian mikroorganisme secara jelas melalui observasi SEM. Peningkatan tekanan dan suhu akan menyebabkan pengaturan kondisi CO_2 dan mempunyai efek yang kuat pada efisiensi mikroba. Namun demikian, dari hasil studi ini, kondisi pengoperasian yang paling sesuai adalah pada tekanan 0.7 MPa dan kisaran suhu 20^{o}C dan 25^{o}C. Adanya sedikit perbedaan pada efek penonaktifan pada air limbah asli dan air limbah buatan menunjukkan bahwa metode ini berpotensi untuk pengolahan air bersih. Desinfeksi sekunder seperti klorin, kloramin atau klorin dioksida dapat digunakan bersama dengan CO_2 bertekanan untuk sistem desinfeksi yang lengkap.

Hasil ini berasal penemuan dari penggunaan tekanan tinggi CO_2 untuk menonaktifkan pathogen di industri makanan. CO_2 aman untuk ditangani (akan menjadi aktif jika terlarut di air, tidak ada campuran spesial atau pipa distribusi plastik dibutuhkan untuk sistem CO_2, kebocoran CO_2 akan menghilang ke atmosfer dengan aman), mudah diterapkan, efisien, relatif rendah toksisitasnya, dan tersedia banyak secara alamiah. Sementara metode desinfeksi yang ada sekarang menghadapi problem dengan produk sampingnya, metode CO_2 bertekanan tidak menghasilkan residu yang toksik. Penelitian ini tidak mengevaluasi faktor yang berhubungan dengan pH intraselular dari sel yang non aktif sebagaimana di sistem kontinyu. Perihal tentang keberhasilan penonaktifan CO_2 pada studi ini menarik untuk dikembangkan lebih jauh pada penelitian-penelitian yang akan datang.

摘要

人们开始越来越担心传统的消毒方法因为其危险的副产物（氯，臭氧），高成本，复杂的设置和高维护费（紫外线，膜，高级氧化工艺）而处于不利的地位。水消毒技术的最新发展必须开拓继承传统消毒方法的优势，并且克服弥补它的缺陷。为此，我们的研究依赖于在食品保鲜中起有效防腐功能的二氧化碳，将其转移应用到污水和饮用水消毒领域。因此，在针对微生物（包括细菌和病毒）由加压的 CO_2 使其失活而达到的消毒效率，以及在其他相关方面的 pH 值下，由溶解 CO_2 引起的灭活机理，温度，压力和环境水样进行了调查。

这些目前的研究结果不仅证实了以前在食品保鲜领域的发现，更提供了证据表明加压 CO_2 在水处理应用方面的可能性。例如，在 0.7 兆帕和室温的相同的处理条件下， 实验结果发现大肠杆菌达到了大于 5.0 log 的减少的 CO_2 消毒效果，噬菌体 T4 为近 4.0 log 的减少，噬菌体 Qβ 为大于 3.3 log 的减少，而噬菌体 MS2 和 ΦX174 为大约 3 log 的减少。溶解的 CO_2 气体的高扩散能力和 pH 值的降低被认为是导致其微生物灭活的最有效因素。此外，SEM 的分析观察到细胞内释放的蛋白质和核酸以及细胞损伤，这个结果清楚的表明微生物已经死亡。通过增加压力和温度调节 CO_2 状态，来影响其杀菌效率。然而，在本研究中发现最合适的操作条件为 0.7 兆帕压力和温度范围 20-25 摄氏度。最后，真实的污水和实验室污水（蒸馏水和人工合成污水）之间的杀菌效果只有微小的差异，这证明该方法在水处理方面有潜在的应用能力。一种次级的消毒剂如氯，氯胺或者二氧化氯可与加压 CO2 联合使用形成完整的消毒体系。

这些发现原本是继承了高压 CO_2 病原体灭活在食品工业的发现。另一方面，二氧化碳易于安全操作（在溶解水里才能被激活，不需要特殊的合金或塑料制的管路配送系统，CO_2 即使泄露也能安全地扩散到大气）容易利用，高效，低毒并且储量丰富。同时，目前的消毒方法主要面临副产品消毒的问题，然而使用加压 CO_2 进行的杀菌过程能够保证没有残留毒性。目前的研究并不是专门设计为了评估有关灭活细胞内的 pH 值的因子，而且不是针对连续的系统。在这项研究中，CO_2 的成功杀菌作用耐人寻味，可能为进一步的研究提供了有益的探索。

บทคัดย่อ

ในปัจจุบัน เป็นที่น่ากังวลว่าวิธีการฆ่าเชื้อโรคในน้ำแบบเดิม มีการปลดปล่อยสารอันตราย (คลอรีน โอโซน) ค่าใช้จ่ายสูง การติดตั้งเครื่องมือมีความซับซ้อน และต้องการการบำรุงรักษาสูง (UV แผ่นกรอง กระบวนการ oxidation ขั้นสูง) การพัฒนาเทคโนโลยีการฆ่าเชื้อโรคในน้ำ ต้องคงคุณสมบัติที่ดีและกำจัดข้อด้อยของวิธีการแบบเดิม การศึกษาของเราเป็นวิธีการใหม่ โดยเลือกใช้เทคโนโลยีการฆ่าเชื้อด้วยคาร์บอนไดออกไซด์ (CO_2) ซึ่งเป็นวิธีการที่ใช้ในการเก็บรักษาอาหาร มาใช้ในการกำจัดเชื้อโรคในน้ำและน้ำเสีย ในงานวิจัยนี้ ได้ทำการศึกษาประสิทธิภาพของการใช้ฟองก๊าซ CO_2 ที่ถูกอัดด้วยแรงดัน (pressurized CO_2 microbubbles) ที่มีต่อประสิทธิภาพในการยับยั้งจุลินทรีย์ (แบคทีเรียและไวรัส) และผลของค่าความเป็นกรดด่างที่เกิดจากการละลายของ CO_2 ต่อกลไกของการยับยั้งจุลินทรีย์ รวมถึงการติดตามค่าอุณหภูมิ ความดันและสิ่งแวดล้อมอื่นๆ ในน้ำ

ผลการทดลองของงานวิจัยนี้ ยืนยันประสิทธิภาพของการใช้ CO_2 ที่ถูกอัดด้วยแรงดันในงานด้านการถนอมอาหาร และยังสามารถนำมาประยุกต์ใช้ในการบำบัดน้ำได้ ตัวอย่างเช่น การใช้ CO_2 ภายใต้ความดัน 0.7 MPa ที่อุณหภูมิห้อง สามารถลดจำนวน *E.coli* มากกว่า 5.0 log ลดจำนวนฟาจ T4 เกือบ 4.0 log ลดจำนวนฟาจ Qβ มากกว่า 3.3 log และลดจำนวนฟาจ MS2 และฟาจ ΦX174 ประมาณ 3.0 log การลดลงของค่าความเป็นกรดในน้ำและการแพร่กระจายของ CO_2 ที่ละลาย ถือเป็นปัจจัยที่มีประสิทธิภาพมากที่สุดในการยับยั้งเชื้อจุลินทรีย์

นอกจากนี้ การตรวจพบโปรตีนและกรดนิวคลีอิกที่ถูกปล่อยออกมาจากเซลล์ และการตรวจพบความเสียหายของเซลล์ภายใต้กล้อง SEM เป็นข้อสนับสนุนอย่างชัดเจนว่าจุลินทรีย์ตายภายหลังเซลล์ถูกอัดด้วย CO_2 การเพิ่มความดันและอุณหภูมิ จะนำไปสู่การปรับสถานะของ CO_2 และส่งผลให้ประสิทธิภาพของการยับยั้งจุลินทรีย์สูงขึ้น อย่างไรก็ตาม สภาวะที่เหมาะสมในการศึกษาครั้งนี้ คือที่ระดับความดัน 0.7 MPa และอุณหภูมิระหว่าง 20 ถึง 25 องศาเซลเซียส จากผลการศึกษาการฆ่าเชื้อโรคในน้ำเสียจริง กับน้ำเสียในห้องปฏิบัติการ (น้ำกลั่นและน้ำเสียสังเคราะห์) พบว่ามีประสิทธิภาพในการยับยั้งจุลินทรีย์ต่างกันเพียงเล็กน้อย จึงสามารถสรุปได้ว่า วิธีการนี้มีศักยภาพในการนำไปใช้ในการบำบัดน้ำ และเพื่อให้ระบบการฆ่าเชื้อโรคมีประสิทธิภาพมากยิ่งขึ้น วิธีการบำบัดน้ำเสียขั้นที่สอง โดยการใช้คลอรีน คลอรามีน หรือ คลอรีนไดออกไซด์ อาจจะนำมาใช้ร่วมกันกับการใช้ CO_2 ที่ถูกอัดด้วยแรงดัน

การค้นพบนี้ได้รับแนวความคิดมาจากการใช้ CO_2 แรงดันสูงในการยับยั้งเชื้อโรคในอุตสาหกรรมอาหาร CO_2 เป็นก๊าซที่มีความปลอดภัย (เมื่อ CO_2 ละลายในน้ำจะทำให้ความเป็นกรดเพิ่มขึ้น ระบบท่อน้ำจึงไม่จำเป็นต้องใช้โลหะผสมแบบพิเศษหรือท่อพลาสติก และCO_2 มีความปลอดภัยเมื่อกระจายออกสู่ชั้นบรรยากาศ) ง่ายต่อการนำมาใช้ มีประสิทธิภาพ ความเป็นพิษต่ำ และพบได้ทั่วไปในธรรมชาติ ในขณะที่วิธีการฆ่าเชื้อในปัจจุบัน มีปัญหาเรื่องสารพิษตกค้างที่เป็นอันตราย แต่การฆ่าเชื้อโรคโดย CO2 ที่ถูกอัดด้วยแรงดันไม่มีการสร้างสารพิษ ในการศึกษาครั้งนี้ไม่ได้ทำการศึกษาประเมินปัจจัยที่เกี่ยวข้องกับความเป็นกรดด่างของเซลล์ที่ถูกทำลายในระบบอย่างต่อเนื่อง ความสำเร็จของการใช้ CO2 ที่ถูกอัดด้วยแรงดันในการยับยั้งจุลินทรีย์ในงานวิจัยชิ้นนี้ จะเป็นประโยชน์สำหรับนักวิจัยที่สนใจต่อไปในอนาคต

CHAPTER I

INTRODUCTION

1.1. PROBLEM STATEMENT

In current years, the drinking water industry and wastewater treatment have focused much attention on waterborne disease. Water security faces to overwhelming water demand, increasing resistance of pathogens to disinfection, increasing population densities and the resulting growth in agricultural, industrial, and human waste discharge that water receives increasingly make clean and safe water a very valuable resource.

Recently, other waterborne emerging pathogens, such as viruses, bacteria, fungi, nematodes, cysts, as well as algae have been shown to have the potential to induce disease in humans. So far, Chlorine is the most widely used disinfectant to treat both water for human consumption and to treat wastewater prior to discharge. Chlorination has become the standard method to removing harmful organisms from water because it is simple, highly reliable, low in cost, easy to use. Especially, chlorine can be employed to every scale of water treatment and has been shown to be extremely effective in inactivating the waterborne pathogens that cause many diseases. However, the by-products from chlorine treatment are the ones that have been most extensively identified and their toxicity assessed. There are alternatives for chlorine: membrane filtration, ultraviolet (UV) radiation, bromine, iodine, ozone, and heat treatment, among others. Each of the used disinfectants has its advantages and disadvantages in terms of cost, efficacy, stability, ease of application and formation of by-products (**Tab 1.1**). For disadvantage examples, disinfection via UV radiation requires the water to be free of turbidity (suspended particles) and the UV-absorbing organic matter; the pH of water is important in application of chlorine; Ozone and chlorine dioxide are both produced on-site because they are unstable for storage. For ozone and UV radiation the cost primarily involves the equipment cost and the power cost for operation of the equipment. For chlorine dioxide the chemical cost, the equipment cost and the power cost have to be taken into consideration. Membrane filtration requires high cost, maintenance and complicated operation.

Table 1. 1. Advantages and disadvantages of conventional disinfectants

Disinfectants	Advantages	Disadvantages
Chlorine	Very effective against bacteria and viruses; stable, very good residual	Not effective against *Cryptosporidium*; forming

	protection; highly economical.	halogenated by-products; gaseous chlorine is a hazardous process; taste and odor problems,
Chloramines	Stability and persistence; lower levels of DBPs; good residual protection, less taste and odor; effective in controlling biofilms.	Weak in inactivation of viruses and protozoa; excess ammonia used cause nitrification.
Ozone	Effective disinfection with less contact time and concentration; no halogenated DBPs (THMs and HAAs); high effectiveness against bacteria, viruses and protozoan cysts; good taste, color and odor control.	Not residual in the distribution system; require secondary disinfection; harmful DBPs with bromates, aldehydes and ketones; high cost for operation and maintenance; maintenance and operator skill; require off-gas destruction;
UV light	High effective against bacteria, *Giardia* and *Cryptosporidium*; less costly than ozone and chlorine dioxide; No concerns with respect to interactions with pipe material; no known formation of DBPs (THMs, HAAs, bromate, aldehydes, ketoacids)	Higher dose is required; no residual protection; difficult to monitor equipment performance and measure germicidal dose.
Chlorine Dioxide	Effective against a wide range of pathogens; Does not form halogenated by-products.	Less stable than other chlorine species; low efficiency at low temperature; must be generated on-site; high chemical costs; be explosive at high temperature and pressure; decomposes on exposure to sunlight and UV;
Membrane filtration	High effective to remove bacteria and other microorganisms, particulate material, natural organic material;	High capital and operating cost and complexity operation and maintenance; High level of pretreatment is required; prone to fouling

For water utilities to continue providing safe drinking water in the future and attempts to overcome the most serious disadvantages of the conventional disinfection, one question is that needs to find new discoveries for killing or removing infectious agents. Many observers have especially drawn attention to high pressure carbon dioxide (HPCD) as a potential solution for wastewater and water treatment despite of earlier attempts investigated for target replacement, such as solar disinfection, ultrasound, hydrodynamic cavitation…

1.2. OBJECTIVES

HPCD is proposed as a promising alternative technology that can inactivate pathogens effectively in water and be an acceptable solution by its common characteristics and successful studies in food preservation and sterilization. According to food researchers, pressurized CO_2 may be more effective in media with high water content. Moreover, CO_2 solubility in water becomes easily and faster if supported under a pressure system. Microbubble technique is assessed to bring back the high contact efficiency between water and gas. From these reasons, this dissertation is investigated to resolve the followings:

- To review the recent research concerning the usefulness of using pressurized CO_2 for removing pathogens in food preservation and water treatment,
- To evaluate and validate inactivation effect of CO_2 against *Escherichia coli* cells in water by a system generating pressurized microbubbles, the role of pH caused by dissolved CO_2, inactivation mechanism of CO_2 treatment,
- To determine whether high pressure CO_2 can remove viruses in water, assessment of temperature and pressure effect by CO_2 treatment,
- To assess the inactivation effect of pressurized CO_2 to environmental water samples; analyze the inactivation rate of CO_2 treatment to *E. coli* and viruses; give a potential base for application via the comparison with other disinfection methods.

1.3. SCOPE OF RESEARCH AND APPROACH METHODS

In limitation of dissertation, this study is designed to accomplish each of above objectives by the following approaches:

- For overview of the area of using high pressure carbon dioxide in disinfection, all articles reviewed for this study are retrieved from many different sources, such as Scopus abstract system, Web of Sciences ^{TM} …
- A pressure batch apparatus to produce microbubbles was developed from the previous studies (Imai et al. 2008; Cheng et al. 2011) to adopt for the operating condition up to 1MPa.
- In order to elucidate the inactivation role of CO_2, the distilled water was used as a media for microorganisms in analysis and water sample for experimental apparatus. Beside that, determining inactivation effect of high pressure carbon dioxide, a series of measurements from other pressurized disinfectants (N_2O, N_2, CO_2) and different indicator

microorganisms (*E. coli* hosts, bacteriophages) was performed for target assessment. The detail characteristics of these agents are showed in **Chapter 3** in this dissertation.

- For counting the cells or phages inhibited by CO_2 treatment and the survival microorganisms, colony-forming units (CFU) method and plaque phage assays (Debartolomeis et al, 1991) using double layers of agar on plates were done.
- To understand how the pH caused from dissolved CO_2 can inactivate microorganisms and give an explanation for inactivation mechanism, a comparison with various acidic pressurized systems was performed, including the neutral media of nitrous oxide (N_2O), a normal acidic environment (the air/ HCl), the buffering system (CO_2/PBS solution) and the only CO_2 treatment. The release of intracellular substances (proteins and nucleic acids) from inactivated cells was measured according to the procedure of identification of UV-absorbing substances used by Kim et al. (2008a). In addition, the photos of scanning electron microscopy (SEM) were taken to observe destroy of cell surface. This method was prepared by adapting the procedure used by Kim et al. (2008b).
- In order to assess the inactivation effect of CO_2 treatment to different environmental water samples, microorganism suspensions in distilled water, artificial ground water, the effluent wastewater were used.

1.4. STRUCTURE OF DISSERTATION

The dissertation structure has been divided into six chapters. The content of each chapter has been organized in the following ways:

- The chapter one first gives a brief introduction why and how this dissertation has been investigated.
- The next chapter begins with the overview of published journals about using high pressure carbon dioxide in the food area and reviews the evidence of their successful investigations and gives the reasons why CO_2 should be continued to study in wastewater and water treatment.
- Chapter 3 describes by laying out the comparison design of CO_2, N_2O and N_2 to inactivation effect against *E. coli*. A mechanism of CO_2 inactivation is also expressed in this section.
- Chapter 4 assesses the disinfection performances of CO_2 treatment against the different bacteriophages, temperature and pressure effect to *E. coli* and phages.

- Chapter 5 describes the potential application of pressurized CO_2 treatment to environmental water samples and shows the inactivation rates to different microorganisms.
- Finally, this chapter summarizes all obtained results in dissertation and gives a further outlook trend.

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CONNECTING TEXT: CHAPTER 1 → CHAPTER 2

The purpose of next chapter is to review recent research into the high pressure carbon dioxide. This section begins with the overview of published journals about using high pressure carbon dioxide in the food area and reviews the evidence of their successful investigations and gives the reasons why CO_2 should be continued to study in wastewater and water treatment.

CHAPTER II
POTENTIAL APPLICATION OF HIGH PRESSURE CARBON DIOXIDE IN WATER AND TREATED WASTEWATER DISINFECTION: RECENT OVERVIEW AND FURTHER TRENDS

2.1. ABSTRACT

Recent disadvantages in conventional disinfection have heightened the need for finding the new solution. Developments in the field of using high dense carbon dioxide for food preservation and sterilization have led to a renewed interest in wastewater and water disinfection. Pressurized CO_2 is one of the most investigated methods of antibacterial techniques and extensively used for decades to inhibit pathogens in dried food and liquid products. This study reviews the literature concerning the usefulness of using CO_2 as a disinfecting agent. In the contents that follow, it will be argued that the successful applications and high effectiveness of CO_2 treatment in liquid foods open a potential opportunity to water disinfection. Moreover, this helps to seek to address overcoming the recent emerging problems in water disinfection.

Keywords: high dense carbon dioxide, high pressure CO_2, inactivation effect, inactivation mechanism, CO_2 microbubble, pressurized CO_2.

Contents

4. Microbicidal mechanism of HPCD

4.1. Disturbance by high dissolved CO_2 in water

4.2. Damage membrane

4.3. Intracellular pH lowering excessively with its buffering capacity

4.4. Metabolism alteration/ Inhibited enzymes

4.5. Restrain intracellular electrolyte stability and cytoplasmic leakage

5. Microbubble techniques for HPCD application in water treatment

6. Conclusions and further outlook

References

2.2. INTRODUCTION

For nearly a century, chlorine has played a major role in standards for water disinfection in Europe, the United States, and other countries around the world. The low cost and effectiveness of chlorination provide it with an advantage over other disinfectants. However, chlorine can combine with other chemicals in water to generate cancer-causing by-products. Another chemical disinfectant, ozone, which has been successfully used for decades to eradicate viruses, Giardia, Cryptosporidium, and other known pathogens is expensive and leads to the formation of disinfection by-products (DBPs) and non-residual disinfectants. Physical disinfectants such as ultraviolet (UV) irradiation, electronic radiation, ultrasound, and heating have been applied to replace conventional methods. UV irradiation has long been considered an effective primary solution for emerging pathogens and DBPs; however, its disinfecting activity depends on water characteristics (turbidity, pathogen population, hardness) and wavelength intensity.

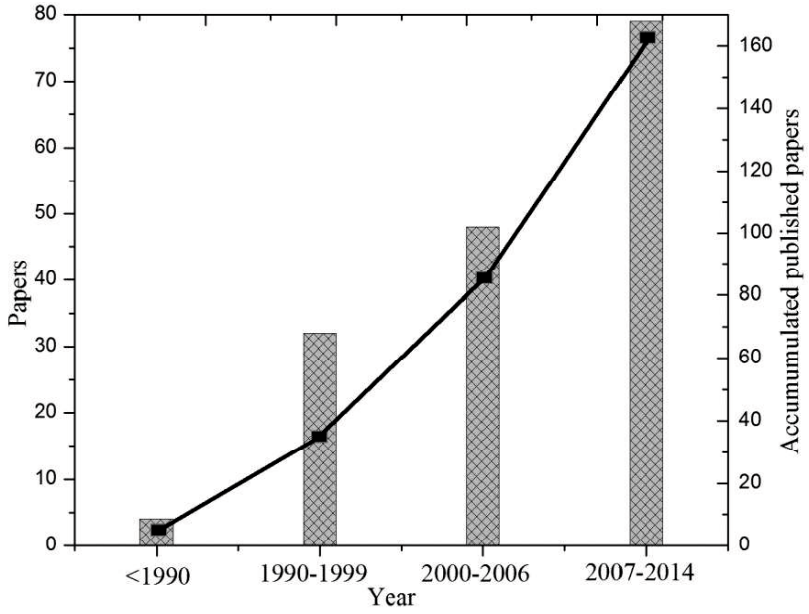

Figure 2. 1. Total studies investigated related to using high pressure carbon dioxide in food disinfection area and water treatment.

Pressurized CO_2 has been applied to inhibit pathogens in food as a cold pasteurization (Garcia-Gonzalez *et al.*, 2007). Numerous studies have explored the bactericidal effect of CO_2 on microbial growth. The discoveries about scientific evidence for HPCD inactivation has rated unabated for more two recent decades, especially from 2007 to present (Fig. 1.1). While there are nearly 200 published journals, this issue has grown in importance in light of recent food preservation.

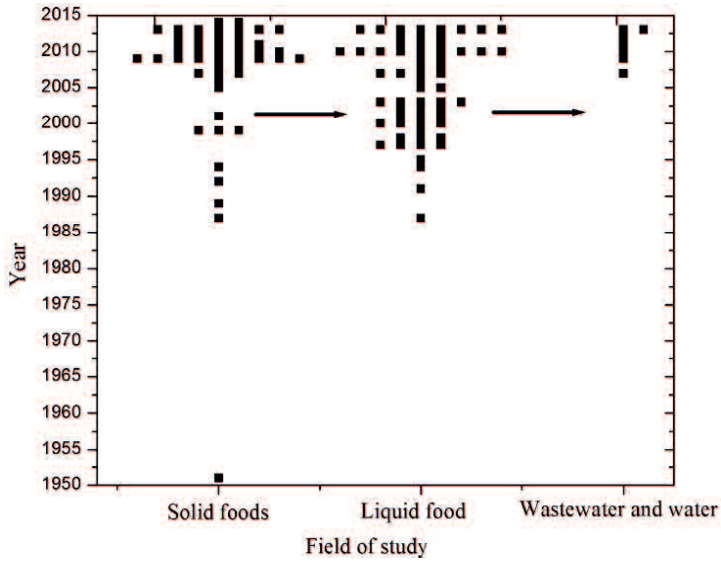

Figure 2. 2. The distribution of all studies investigated related to high pressure carbon dioxide inactivation in dry media, liquid and water.

Most studies in using pressurized CO_2 have been carried out in three separate areas: dried food, liquid food and water treatment (Fig. 2.2). On the figure 2.3, the research to 2009 has tended to focus on food sterilization rather than water disinfection. So far this method has only been applied to food. However, far too little attention has been paid to water disinfection since the first study of Kobayashi. (2007) has successfully attained in an attempt of transferring from food disinfection to water disinfection. In recent years, there has been an increasing interest in water treatment (Kobayshi et al. 2007, 2009, 2010; Cheng et al. 2011) and using pressurized CO_2 has subscribed to the belief that use the advantages of conventional methods while eradicating their adverse effects should be developed.

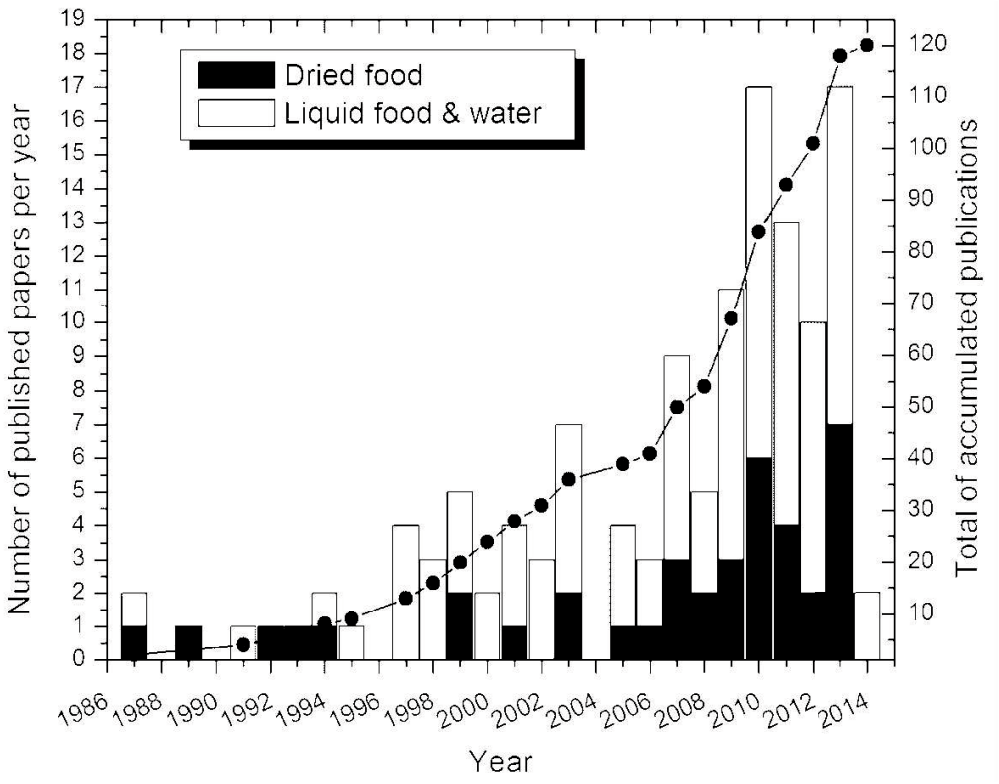

Figure 2.3. The trend forecast of studies relative to HPCD application.

2.3. EFFECT OF HIGH PRESSURE CARBON DIOXIDE (HPCD) ON MICROBIAL DISINFECTION

Many species of microorganisms including gram-negative and gram-positive bacteria, and bacterial spores have been subjected to CO_2 treatment under various operating conditions. The use of supercritical CO_2 is also of great interest for the inactivation of microorganisms (Kuhne and Knorr. 1990). Pressurized CO_2 has been found to inhibit various microorganisms (bacteria, molds, yeast) (Haas *et al.* 1989).

2.3.1. *Escherichia coli* inactivation.

Since *E. coli* has successfully been inhibited by pressurized CO_2 in the first study (Fraser. 1951), numerous investigations, at least 20 studies, have attempted to explain the relationship the inactivation effect of CO_2 and the cell death of *E. coli* (Tab. 2.1). Kamihira *et al.* (1987) found that *E. coli* suspended in distilled water was killed to 5.1 log by high-pressure CO_2 treatment at 20 MPa and 35°C for 120 min, while Haas *et al.* (1989) found that same treatment time this method killed to 6.3 log of *E. coli* cell suspended in culture broth. Dillow *et al.* (1999) confirmed the complete and high inactivation of a wide variety of bacterial organisms, especially *E. coli*, in response to supercritical fluid CO_2 applied in the absence of organic solvents or irradiation. Schmidt et al. (2005) and Cinquemani et al. (2007) have found that *E. coli* was completely inhibited with the 5-7 MPa in only 20 min. Moreover, an increase of pressure, temperature, or treatment time enhanced the antimicrobial effect of CO_2 under pressure against *Escherichia coli* (Kamihira *et al.* 1987; Dillow *et al.* 1999; Wu et al. 2007; Kobayashi et al. 2007, 2009; Garcia-Gonzalez et al. 2010). So far using of pressurized CO_2 has been widely investigated (Wu et al. 2007; Kobayashi et al. 2007, 2009; Jung et al. 2009; Garcia-Gonzalez et al. 2010; Klangpetch et al. 2011, 2012; Cheng et al. 2011).

Table 2. 1. Effect of high pressure carbon dioxide on *Escherichia coli* (a negative gram bacteria) disinfection

No.	Pressure, MPa	Tempt., °C	Time, min	Reduction, log	Solution	References
1	3.5	37-38	3	1.6	Synthetic medium	Fraser (1951)
2	4 10	20 35	120 120	3.9 4.2	Distilled water	Kamihira *et al.* (1987)

	20	35	120	5.1		
3	6.2	Room Tempt.	120	6.3	Nutrient broth	Haas *et al.* (1989)
4	31.03	35	40	3.5	PBS	Sirisee *et al.* (1998)
	31.03	42.5	180	1	Ground beef system	
5	5	Room Tempt.	200	4	a solid hydrophilic medium	Debs-Louka *et al.* (1999)
6	20.5	42	20	9	Growth medium	Dillow *et al.* (1999)
	11	38	45	8.6		
	14	34	60	8	GM (no water)	
	14	34	30	8	GM (with water present)	
7	10	30	50	7.5	Nutrient broth	Erkmen *et al.* (2001)
8	20	34	10	2.5	Sterile water	Spilimbergo *et al.* (2003)
9	7	20	15	complete	Cotton fabric impregnated with water	Schmidt *et al.* (2005)
10	5	20	60	complete	Textile in water condition	Cinquemani *et al.* (2007)
11	7.8	45	60	8.5	Aqueous solution	Wu *et al.* (2007)
	7.8	35	60	8		
	7.8	25	60	5.5		
	7.8	35	30	4.3		
	4.9	35	60	5		
	2	35	60	2.3		
12	10	35	13.3	8	Drinking water	Kobayashi *et al.* (2007)
13	2	40	60	6	PS	Kobayashi *et al.* (2009)
	1	40	60	5		
	0.5	40	60	4.5		
	atmosphere	40	60	0.2		
	2	40	30	6		
	2	35	30	4.5		
	2	30	30	1.4		
	2	25	30	0.2		
14	20	45	15	7		Jung *et al.* (2009)
15	10.5	35	20	3	Microbial suspension	Garcia-Gonzalez *et al.* (2010)
	10.5	35	10	1.5		

	21	35	10	3.5		
	21	45	10	4		
16	1	60 (heating)*	15	4.5		Noma *et al.* (2010)
17	1	55	1	3.5	Cell suspension (additive: glucose)	Klangpetch *et al.* (2011)
	1	55	1	0.5	without glucose	
18	0.3	RT	20	4.8	DW	Cheng *et al.* (2011)
19	1	61 (heating)*	1	5	Cell suspension	Klangpetch *et al.* (2012)

PS Physiological Saline, DW Distilled water, RT Room Temperature
* low pressure carbon dioxide treated suspension and heating in 1 min

2.3.2. Gram-positive bacteria inactivation

Several studies have revealed that high pressure carbon dioxide is effective not just gram-negative bacteria (*E. coli*) that acts on variety of gram-positive bacteria (Tab. 2.2). The cell deaths of *Listeria monocytogenes, Bacillus cereus* and *S. aureus* (from 7 to 9 log) caused by high-pressure CO_2 were conducted by many studies (Wei et al. 1991; Lin et al. 1994; Ishikawa et al. 1997; Sirisee et al. 1998; Erkmen. 2001; Spilimbergo et al. 2002, 2003, 2010; Kim et al. 2008). Kobayashi et al. (2012) draws the attention to inactivation effect by pressurized CO_2 bubbles. In his major study, Kobayashi identifies *Lactobacillus fructivorans* was inhibited to 6 log with the only 2 MPa, 40ºC in 50 min.

Table 2. 2. Effect of HPCD on gram-positive bacteria inactivation

No.	Pressure, MPa	Tempt., °C	Time, min	Gram-positive bacteria	Reduction, log	Solution	References
1	6.18	35	120	*Listeria monocytogenes*	8.9	DW	Wei *et al.* (1991)
2	6.9	45	8	*Listeria monocytogenes*	9.9	Growth medium	Lin *et al.* (1994)
3	5	35	15	*Lactobacillus brevis*	2	PS	Ishikawa *et al.* (1995)
4	5.9	60	144	*Bacillus megatarium*	5.8	DW	Enomoto *et al.* (1997a)
5	30	55	60	*Bacillus subtilis*	6	PS	Ishikawa *et al.* (1997)
	30	50	60	*Bacillus cereus*	6		
	30	60	30	*Bacillus cereus*	6		

6	31.03	35	30	*S. aureus*	7	PBS	Sirisee *et al.* (1998)
	31.03	42.5	38	*S. aureus*	1	Ground beef system	
7	5	RT	200	*Enterococus faecalis(+G)*	1		Debs-Louka *et al.* (1999)
8	6.05	45	15	*Enterococcus*	8	PS (pH: 6.15)	Erkmen *et al.* (2000)
9	6	45	60	*L. monocytogenes*	7	PS (pH: 6.8)	Erkmen *et al.* (2001)
	6.05	25	50	*L. monocytogenes*	7		
10	7.4	38	2.5	*Bacillus subtilis*	7	PS	Spilimbergo *et al.* (2002)
11	20	34	10	*S. aureus*	3.5	Sterile water	Spilimbergo *et al.* (2003)
12	6.5	35	10	*Bacillus coagulans*	6	DW	Watanabe *et al.* (2003)
	6.5	35	10	*Bacillus licheniformis*	7		
	30	95	120	*Geobacillus stearothermophilus*	5		
13	20	40	900	*B. cereus*	1.5	PS	Spilimbergo *et al.* (2003)
	20	40	1440	*B. cereus*	3		
14	27.5	50	240	*Bacillus pumilus*	4.6	Spores trips + H_2O_2	Zhang *et al.* (2006)
	27.5	60	120		4.5		
	27.5	60	240		6.3		
15	20	40	1.7	*Lactobacillus fructivorans*	6		Tanimoto *et al.* (2007)
16	5	65	60	*Micrococcus luteus*	Complete	Textile	Cinquemani *et al.* (2007)
17	8-15	35-45	10-50	*Listeria monocytogenes*	8*	PBS	Kim *et al.* (2008)
18	6.8	25	10-20	*Listeria monocytogenes*	3		Spilimbergo *et al.* (2010)
19	2	40	60	*Lactobacillus fructivorans*	5	PBS (pH:4)	Kobayashi *et al.* (2012)
	2	40	50		6	PS	
	2	40	60		5	Sake	

[RT] Room Temperature, [PS] Physiological Saline, [DW] Distilled water, [PBS] Phosphate buffer solution
[*] max reduction log

2.3.3. Saccharomyces cerevisiae (yeast) inactivation.

Many studies investigating high pressure carbon dioxide inactivation has been carried out on *Saccharomyces cerevisiae,* one kind of common yeast (Tab. 2.3). Nakamura *et al.* (1994) developed a novel sterilization method in which CO_2 completely destroyed wet cells of baker's yeast when applied at 4 MPa and 40°C for more than 3 h. It is noteworthy

that at the room temperature, 2 log of *S. cerevisiae* was killed by CO_2 treatment under 5 MPa (Debs-Luoka et al. 1999). A significant reduction ratio of 8 log was attained at 6 MPa, 40ºC (Kumugai et al. 1997), 7.8 MPa at 35ºC (Wu et al. 2007), 13 MPa at 50ºC (Ferrentino et al. 2010). In 2010, Ferrentino et al, published a paper in which they described inactivation effect at different temperatures (35-50 ºC) and pressures (7.5-13 MPa) and the results show that high effectiveness of inhibition is enhanced under higher temperature and pressure.

Table 2. 3. Effect HPCD on *Saccharomyces cerevisiae* disinfection

No.	Pressure, MPa	Tempt., °C	Time, min	Reduction, log	Solution	References
1	4	20	120	0.1	DW	Kamihira *et al.* (1987)
	10	35	120	3.9		
	20	35	120	6.3		
2	6.9	35	15	7	Growth medium	Lin *et al.* (1992)
3	4	40	180	8	Water	Nakamura *et al.* (1994)
4	5	35	15	3	PS	Ishikawa *et al.* (1995)
5	6	40	240	8	Water	Kumugai *et al.* (1997)
	15	40	60	8		
6	4	40	240	6.8	DW	Enomoto *et al.* (1997)
7	6	35	15	5.7	Physiological Saline	Shimoda *et al.* (1998)
8	5	RT	200	2	Hydrophilic filter paper disks	Debs-Louka *et al.* (1999)
9	7.4	38-40	10	5.8*	PBS	Spilimbergo *et al.* (2003)
10	6.9	35	5	3.3	Grape juice	Gunes *et al.* (2005)
11	10	36	30	3.4	Apple juice	Spilimbergo *et al.* (2007)
	20	36	30	4		
12	7.8	35	30	4.6	Aqueous solution	Wu *et al.* (2007)
	7.8	35	60	8.8		
13	10	36	10	1	Peptonated sterile water	Spilimbergo *et al.* (2009)
	10	36	30	3		
14	7.5	35	20	2.5	DW	Ferrentino *et al.* (2010)
	10	35	20	3		
	13	35	20	4		
	7.5	40	20	4.5		

10	40	20	6	
13	40	20	7.5	
7.5	50	20	5	
10	50	20	7	
13	50	20	8	
7.5	50	20	3	PBS
7.5	50	20	5	DW

[RT] Room Temperature, [PS] Physiological Saline, [DW] Distilled water, [PBS] Phosphate buffer solution

[*] max reduction log

2.4. FACTORS AFFECTING TO INACTIVATION EFFECT OF HIGH PRESSURE CO_2 TREATMENT

2.4.1. Influence of pressure and temperature on HPCD treatment

Pressure and temperature changed leads to the adjustment of CO_2 state and have a strongly effect on the microbicidal efficiency. Increased pressure accelerates the CO_2 diffusivity into cell membrane and its solubility in cell cytoplasm. At the same effect of reduction ratio of microorganism, increasing working pressure made a shorter exposure time to treatment process (Kumugai *et al.* 1997; Erkmen. 2000c; Garcia-Gonzalez *et al.* 2010). However, the exceed pressure does not increase strongly to bacterial deaths due to saturation limitation of CO_2 in suspension phase (Spilimbergo *et al.* 2003). Whereas, temperature plays an important role in enhancing to contact efficiency between CO_2 and cell membrane, high temperature makes a major change the physical state in CO_2 transportation, such as low viscosity and high fluidity through the cells (Oulé *et al.* 2006). Moreover, at high temperature proteins are easy to be denatured and the components of external membrane are disintegrated and broken down, CO_2 molecules are facilitated to penetrate into lipid phase and cytoplasm. Most of recent studies found that increasing temperature lead to a high effect on microorganism inactivation (Kamihira *et al.* 1987; Dillow *et al.* 1999; Zhang *et al.* 2006; Wu *et al.* 2007; Kobayashi *et al.* 2009; Garcia-Gonzalez *et al.* 2010; Ferrentino *et al.* 2010). However, rising temperature makes the CO_2 solubility in water decrease, an increase in temperature may be considered in the point of optimum operation condition. Both temperature and pressure need to be determined depending on real state and each target microorganism via experiment.

2.4.2. Water effect on HPCD treatment.

Water makes cell bodies distend and CO_2 penetrate into cell membrane easily. In addition, water hydrates CO_2 to carbonate, bicarbonate and hydrogen ions changing the characteristics of CO_2 molecules leads to increase inactivation effect strongly. Membranes and cell wall may be enlarged due to presence of water to attract CO_2 gas and this improves the modification of biological obstacles. It is found that water under 0.2 g/g dry matter cause no cell death of *S. cerevisae* and the inactivation rate increases with high water content in suspension (Kumugai *et al.* 1997).

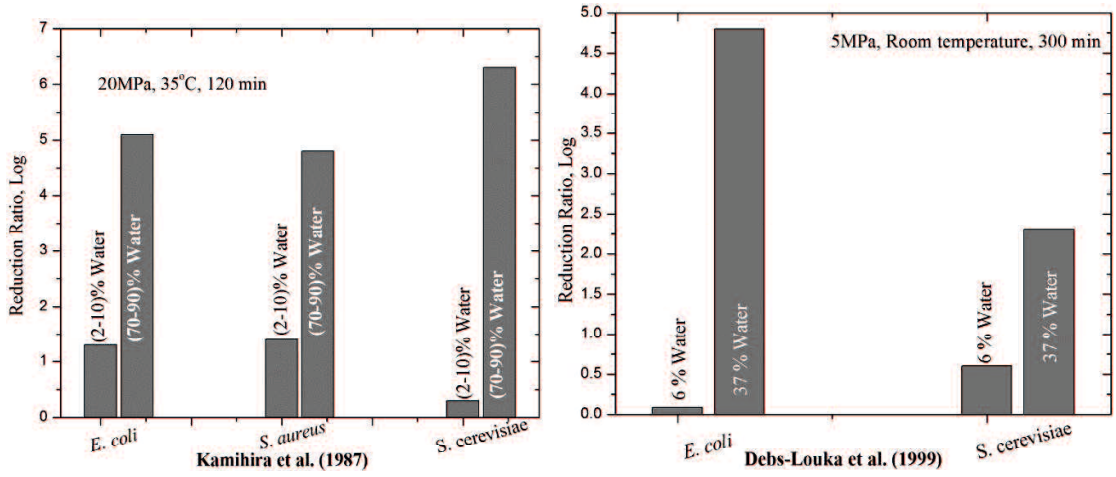

Figure 2. 4. Inactivation effect of HPCD on different bacteria by Kamihira *et al.* (1987) and Debs-Louka *et al.* (1999).

Microbicidal effect rises significantly with addition of more water volume. For example, Kamihira *et al.* (1987) found that microorganisms were inhibited nearly 4 log (*E. coli*, *S. aureus*) and 6 log (*S. cerevisiae*) when water content changed from 2-10 % to 70-90% (Fig. 1.4). Whereas, a little change of water content (from 6% to 37%) in study of Debs-Luoka *et al.* (1999), inactivation ratio of *E. coli* and *S. cerevisiae* increased strongly, 4.5 log and 2.5 log, respectively. Table 2.4 shows the results of other studies that inactivation effect of pressurized CO_2 was proved to be the most effective in a combination of water.

Table 2. 4. Influence of water on inactivation effect of HPCD.

Pressure, MPa	Tempt, °C	Time, min	Bacteria	Water status	Reduction effect	References
6.2	RT	120	*E. coli*	61% 91%	75% 99.96%	Haas *et al.* (1999)
			S. aureus	61%	75%	

				91%	99.99%	
14	34	60 30	*E. coli*	no water with water	8 8	Dillow *et al.* (1999)
7	20	15	*E. coli*	dry with water	small complete	Schmidt *et al.* (2005)
5	20	60	*E. coli*	dry (textile) with water	Small complete	Cinquemani *et al.* (2007)
			Micrococcus luteus	dry with water	small complete	

[RT] Room Temperature

2.4.3. Effect of combination processes and pressurized systems

The lethal effect of high pressure CO_2 may be improved when treated with the combination of other methods (Tab. 2.5), such as: pulsed electric field (Spilimbergo *et al.* 2003b), high hydrostatic pressure (Park *et al.* 2002), additives (Lin *et al.* 1992; Zhang *et al.* 2006), temperature…. These combinations accelerate to inactivate injured cells, reduce the treatment time to the determined level of microorganism.

Table 2. 5. Effect of pressurized CO_2 treatment with other preservation methods.

References	Individual treatment	Effect	Combination treatment with	Effect
Spilimbergo *et al.* (2003b)	Pulsed electric field at 25 KV/cm (20 pulses)	1.3 log (*B. cereus*)	Dense phase CO_2 20 MPa, 40°C, 15h	3 log (*B. cereus*)
Park *et al.* (2002)	High pressure CO_2 at 4.9 MPa	4 log (Aerobes)	High hydrostatic pressure at 300 MPa	Completely inactivated (8 log Aerobes)
Lin *et al.* (1992)	Pressurized CO_2	Remain the survival cells of *S. cerevisae*	Sulphur dioxide (30 ppm)	Complete inactivation
Zhang *et al.* (2006)	Supercritical CO_2 at 27.5 MPa, 60ºC, 4h		H_2O_2(200 ppm), ethanol	Complete destruction (6.28 log *B. pumilus*)

Pressurized systems make a highly efficient contact between CO_2 and water. Rapid saturated time of dissolved CO_2 is more effective in microbial inactivation. The continuous-flow system, pressurized CO_2 in a semi continuous process or micro-bubble reactor were found to achieve a greater efficiency in microorganism inactivation than the batch systems (Ishikawa *et al.* 1995; Shimoda *et al.* 1998; Debs-Luoka *et al.* 1999).

2.4.4. Treatment time, pressure cycling, microbial type, depressurization rate.

- **Treatment time**: Inactivation rate increases with an increase of exposure time to treatment. Curves of the relationship between microbial inactivation and treatment time are usually expressed for this. For examples, *E. coli* and *E. faecalis* inactivation were indicated to be linear correlation with pressurized CO_2 (Debs-Luoka *et al.* 1999). Whereas, a two - stage inactivation kinetics, low in first stage and fast in second stage, was found to inactivate microorganisms (Lin *et al.* 1991 & 1992; Erkmen. 2000 & 2002; Ballestra and Cuq. 1998; Enomoto *et al.* 1997b).

- **Pressure cycling:** Pressure cycling is relative to repeated process of release and compression and a promising method to enhance deactivation of microorganism. While release cycle enhances the cell rupture, compression cycle enhances to transfer CO_2 into the cell membrane. The cells can be burnt due to explosive mechanism to nearly 1 log only after 2 pressure cycles (Fraser. 1951). Inactivation effect attained a significant reduction from 3 log (after 3 cycles) to 9 log (after 6 cycles) at 20.5 MPa, 34C in 0.6 h (Dillow *et al.* 1999). Spilimbergo *et al.* (2002) found that at pressure cycling of 30 cycles/h, P= 8 MPa, at 36ºC for 30 min, a 3.5 log reduction of *B. subtilis* spores was achieved, while a treatment at 36ºC, 7,5 MPa for 24h only resulted in 0.5 log reduction without pressure cycling.

- **Microbial type**: Different cell wall structures of microorganisms may influence and differ in their resistances to inactivation effect by CO_2 treatment (Mun *et al.* 2011, 2012; Debs-Luoka *et al.* 1999). Gram positive bacteria having thicker peptidoglycan layer than gram negative bacteria is less susceptible to CO_2 (Dillow *et al.* 1999; Zhang et al, 2006).

- **Depressurization rate**: Depressurization concerns to sudden change of working pressure and this modifies physically to the psychology of cells leading to bacterial deaths or injure. Some authors concluded that decompression rate is one important factor to inactivation due to expansion of CO_2 into the cells (Fraser *et al.* 1951; Lin *et al.* 1992a & 1992b; Kumugai *et al.* 1997; Cheng *et al.* 2011). While others considered that it leads no significant effect to bacterial inhibition and the mechanical cell bursting did not happen (Li *et al.* 2013; Debs-Luoka *et al.* 1999; Nakamura *et al.* 1994). Enomoto *et al.* (1997b) found that explosive depressurization with over 4 MPa has a strong effect to inhibition but not under 4 MPa.

2.5. MICROBICIDAL MECHANISM OF HPCD

2.5.1. Disturbance by high dissolved CO_2 in water

Water will dissolve most molecular covalent substances like carbon dioxide. CO_2 has nonpolar due to symmetry but there is the possibility of fairly strong interactions with water due to each oxygen's two lone pairs. These can donate electron density to the positive hydrogen ions on the water molecule in an analogous way to how water molecules hydrogen bond to each other.

Solution of air in water follows Henry's Law - "*the amount of air dissolved in a fluid is proportional with the pressure of the system*" - and can be expressed as:

$$C = P_g . K_H^T$$

Where, C : Solubility of dissolved gas, mol/L

K_H^T : Proportionality constant (Henry's Law constant) depending on the nature of the gas and the solvent, (mol/L).MPa^{-1}.

P_g : Partial pressure of the gas, MPa.

Henry's Law constant, K_H^T (CO_2 gas, water solution) can be determined as:

$$K_H^T = K_H^0 . e^{\frac{d(Ln(K_H))}{d(\frac{1}{T})}(\frac{1}{T}-\frac{1}{298,15})}$$

Where, K_H^0: Henry's law constant for solubility in water at 298.15 K (mol/kg*MPa)

$\frac{d(Ln(K_H)}{d(\frac{1}{T})}$: Temperature dependence constant (K)

According to Lide and Frederikse (1995), K_H^0 at 25°C (298°K) is 0.35 M/MPa and $\frac{d(Ln(K_H)}{d(\frac{1}{T})}$ is 2400 (°K)

But the dissolving of CO_2 in water is actually more than just dissolving, it forms a equilibrium with water molecules to form carbonic acid H_2CO_3 and this also has equilibria with hydrogen carbonate HCO_3^- and carbonate CO_3^{2-}. These ions have strong attractions to

water molecules through hydration spheres, the same as any soluble ion. It is really this set of equilibria that gives CO_2 its solubility, the actual concentration of CO_2 (l) is quite low.

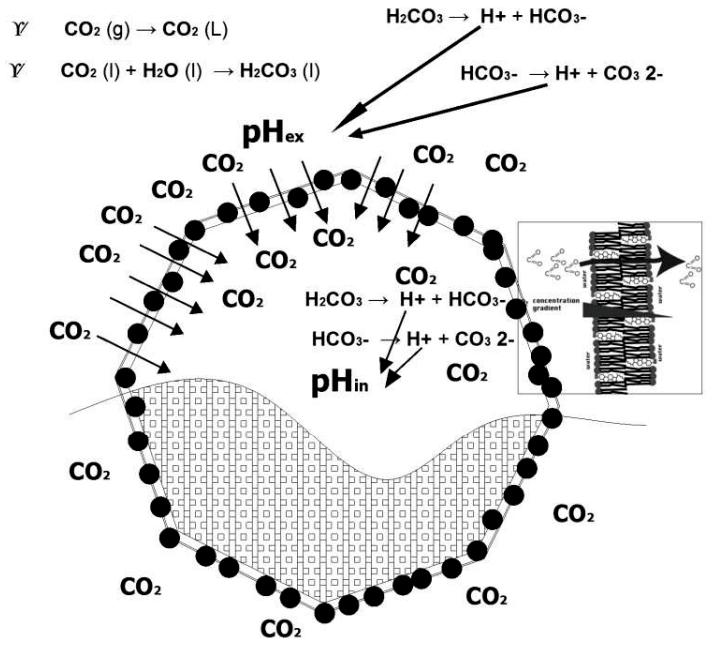

Figure 2. 5. High dissolved carbon dioxide affecting to the microbial resistance.

Equilibrium is established between the dissolved CO_2 and H_2CO_3, carbonic acid.

(1) CO_2 (g) ⇔ CO_2 (l)

(2) CO_2 (l) + H_2O (l) ⇔ H_2CO_3 (l)

This reaction is kinetically slow. At equilibrium, only a small fraction (0.2 - 1%) of the dissolved CO_2 is actually converted to H_2CO_3. Most of the CO_2 remains as solvated molecular CO_2. As equation:

$$K_r = \frac{[H_2CO_3]}{[CO_2]_l} \approx 1.7.10^{-3}$$

Carbonic acid is a weak acid that dissociates in two steps:

(3) $H_2CO_3 + H_2O \Leftrightarrow H_3O^+ + HCO_3^-$ $\quad pK_{a1}$ (25 °C) = 6.37

(4) $HCO_3^- + H_2O \Leftrightarrow H_3O^+ + CO_3^{2-}$ $\quad pK_{a2}$ (25 °C) = 10.25

It is worth bearing in mind that the solubility of CO_2 is strongly affected by temperature and pressure, less soluble in high temperatures and low pressures. Overall, formed H^+ hydrogen ions make the chemical characteristics change and the lowered extracellular pH

may prevent microbial growth (Hutkins and Nannen. 1993). External pH change may also reduce microbial resistance to inactivation because of enhanced energy demand to support pH homeostasis by the proton motive force (Hutkins and Nannen. 1993; Hong and Pyun. 1999).

2.5.2. Damage membrane

Membrane which prevents harmful agents from its environment to keep bacteria survival consists of the basic compounds: lipopolysaccharides and phospholipids and becomes stable due to Mg^{2+} and Ca^{2+} cations. Driving the substance from one side of the membrane to the other is the force of diffusion. This must penetrate the hydrophobic core of the phospholipid bilayer. CO_2 is a potential candidate for such a substance.

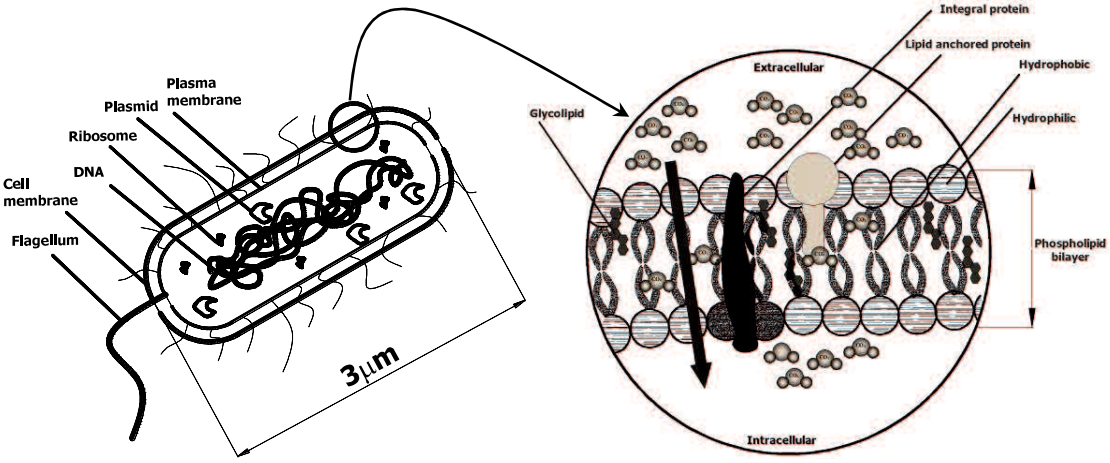

Figure 2. 6. The penetration of pressurized CO_2 through the cell membrane.

Once the ionized disinfecting molecules like CO_2, a non-polar gas, pervade into membrane, lipid phase may be dissolved and disturbed by these ions. The carbon dioxide penetrates the phospholipid bilayer without the aid of an intermediary molecule. The carbon dioxide molecules (3.4 Angstroms) are much smaller than the phospholipids (approximately 20-50 Angstroms). CO_2 molecules are very lipid soluble and transfer through cell membranes easily because formation of H^+ and HCO_3^- causes acid-base changes. This agreed with findings of Kim *et al.* (2007, 2008), pressurized CO_2, which was lipophilic nature, and easy to diffuse into the lipid bilayer with a low viscosity and high diffusivity, and then disordered the cell cytoplasm. This ease of CO_2 movement does not cause differences in pH on the two sides of the cell membrane. Another factor of high pressure

CO_2, characteristics of cellular lipid extraction, led to the cell membrane surface collapsed and changed (Li *et al.* 2013).

2.5.3. Intracellular pH lowering excessively with its buffering capacity

Another cause leading to bacterial death is the lowered intracellular pH. After CO_2 molecules getting into through cell wall and damaging the membrane structure of lipid, they continue to be accumulated inside and make the rapidly decreased intracellular pH. Most cells control pH inside themselves, they develop to adjust extracellular environment change due to buffering capacity of cytoplasmic interior, proton pumping system, bases and acids producing modification (Hutkins and Nannen. 1993). Once CO_2 gas appears to a saturated level and hydrongen H^+ ions are formed excessively, the homeostatic system of microorganism is changed, proton pumping to outside works so hard, cytoplasmic buffering capacity is limited, the cells does not produce bases to balance with H^+ ions. Many authors indicated the lowered intracellular pH is an important factor to inactivation mechanism of microorganisms by CO_2 treatment (Hutkins and Nannen. 1993; Hong and Pyun. 1999; Spilimbergo *et al.* 2005; Garcia-Golzalez *et al.* 2007).

2.5.4. Metabolism alteration/ Inhibited enzymes

Pressurized CO_2 causing the lowered internal pH has a biocidal effect on the physiology change of the cells. The appearance of excessive CO_2 breaks the metabolic chain in the decarboxylases and the vital biological processes such as glycolysis, H^+-ATPase bounding with the membrane, amino acids and peptide transport, ion transport are inhibited significantly (Hutkins and Nannen. 1993; Haas *et al.* 1999; Hong and Pyun. 1999; Spilimbergo *et al.* 2002). Moreover, after CO_2 molecules penetrating the intracellular cytoplasm, several catalytic enzymes are sensitive to this change and their activities are inhibited sharply (Garcia-Gonzalez *et al.* 2007). A lowering of intracellular pH may lead to precipitate some enzymes with an acidic isoelectric point (Ballestra and Cuq. 1998). The enzymes of lipases, several phosphatases, dehydrogenases, oxidases, amylases are considered to be well-reacted with pressurized CO_2 leading to denaturation and loss of activity (Wimmer and Zarevucka. 2010).

2.5.5. Restrain intracellular electrolyte stability and cytoplasmic leakage

Intracellular inorganic electrolytes (Mg^{2+}, Ca^{2+}…) are the important regulators for the cell activities, such as maintaining acid-base balance in the cell, the osmotic relationship

between cells and extracellular media, generating action potential and graded potentials. Once the accumulation of CO_2 increases, the essential formation of CO_3^{2-} converted from HCO_3^- is precipitated. This leads the cell activities inhibited and these vital components become inactive for their growth (Lin *et al.* 1993). Hong and Pyun. (2001) indicated that a large number of the intracellular ions (Mg and K) and UV-absorbing substances from *L. plantarum* cells lost and released under CO_2 treatment. To confirm this, Li *et al.* (2013) measured the electrical conductivity of supernatant from CO_2 treatment. The result showed a significant increase of the conductivity due to contribution of Ca^{2+}, Mg^{2+}, Na^+, K^+ ions leaked from the cells.

2.6. MICROBUBBLE TECHNIQUES FOR HPCD APPLICATION IN WATER TREATMENT

Recent years, many studies investigated on using high pressure carbon dioxide inactivation in a combination with microbubble producing techniques. The antimicrobial effects of the dissolved CO_2 are enhanced with pressurizing microbubbles (Ishikawa et al, 1997; Yoshimura *et al.* 2002; Shimoda *et al.*2002; Kobayashi *et al.* 2007, 2009, 2010, 2012). While an explosive mechanism of microbubbles was recommended by Cheng *et al.* (2011) that under sudden discharge, sharply collapsed working pressure burst the cell membranes. Takahashi *et al.* (2007) found an interesting explanation that under acidic conditions tiny microbubbles collapsed and generated the hydroxyl radicals (OH*). This is a strong oxidant. The promising discovery of this bactericidal effect should be continued to investigate in water disinfection.

2.7. CONCLUSIONS AND FURTHER OUTLOOK

This assignment has given an account of and the reasons for the widespread investigation of high pressure carbon dioxide in food preservation and sterilization. These findings reviewed in this study enhance our understanding of inactivation capacity of pressurized CO_2 to microorganisms. CO_2 used for food disinfection can be applied to dried food and liquid products. These findings from models with different operating conditions (pressure, temperature, microorganism, water content, media…) suggest that in general most of microorganisms were successfully inhibited under CO_2 treatment. It was also shown that the reasons causing the bacterial deaths are explained by many different ways even though there has not given a specific unification for clearly inactivation mechanisms yet. One of the more significant findings to emerge from these studies is that the role of water is relative to

the cell deaths. Food researchers provide important evidences with respect to the area of water disinfection that inactivation effect of HPCD seems to be better in high water content. The relevance of water disinfection by pressurized CO_2 is clearly supported by high diffusivity of CO_2 in wet media, well solubility under high pressure, low viscosity under high temperature. Microbubble producing technique in water is promising to become a great combination method with pressurizing CO_2 for target disinfection.

Finally, the current investigations were not specifically designed to evaluate factors related to water disinfection. Therefore, their successful results in food area open a potential opportunity for other fields. What is now needed is further studies involving wastewater and water treatment. These findings provide the following insights for future research:

- Elucidating the inactivation effect of CO_2 nature in water.
- Current findings have thrown up many questions in need of further investigation about clear mechanisms.
- Research is also needed to determine the role pH caused by pressurized CO_2 to the cell deaths.
- It would be interesting to compare inactivation effect of different gases within the same disinfecting agents.
- A further study could assess water disinfection effect of CO_2 against virus
    - A future study investigating pressure processes with producing CO_2 microbubbles may enhance inactivation effect.
    - Considerably more work will need to be done to determine the CO_2 effect in different environmental water samples (distilled water, buffered water, effluent wastewater, ground water, surface water…).
    - Future trials should assess a disinfection performance of high pressure CO_2 including biosolids.

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CONNECTING TEXT: CHAPTER 2 → CHAPTER 3

One question that needs to be referred, however, is whether pressurized CO_2 can be effective to inactivate pathogens in water or not? From the objectives at Chapter 1 and the evidences and successful investigations found in the previous chapter, next chapter describes by laying out the comparison design of CO_2, N_2O and N_2 to inactivation effect against *E. coli* in distilled water. A mechanism of CO_2 inactivation is also expressed for the cell deaths in this section

CHAPTER III

COMPARISON OF DISINFECTION EFFECT OF PRESSURIZED GASES OF CO_2, N_2O, AND N_2 ON *ESCHERICHIA COLI*

3.1. ABSTRACT

Based on the production of gas bubbles with the support of a liquid film-forming apparatus, a device inducing contact between gas and water was used to inactivate pathogens for water disinfection. In this study, the inactivation effect of CO_2 against *Escherichia coli* was investigated and compared with the effects of N_2O and N_2 under the same pressure (0.3–0.9 MPa), initial concentration, and temperature. The optimum conditions were found to be 0.7 MPa and an exposure time of 25 min. Under identical treatment conditions, a greater than 5.0-log reduction in *E. coli* was achieved by CO_2, while 3.3 log and 2.4 log reductions were observed when N_2O and N_2 were used, respectively. Observation under scanning electron microscopy and measurement of bacterial cell substances by UV-absorbance revealed greater cell rupture of *E. coli* following treatment with CO_2 than when treatment was conducted using N_2O, N_2 and untreated water. The physical effects of the pump, acidified characteristics and the release of intracellular substances caused by CO_2 were bactericidal mechanism of this process. Overall, the results of this study indicate that CO_2 has the disinfection potential without undesired by-product forming.

Keywords: *Escherichia coli*, nitrous oxide, high pressure CO_2, bactericidal effect, water disinfection

3.2. INTRODUCTION

Various studies have been conducted to investigate the use of different disinfectants for inactivation of pathogens in wastewater and water treatment. For nearly a century, chlorine has played a major role in standards for water disinfection in Europe, the United States and other countries around the world. The economic and effective characteristics of chlorination disinfection make it a better choice for treatment than other disinfectants. Unfortunately, during chlorination, the chlorine combines with organic matter to generate carcinogenic by-products. Accordingly, the risks posed to human health by the use of chlorinated drinking water are uncertain at this time. Alternative techniques to improve by-products releasing from the reaction of residual chlorines and ozone with organic materials

during conventional disinfection have also been investigated (Richardson, 2011; Steve, 2009). UV disinfection requires a preventive maintenance program and ozone treatment generates undesired disinfection by-products (DBPs) (Guus et al., 2007; Singer et al., 1993), while the membrane filtration process does not produce DBPs, but is a complicated disinfection process and quite expensive (EPA, 2001a). Recently, solar disinfection (David et al., 2012; Lee et al., 2009), ultrasound (Ayyildiz et al., 2011) and hydrodynamic cavitation (Mezule et al., 2010) have been reported as potential treatment technologies; however, further studies of alternative disinfectants or disinfection methods are needed. The latest technologies for water disinfection should enhance the advantages of conventional methods and eradicate their shortcomings. This study attempted to accomplish this by transferring the antiseptic properties of carbon dioxide used in food preservation to wastewater and water disinfection.

High pressure CO_2 (4.0–50.0 MPa) has been found to effectively inactivate many types of pathogens (Ballestra et al., 1996; Dillow et al., 1999; Erkmen et al., 2001; Haas et al., 1989; Kamihira et al., 1987). This method is dependent on contact between a liquid-film and the air (Imai et al., 2008), and involves the application of carbon dioxide to inactivate pathogens as a new disinfectant producing no by-products. More than 100 published journals in the area of food preservation have reported that high pressure CO_2 caused efficient bactericidal effects, but an inactivation mechanism has not clearly been understood (Zhang et al., 2006). Thus, few studies have been conducted to investigate the use of pressurized CO_2 to enhance antimicrobial treatment of wastewater and water. Kobayashi (2007, 2009) conducted one of the first studies to investigate the use of CO_2 microbubbles to inactivate *E. coli* and coliforms within 13.3 min, but a supercritical pressure of 10 MPa and high temperature range (35–55°C) were needed to achieve the effective results. On the other hand, *E. coli* disruption by the combination of high temperature (55°C) with low pressure carbon dioxide at 1 MPa was reported by Klangpetch et al. (2011). However, these studies did not alleviate the need for conventional heat pasteurization. Enomoto (1997) concluded that the depressurization rate at less than 4 MPa led to no mechanical cell rupture, while the only 0.3 MPa was found to cause cell death (Cheng et al., 2011). Therefore, these findings did not reveal whether the chemical nature of CO_2 or depressurization was related to the death of the *E. coli* cells. Low pH caused by CO_2 dissolution is believed to have a bactericidal effect (Garcia- Gonzalez et al., 2007), and acidified CO_2 has been found to more

easily penetrate cell walls and the intracellular environment of bacteria to inhibit microbial growth (Haas et al., 1989; Hong and Pyun, 2001; Spilimbergo et al., 2002).

This study was conducted to investigate the relationship between the reduction in pH of the liquid environment induced by CO_2 applied at 0.2–1.0 MPa and pathogen inactivation. In addition, nitrogen (N_2) and nitrous oxide (N_2O) were used to provide a basis for comparison of bactericidal mechanisms involved in CO_2 disinfection. Both N_2O and CO_2 have strong solubility in water and similar characteristics; however, CO_2 leads to acidification of the solution and N_2O leads to neutralization. Moreover, N_2 has weak solubility in water. Therefore, these compounds were compared to determine whether CO_2 or N_2O led to its inactivation effects. In addition, the weak solubility of N_2 was confirmed. The results of this study could facilitate the application of low pressure CO_2, an innovative bactericidal disinfectant technique, to wastewater and water disinfection.

3.3. MATERIALS AND METHODS

3.3.1. Microorganism preparation and enumeration

Escherichia coli (ATCC 11303) from stock cultures (American Type Culture Collection, Manassas, VA, USA) was used as a representative pathogen in this experiment. *E. coli* was propagated in flasks containing 100 ml Luria-Bertani (LB) broth (Wako Chemical Co. Ltd., Osaka, Japan) at 37°C with continuous shaking for 16–18 h at 150 rpm. The cell concentrations were then determined by plating aliquots of the culture onto LB agar (Wako) and incubating the samples at 37°C overnight. The number of colony-forming units (CFU) was subsequently counted on plates that contained 25–300 CFUs/plate. The initial enumeration was approximately 10^9–10^{10} CFU·mL^{-1} and cell suspensions were maintained in 20% glycerol at -80°C. All stock cultures were used within one month, and 1–100 mL of *E. coli* that had been incubated at 37°C and 150 rpm for 12–18 h was used for each experiment.

3.3.2. Apparatus and procedure for inactivation

The apparatus used for the experiment was designed to provide a high contact efficacy between the treatment gas and liquid (Imai et al., 2008) (Fig. 3.1). A nozzle and shield were set up inside the apparatus to strongly agitate the influent water. Highly dissolved treatment gas in water obviously developed inside the device. The initial temperature of 20–22°C was maintained throughout the experiment.

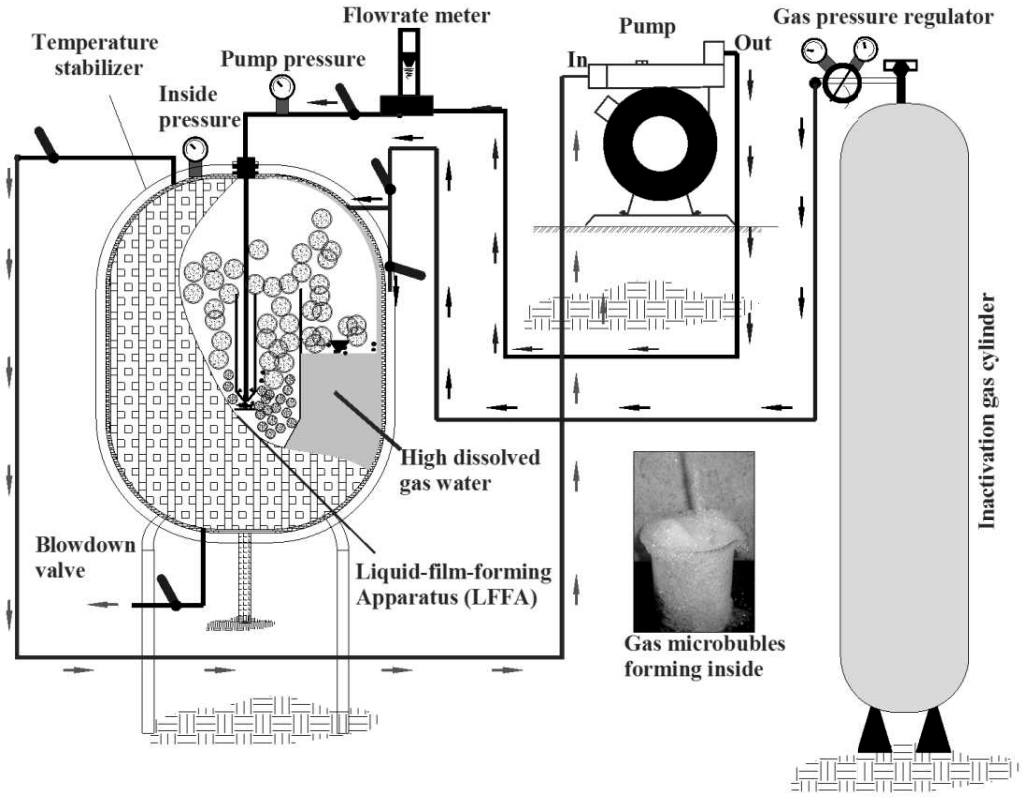

Figure 3. 1. Schematic diagram of apparatus used for microbial inactivation.

Microbial suspensions of low (100–200 μL), medium (1–5 mL) or high (50–100 mL) concentration and 7000 mL of distilled water were mixed at room temperature to give the desired concentrations (low: 10^3–10^4 CFU/mL, medium: 10^5–10^6 CFU/mL and high: 10^7–10^8 CFU/mL), after which these mixtures were used as water samples that have been subjected to microbial contamination. Approximately 7000 mL of this wastewater was pumped into the device. During treatment, the flow rate was 13–15 l·min^{-1} and the contact time was 25 min. At the beginning of the experiment, the treatment system, which can tolerate up to 1.0 MPa, was filled with treatment gas at 0.2 to 1.0 MPa. A blow down valve designed for low or rapid depressurization was used to collect the samples. Performance was judged based on the inactivation of *E. coli* at various pressures and concentrations. All experiments were conducted in triplicate.

3.3.3. Inactivation mechanism assessment

The bactericidal mechanism was judged by examining the pressurized microbubbles of N_2, N_2O and CO_2 on *E. coli* leading to their different inactivation effects. As shown in Table 3.1, the solubility and nature of N_2 are much different from those of CO_2, whereas N_2O and

CO_2 have similar properties in terms of molecular weight, gas density, specific volume, critical pressure and temperature and solubility in water. N_2O and CO_2 have very similar features, with the only major difference being that a reduction in pH is caused by CO_2 ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow 2H^+ + CO_3^{2-}$) but not N_2O ($N_2O + H_2O \leftrightarrow H_2N_2O_2$). UV-absorbance and scanning electron microscopy (SEM) were used to evaluate the disinfection mechanism of CO_2.

Table 3. 1. Physical and chemical characteristics of inactivation gases.

Properties	CO_2	N_2O	N_2
[a] Molecular weight, g/mol	44.01	44.013	28.013
[a] Gas density, 1.013bar at 15°C, kg/m^3	1.87	3.16	1.185
[a] Specific volume, 1.013bar at 21°C, m^3/kg	0.574	0.543	0.862
[a] Critical temperature, °C	31.1	36.42	-147.0
[a] Critical pressure, MPa	7.3825	7.245	3.3999
[a] Solubility in water, 0.1013MPa, (25°C), g/L	1.80	1.20	0.018
[a] Diffusivity, cm^2/s	2.47.10^{-5}	4.89.10^{-5}	

a Cited from Murat and Giovanna. 2012.

3.3.4. Measurement of UV-absorbing substances

E. coli cells destroyed by CO_2 released various substances, including nucleic acids and proteins. Therefore, treated samples were centrifuged at 1000×*g* for 10 min, after which the absorbance of the supernatant at 260 nm and 280 nm was measured by spectrophotometry (Hitachi, Tokyo, Japan) to determine the levels of nucleic acids and proteins, respectively (Kim et al., 2008a).

3.3.5. Scanning electron microscopy

Treated and untreated samples were centrifuged at 8000 rpm for 10 min, after which the supernatant was discarded and the pellet was washed with PBS buffer three times. The samples were then fixed with 2.5–3.0% glutaraldehyde (Wako) in PBS buffer (pH=7.2) overnight at 4°C, after which they were immersed in 1% osmium tetroxide and cacodylate buffer for 90 min at room temperature and then dehydrated at 4°C with sequences of ethanol at 50% (twice for 10 min each), 70%, 80%, 90%, 95% and 100% (three times for 15 min each), followed by EtOH/t-butyl alcohol (v/v=1/1 for 30 min) (Kim et al., 2008b). Finally, the samples were washed in fresh t-butyl alcohol twice for 1 h each, freeze-dried under low temperature for 3 h (VFD-21S t-BuOH free dryer), covered with gold-palladium and observed by scanning electron microscopy (SEM, Quanta^{TM} 3D, FEI Co.).

3.4. RESULTS AND DISCUSSION

3.4.1. Inactivation effect of CO_2, N_2 and N_2O against *E. coli*

In this study, CO_2, N_2 and N_2O were evaluated for their inactivation performance. The inactivation efficiency of gases was compared under various pressures. As shown in Fig. 3.2, the bactericidal effect of CO_2 was higher than that of N_2O and N_2 at every operating pressure. When CO_2 was used, the reduction ratios of *E. coli* at 0.3 MPa and 0.5 MPa (Fig. 3.2a & 3.2b) were nearly 2.8 log within 25 min, while they were only 0.62–0.90 log and 0.85–1.44 log when N_2 and N_2O were used, respectively. When higher pressure conditions of 0.7–0.9 MPa (Fig. 3.2c & 3.2d) were employed, most gases showed greater *E. coli* inactivation. Specifically, CO_2 inactivation reached 5.2 log at 0.7 MPa and 4.7 log at 0.9 MPa. Surprisingly, N_2 induced 2.4 and 2.8 log reductions in *E. coli* at 0.7 MPa and 0.9 MPa, respectively. N_2O, which has the same molecular weight, critical temperature and pressure, solubility in water and diffusivity as CO_2, but does not change the pH, induced an inhibition effect of 3.33 (0.7 MPa) and 3.69 log (0.9 MPa). As shown in Fig. 3.3, the change in the pH of water differed in response to treatment with CO_2, N_2O and N_2. Specifically, during the first minute of inactivation, the pH decreased from 8.4 to 4.9 in response to CO_2 treatment, while that of N_2O and N_2 treated water remained stable or increased slightly. These findings indicate that CO_2 may play a crucial role in attenuation of microbial growth.

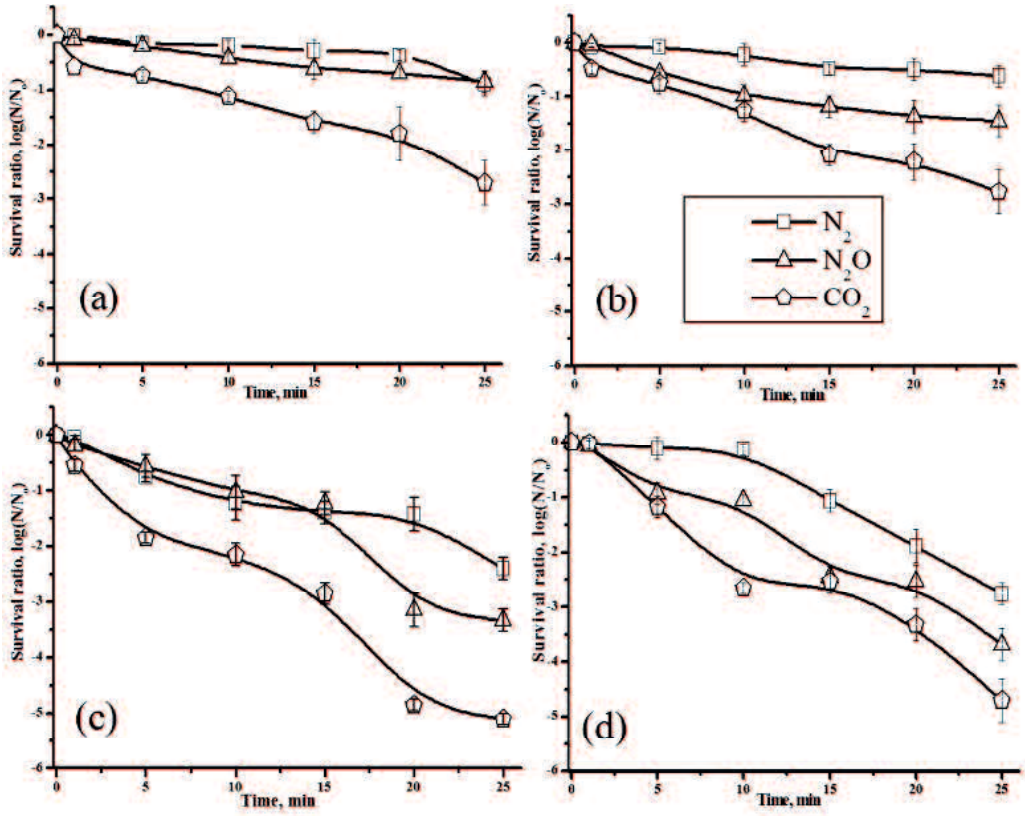

Figure 3. 2. Comparison of bactericidal performance of N_2, N_2O and CO_2 against *E. coli* (ATCC 11303, initial concentration: 10^5–10^6 CFU/mL) inactivation at (a) 0.3 MPa, (b) 0.5 MPa, (c) 0.7 MPa and (d) 0.9 MPa.

Overall, the results of this study indicate that the CO_2 inactivation mechanism was as follows. The production of gas microbubbles and high pressure enables CO_2 to easily penetrate the cell membrane and change the physiological features of *E. coli*. When non-polar CO_2 molecules enter the cell, they impact the structure of the cell wall (Isenschmid et al., 1995). Moreover, too much dissolved CO_2 continuously pumped into a layer of phospholipids may disrupt and change the stability of lipid chains. The accumulation of CO_2 also leads to a rapid decrease in intracellular pH (Spilimbergo et al., 2005). The buffering capacity of bacteria is limited and increases the proton pumping system (Hutkins and Nannen, 1993), which leads to restraint of the cellular metabolism and important enzymes (Hong and Puyn, 2001, Hutkins and Nannen, 1993, Spilimbergo et al., 2002).

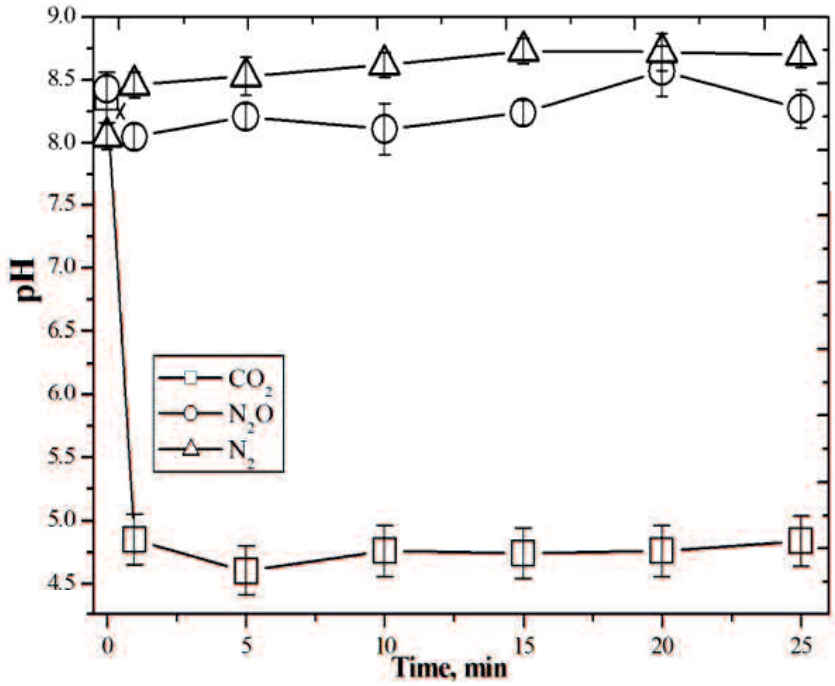

Figure 3. 3. pH change in water in response to CO_2, N_2 and N_2O at 0.7 MPa.

Many studies of food preservation and water disinfection have shown that CO_2 has the potential to inhibit pathogens (Ishikawa et al., 1995; Haas et al., 1989; Kamihira et al., 1987; Kobayashi et al., 2007, 2009; Enomoto et al., 1997). However, the importance of cell rupture and the physiological mechanism behind such inhibition have been extensively debated. In this study, the bacterial inhibition by CO_2 was investigated by comparison with the effects of N_2 and N_2O treatment. The differences in the following parameters between N_2 and CO_2 ($T_c(N_2)$ = -147°C, $P_c(N_2)$ = 3.39 MPa versus $T_c(CO_2)$ = 36.5°C, $P_c(CO_2)$ = 7.24 MPa) led to various bactericidal effects. N_2 induced little or no bactericidal effect at low pressure (0.2–0.6 MPa), but did show a bactericidal effect at 0.7 MPa. Nevertheless, these effects were much lower than those induced by CO_2 under the same conditions. The solubility of N_2 may prevent it from modifying bacterial cells during treatment, which would explain the greater effect observed at higher pressures. Indeed, SEM analysis confirmed that some cells were sheared by high pressure forces, but that the shapes of *E. coli* were unchanged.

Conversely, the inactivation effect of N_2 was merely due to physical factors (pressure and pump cycling), while that of N_2O was primarily in response to a combination of physical

factors, and to a lesser degree, its ability to penetrate and dissolve the cells via its chemical properties.

3.4.2. Inactivation effect of CO_2 at various pressures against *E. coli*

Owing to the superior bactericidal performance of CO_2, the effects of CO_2 at pressures of 0.2 MPa to 1 MPa were investigated. Fig. 3.4 shows the reduction of *E. coli* after 25 min, a common period used for water disinfection, for example with chlorine (EPA, 2001b). In contrast to tests conducted at low pressures (0.2–0.6 MPa), which showed a maximum decrease in *E. coli* of 3.2 log at 0.6 MPa and a minimum decrease of 2.5 log at 0.4 MPa, those at high pressures (0.7–1.0 MPa) showed decreases of 4.2 to >5.2 log. The greatest decrease in *E. coli* was observed at 0.7 MPa; therefore, subsequent experiments were conducted using 0.7 MPa.

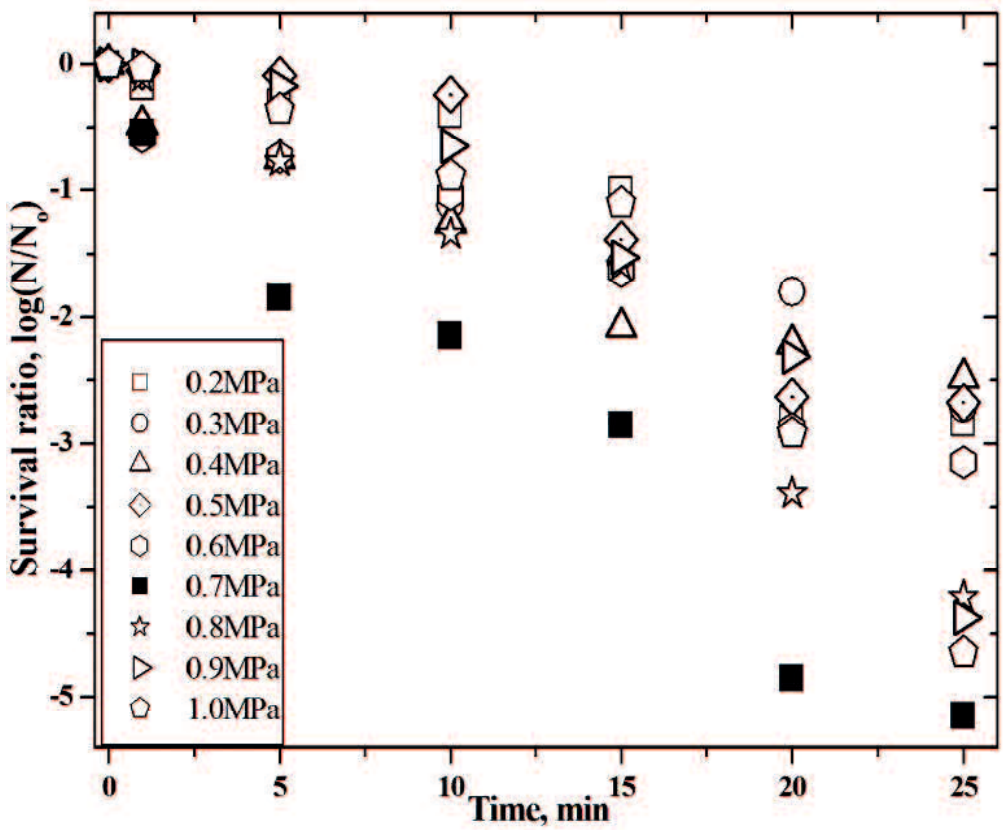

Figure 3. 4. Inactivation effect of CO_2 at various pressures against *E. coli* (ATCC 11303 - initial concentration: 10^5–10^6 CFU/mL).

3.4.3. Bactericidal effect of CO_2 against *E. coli* at 0.7 MPa and UV-absorbance of *E. coli* cell supernatant

As shown in Fig. 3.5, the inhibition of *E. coli* reached 4.7–5.2 log after 25 min at 0.7 MPa. We previously found 20 min to be the most effective period for inactivation (Cheng et al., 2011); however, in the present study, the reduction of *E. coli* at 20 min was not steady; therefore, 25-minutes was used for subsequent experiments. The decrease in pH caused by CO_2 (Fig. 3.3) was considered to be a reason for cell death (Spilimbergo et al., 2002). To confirm that the cells had been lysed, the levels of nucleic acids and proteins were measured based on the absorbance of samples at 260 nm and 280 nm, respectively. As shown in Fig. 3.5, within the first 10 minutes, the absorbance increased only slightly, indicating a low inactivation effect. However, the absorbance peaked at 25 minutes, corresponding to the maximum inactivation.

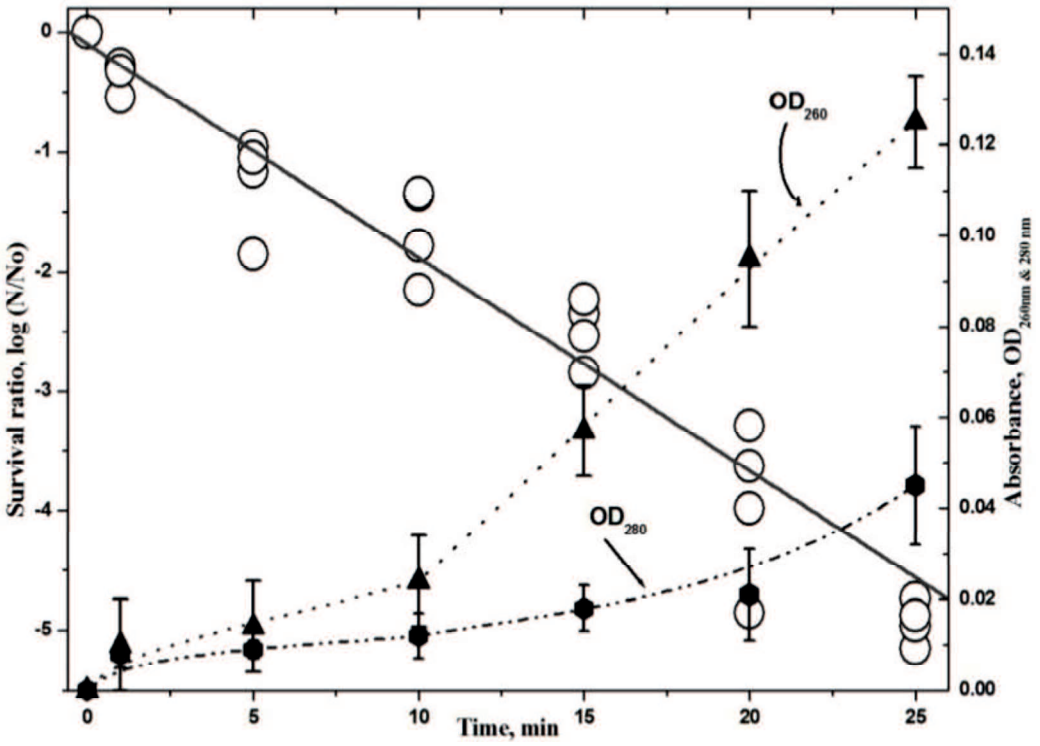

Figure 3. 5. Inactivation effect of CO_2 against *E. coli* (ATCC 11303- initial concentration: 10^5–10^6 CFU/mL) at 0.7 MPa and UV-absorbance of *E. coli* cell supernatant over time. Light absorbance (OD: optical density) at 260 nm for nucleic acids and 280 nm for proteins.

3.4.4. Inactivation performance of CO_2 at 0.7 MPa against *E. coli* in samples with different initial concentrations

Figure 3.6 shows the inactivation of *E. coli* under different initial concentrations at 0.7 MPa. When the initial concentration was low (10^3–10^4 CFU/mL), no surviving cells were detected after 20 min, whereas samples with moderate initial concentrations showed a decrease of 5.2 log within 25 min. When high initial concentrations (10^7–10^8 CFU/mL)

were used, the rate of cell reduction only reached 4.5 log after 25 min, but this rate grew steadily for 10 min to over 6.5 log, indicating an approximately 1.0-log reduction/5 min. In general, the bactericidal effectiveness was best at medium concentration.

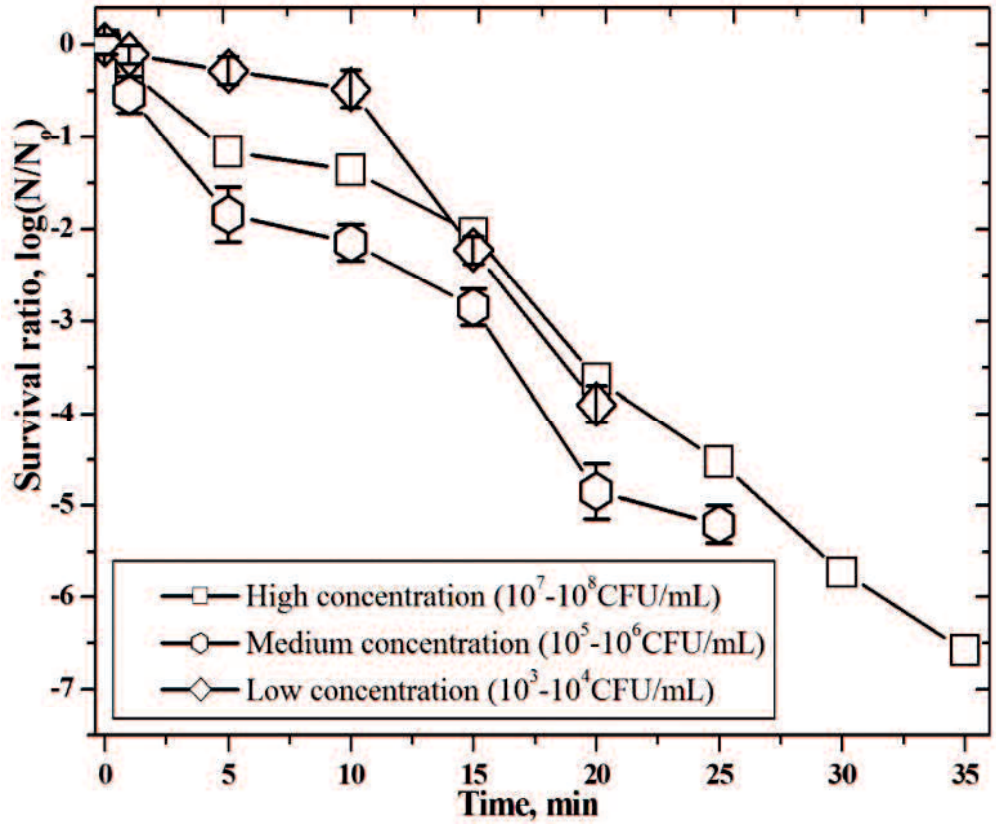

Figure 3. 6. Inactivation performance of CO_2 at 0.7 MPa with different initial concentrations of *E. coli* (ATCC 11303).

3.4.5. SEM observation

Cell modifications were observed by SEM analysis of cells treated with N_2, N_2O and CO_2 for 25 min. As shown in Fig. 3.7a, the *E. coli* initially appeared healthy. After treatment at 0.7 MPa with pressurized N_2, no or only a few cells appeared broken (Fig. 3.7b). Conversely, cells treated with N_2O had rough surfaces and many had broken cell walls (Fig. 3.7c). No cells could be identified after treatment with CO_2, indicating that they had all been lysed (Fig. 3.7d).

N_2O has a similar molecular weight, solubility in water, critical temperature, and critical pressure as CO_2. Despite these similarities, treatment with N_2O produced less effective inactivation than treatment with CO_2. N_2O did not acidify the treated water, while CO_2 reduced the pH to nearly 4 during the first minute. Nevertheless, N_2O had a greater

bactericidal effect than N_2. The anesthesia and non-polar characteristics of N_2O enable it to be easily dispersed into the phospholipid layer of cell membranes with the support of high pressure (Spilimbergo et al., 2002). This may lead to dissolution of fatty sections, changes in the activity of the cells and obstruction of the bacterial growth. Indeed, *E. coli* cells were peeled and lysed in response to treatment with N_2O (Fig. 3.7c).

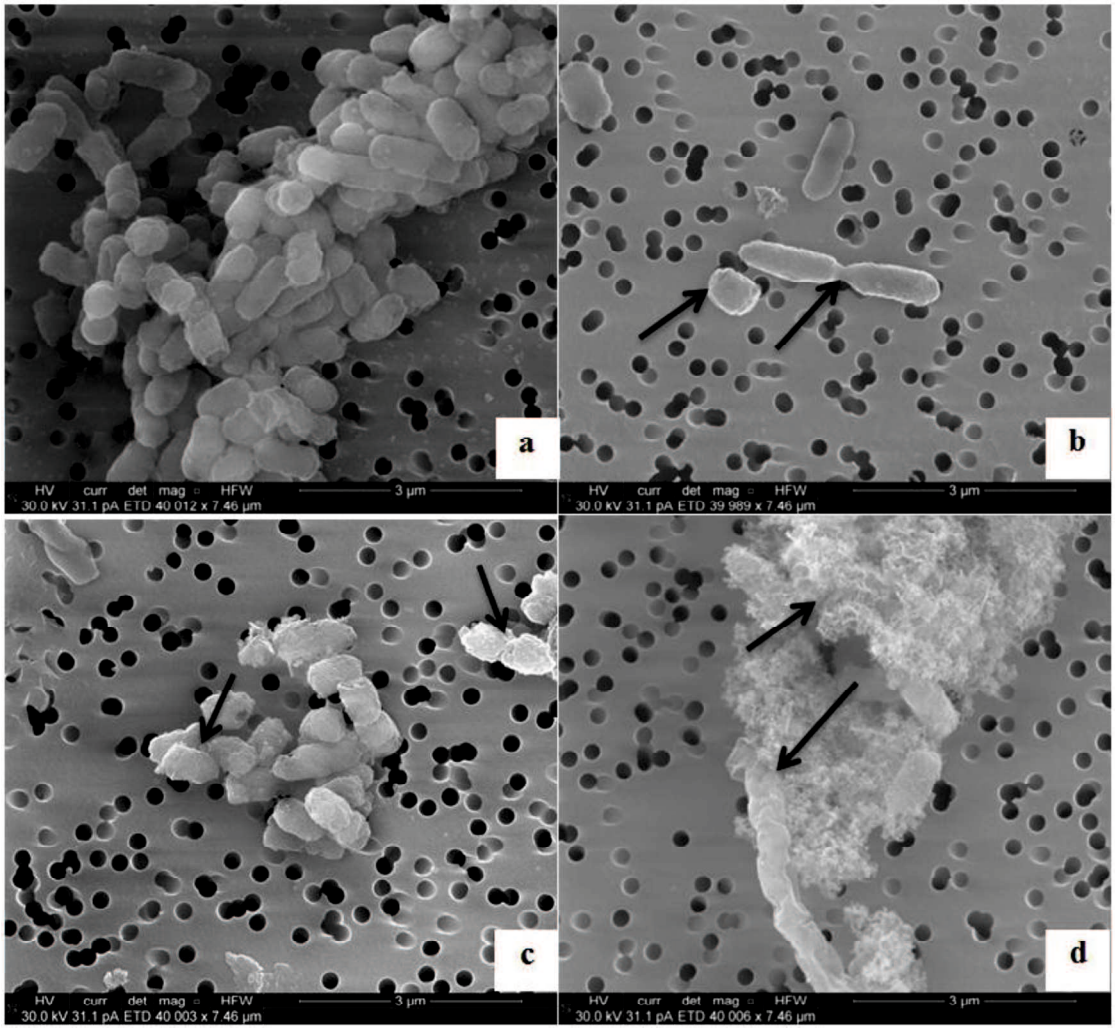

Figure 3. 7. *E. coli* cells under SEM observation (a) untreated (b) N_2 treated (25 min, 0.7MPa), (c) N_2O treated (25 min, 0.7MPa) and (d) CO_2 treated (0.7MPa, 25 min).

The reduction in pH induced by treatment with CO_2 was likely the mechanism through which CO_2 attenuated *E. coli*. The bacterial deaths caused by CO_2 were inhibited to the same degree. SEM images of treated cells affirmed the superior treatment performance of CO_2. The cell membranes of *E. coli* were severely damaged and their initial structures were unrecognizable.

Analysis of the absorbance of the samples revealed that nucleic acids and proteins had been extracted from the *E. coli* cells. These findings are in accordance with those of previous investigations in the field of food preservation (Erkmen et al., 2001; Ishikawa et al., 1995; Haas et al., 1989; Kamihira et al., 1987) and were further confirmed in water disinfection by comparison with the results of the N_2 and N_2O experiments. After exposure for a sufficient time, bacterial cells were easily damaged and lysed (Figure 7d). Although the discharge of water appeared to change the pressure of the cells, this was likely not responsible for most bacterial deaths, and rapid or slow depressurization was not the principal factor involved in the inactivation effect (Enomoto et al., 1997).

The optimal conditions for CO_2 treatment were found to be 0.7 MPa and 25 min at room temperature. Cheng et al. (2011) found that a 20-minute period was sufficient for bacterial inactivation, but in the present study, the cells were lysed after extending treatment time to 25 minutes because this was determined to be the point at which CO_2 accumulation inside the cells surpassed their limitations. Residual CO_2 after treatment may diffuse to air and gradually recover neutral pH later.

3.5. CONCLUSIONS

Using microbubbles of pressurized CO_2, N_2 and N_2O to inactivate *E. coli* (ATCC 11303) revealed the following:

- When compared with those of N_2O and N_2, the bactericidal effect of CO_2 was much greater. Additionally, operation of the apparatus at higher pressure (0.7–1.0 MPa) led to a more prominent reduction of *E. coli*, as compared with operation at 0.2–0.6 MPa.
- The decrease of pH in water induced by treatment with CO_2 is considered to be the most effective factor leading to its bactericidal effects.
- A pressure of 0.7 MPa, room temperature and an exposure time of 25 minutes were determined to be the optimum operating conditions for the treatment of artificial wastewater when *E. coli* were the target pathogens.

Overall, CO_2 has the potential for use as a disinfectant of wastewater and drinking water with low and medium concentrations of *E. coli*. Furthermore, this method does not produce disinfection by-products, resulting in reduced health risks and operation costs. Further research is needed to confirm the disinfection effect of CO_2 toward bacteriophages and to fully elucidate the role of intracellular pH.

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CONNECTING TEXT: CHAPTER 3 → CHAPTER 4

The findings of the previous chapter indicate that pressurized CO_2 attained a high effective to inhibit *E. coli* cells in distilled water. For this reason, Chapter 4 assesses the disinfection performances of CO_2 treatment against the different bacteriophages, the role of temperature and pressure to inactivation effect of *E. coli* and phages.

CHAPTER IV
DISINFECTION USING PRESSURIZED CARBON DIOXIDE TO INACTIVATE *ESCHERICHIA COLI* AND BACTEROPHAGES

4.1. ABSTRACT

This study investigated the potential application of pressurized CO_2 for water disinfection. Under supporting high pressure, a high volume of CO_2 microbubbles were produced in a liquid environment. Specifically, the inactivation effects of CO_2 against *Escherichia coli*, bacteriophage MS2, T4, Qβ and ΦX174 were examined at equal pressures (0.3–0.9 MPa) and temperatures. The optimum conditions were found to be 0.7 MPa and an exposure time of 25 min. Under identical treatment conditions, a greater than 5.0 log reduction in *E. coli* was achieved, while approximately 3.0 log reduction was observed for phage MS2 and phage ΦX174. For phage T4 and phage Qβ, a reduction of nearly 4.0 log in the former and more than 3.3 log in the latter were achieved by CO_2. Comparison of the inactivation effect of CO_2, N_2O, a common acid and buffer solution against phage MS2, revealed that the change in pH caused by CO_2 plays an important role in its virucidal effects. Moreover, the pumping cycle and depressurization rate contributed to the inhibition of microorganisms. Overall, the results of this study indicate that CO_2 has the potential for use as a disinfectant without the formation of by-products.

Keywords: bacteriophage, *Escherichia coli*, microbubbles, pressurized CO_2, viricidal effect, water disinfection.

4.2. INTRODUCTION

For several decades, water and wastewater treatment plants have primarily relied on the use of chlorine for disinfection. Chlorine is a well-known economical disinfectant for protection against waterborne diseases. However, chlorination has numerous disadvantages. Specifically, chlorination can produce chlorinated hydrocarbons that are considered health hazards, and can be corrosive. Compared to chlorination, ozone has greater inactivation effectiveness against bacteria, viruses, *Giardia* and *Cryptosporidium* (Rennecker et al, 1999; Driedger et al, 2001; Haas and Kaymak, 2003). However, ozone disinfection is corrosive, has a high initial cost and requires high electricity consumption. Ultraviolet irradiation and membrane filtration are potential alternatives to chemical disinfection, but require intensive maintenance, high costs and complicated setups. For these reasons, alternative water

disinfectants need to be investigated. Our group developed a novel method for the application of an existing technique (high pressure CO_2 treatment) that has been successfully implemented in the preservation of food to the treatment of water and wastewater. A wide variety of bacterial pathogens in liquid foods have been shown to be inactivated in previous studies (Nakamura et al, 1994; Ballestra et al, 1996; Dillow et al, 1999; Hong and Pyun, 1999). Several studies recently investigated the application of high pressure CO_2 disinfection to water treatment. Supercritical pressure up to 10 MPa and high temperature (55°C) were reported to effectively inactivate *E. coli* and coliforms (Kobayashi et al, 2009). However, Cheng (2011) concluded that pH was not related to the inactivation mechanism of dissolved CO_2, which differed from the results of previous studies (Ballestra et al, 1996; Dillow et al, 1999; Hong and Pyun, 1999; Garcia-Gonzalez et al, 2007).

Therefore, this study was conducted to investigate the inactivation of pathogenic bacteria (*E. coli*.) and indicator viruses, including T4 (double-strained DNA), MS2 (single-strained RNA), Qβ (single-strained RNA) and ΦX174 (single-strained DNA) by CO_2. First, the use of CO_2 treatment at different pressures for the inactivation of phage T4, MS2, phage Qβ and ΦX174 was investigated, after which treatment of samples containing *E. coli*, MS2 and phage ΦX174 was conducted at various temperatures and with and without buffer solution to confirm the thermal and pH roles. Finally, a comparison of the inactivation effects of CO_2, nitrous oxide (N_2O) and common acid on phage MS2 and phage Qβ was conducted to elucidate the chemical nature of CO_2.

4.3. MATERIALS AND METHODS

4.3.1. Microorganism preparation and enumeration

Escherichia coli (ATCC 11303) from stock cultures (American Type Culture Collection, Manassas, VA, USA) was propagated in flasks containing 100 mL Luria-Bertani (LB) broth media (Wako Chemical Co. Ltd., Osaka, Japan) and incubated at 37°C with continuous shaking for 16–18 h at 150 rpm. The cell concentration was determined by spreading aliquots on LB agar plates (Wako), incubating the samples overnight at 37°C, and then determining the number of colony-forming units (CFU) from plates containing 25–300 colonies. The initial concentration was estimated to be approximately 10^7-10^9 CFU/mL. Cell suspensions were maintained in 20% glycerol at –80°C and were used within 1 month. For

each experiment, 100 mL of *E. coli* stock inoculated LB was incubated at 37°C and 150 rpm for 12–18 h.

4.3.2. Bacteriophage propagation

T4 (ATCC 11303-B4^{TM}), MS2 (ATCC 15597-B1^{TM}), Qβ (ATCC 23631-B1^{TM}) and ΦX174 (ATCC 13706-B1^{TM}) were grown to high titers by overnight incubation at 37°C in *E. coli* hosts ATCC 11303, 15597, ATCC 23631 and 13706, respectively. The remaining cells and cell debris were eliminated by centrifugation at 2,000×*g* for 10 min. The supernatant, including the phage, was then filtered through a membrane filter with a pore size of 0.20 μm (Millipore, Carrigtwohill, County Cork, Ireland). Virus suspensions with initial concentrations of 10^7–10^9 PFU/mL were stored in 20% glycerol. For storage, samples were initially refrigerated at -20°C for 24 h, then reduced to -80°C to prevent temperature shock.

4.3.3. Bacteriophage titer

Surviving infectious T4, MS2, Qβ and ΦX174 were enumerated by forming lawns of sensitive strains of *E. coli* hosts (Debartolomeis et al, 1991) and then conducting plaque phage assays using double layers of agar on the plates. Initially, 0.1 ml phage suspension was mixed with 0.2 ml *E. coli* host culture and incubated at 37°C (50 rpm) for 30 min. This mixture was then blended directly in a test tube containing 5 ml of top layer of liquefied Tryptic Soy Agar (TSA) 0.7% [wt/vol] (Wako) and poured rapidly onto a Petri dish containing TSA at 1.5% [wt/vol]. Plaque-forming units (PFU) were determined after overnight incubation at 37°C based on plates containing 30–300 PFU.

4.3.4. Apparatus and procedure for activation

The apparatus erected for the experiment was designed to produce a lot of microbubbles of pressurized CO_2 (Fig. 4.1). A nozzle and shield were placed inside the apparatus to powerfully disturb the influent water. Highly dissolved CO_2 in water formed inside the device. The initial temperature was set by warming or cooling the distilled water to the desired temperature using a heat exchanger contacting the outside of the device.

Microbial suspensions and 7000–8000 mL of distilled water were mixed at the identified temperatures to produce synthetic wastewater with the desired concentration of microbes, after which approximately 7000 mL of this wastewater was pumped into the device. During treatment, the flow rate was 13–15 L/min and the contact time was 25 min. At the beginning of the experiment, the treatment system, which could tolerate up to 1.0 MPa, was filled with

treatment gas at 0.6 to 0.9 MPa. A blowdown valve was designed to collect the samples at the expected times. Inactivation results were judged based on the survival ratio of microorganisms at various pressures and temperatures. All experiments were conducted in triplicate.

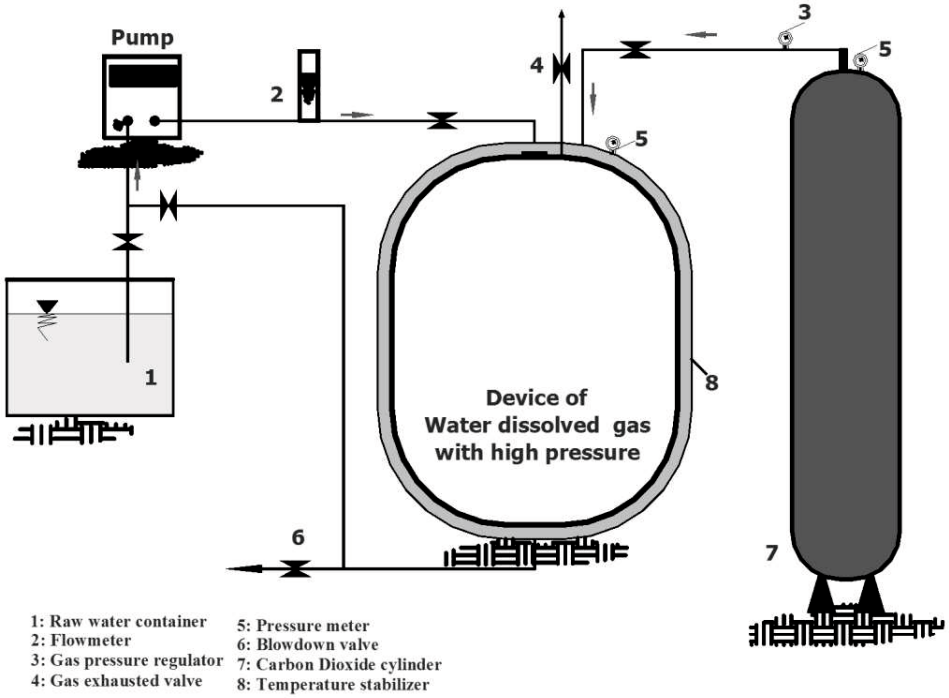


Figure 4. 1. Schematic diagram of the experimental apparatus used for microbial inactivation.

4.3.5. Inactivation mechanism assessment

It was not possible to investigate the significant relationships of the inactivation effect and intracellular pH because the cell size was too small. In this study, a series of disinfectants was performed against *E. coli* and phage MS2 to understand how pH caused by pressurized CO_2 bubbles regulates with the inactivation effect. The role of pH has been examined in four different ways. All wastewater samples used were prepared from the distilled water contaminated by microorganism. First, the only CO_2 treatment at 0.7 MPa was conducted. After that, the experiment was run under the same pressure using N_2O for inactivating process. N_2O was chosen since both N_2O and CO_2 have analogous properties. The only distinction is that CO_2 lowers the pH in water but N_2O does not (Vo et al, 2013). For the third disinfectant, neither CO_2 nor N_2O was used, instead compressed air (from atmosphere) at 0.7 MPa was prepared for disinfection. However, the initial pH of the water sample was adjusted to around 4.0 by 0.1M HCl. Lastly, the distilled water was autoclaved

with phosphate-buffered saline, PBS, pH 7.4 (Wako) at 121°C for 15 minutes and contaminated with microorganism at room temperature. This sample was treated with pressurized CO_2. All experimental conditions are shown in Table.4.1. The microbial inactivation was also considered based on the exposure of *E. coli* and T4 to CO_2 bubbles under different pressures (0.6 to 0.9 MPa) and temperatures (13°C, 20°C, 27°C).

Table 4. 1. Experimental conditions.

Disinfectants	Samples	Pressure, MPa	Characteristics
CO_2	Distilled water	0.7	Low pH
N_2O	Distilled water	0.7	Stable pH
Air	Distilled water + 0.1M HCl	0.7	Low pH
CO_2	Distilled water + PBS buffer	0.7	Stable pH

4.4. RESULTS AND DISCUSION

4.4.1. Inactivation effect against T4, MS2, Qβ and ΦX174

To identify the effects of pressure on viruses, a series of tests investigating the inactivation of bacteriophages T4, MS2, ΦX174 and Qβ by pressurized CO_2 were conducted at 0.6–0.9 MPa (Fig. 4.2 & Fig. 4.3). A slight variation in the reduction ratio of both T4 and MS2 was observed at operating pressures of 0.6–0.8 MPa. The reduction of T4 was 3.5–4.5 log, while that of MS2 was 2.6–3.7 log at 0.6–0.8 MPa. The bacteriophage inactivation of T4 and MS2 increased to approximately 5.5 log reductions when the pressure increased to 0.9 MPa.

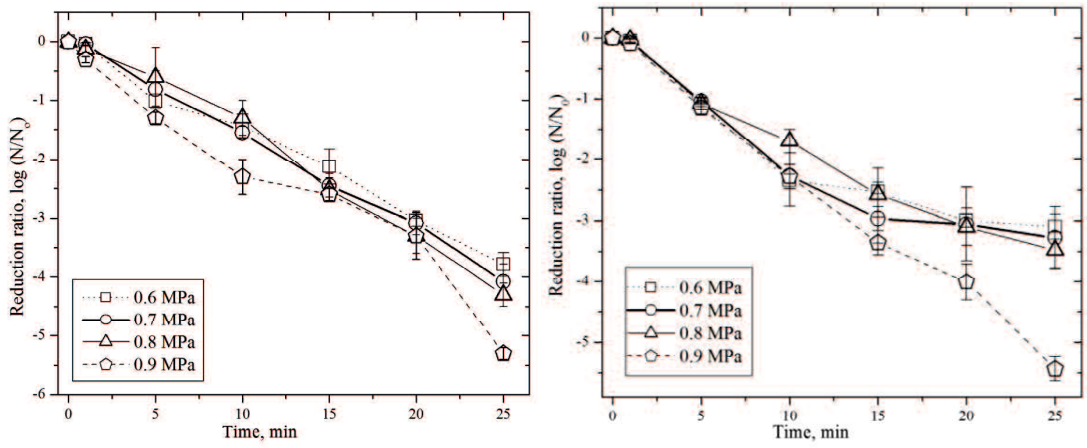
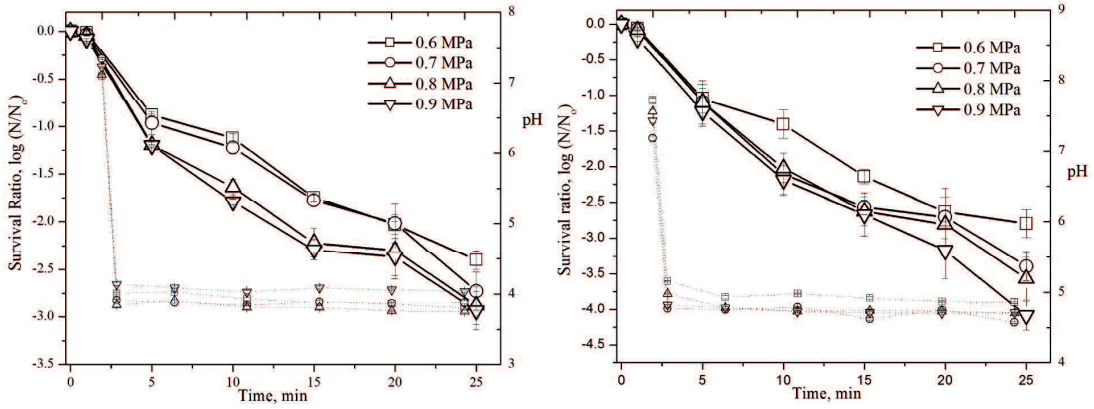


Figure 4. 2. Inactivation effect over time during treatment with pressurized CO_2 at different pressures against (a) phage T4, and (b) phage MS2. Initial concentrations: 10^7–10^9 PFU/mL. The operating temperature is around 22.0°C. Each data point shows the average of independent experiments and error bars represent the standard deviation from the mean.

As shown in Fig. 4.2b, at approximately 0.7 MPa, phage T4 (Fig. 4.2a) showed a higher reduction (>4.0 log) than phage MS2 (approximately 3.2 log). One possibility for these findings is that DNA-based viruses such as T4 are more sensitive to dissolved CO_2 than RNA-based viruses such as MS2. MS2 has been shown to survive better than other bacteriophages in acidic environments (Feng et al, 2003). Moreover, Mamane (2007) found that nucleotide bases of RNA are more resistant than nucleotide bases of DNA using advanced oxidation for the inactivation process.

Although these findings indicate that at an operating pressure of 0.9 MPa a high inactivation ratio of bacteriophages is attained, this pressure exceeds the limitations of normal conditions in water pipelines. At 0.7 MPa, which does not exceed the maximum operating value of water pipelines under normal conditions (Saskatchewan Environment, 2004), the inactivation effect of CO_2 was equal to that at 0.8 MPa (Fig. 4.2). These findings are in accordance with those of a previous study (Vo et al, 2013); therefore, an operating pressure of 0.7 MPa was used for subsequent experiments.



(a) (b)

Figure 4. 3. Inactivation effect over time during treatment with pressurized CO_2 against bacteriophage ΦX174 (a) and Qβ (b) at different pressures. Initial concentrations: 10^7–10^9 PFU/mL. The dotted lines demonstrate the pH change.

As showed on Fig. 4.3, a minor variation in the reduction ratio of ΦX174 was observed at pressures in the range of 0.7–0.9 MPa (2.8–3.2 log), while the variation was only 2.4 log at 0.6 MPa (Fig. 2). The pH decreased to 3.5–4.0 in the first minute of CO_2 treatment. The survival ratio of Qβ treated with CO_2 for 25 min differed with pressure. Specifically, only 2.7 log of phage Qβ were deactivated at 0.6 MPa, whereas approximately 3.6 log were deactivated at pressures of 0.7 and 0.8 MPa. When the operating pressure

increased to 0.9 MPa, the reduction ratio increased to nearly 4.2 log. However, it should be noted that these values of pressure (0.8 and 0.9 MPa) exceed the limitations of normal conditions in water pipelines. These findings agree with those of a previous study (Vo *et al.*, 2013); therefore, an operating of 0.7 MPa was used for subsequent experiments.

The inactivation effect of bacteriophage ΦX174 increased insignificantly when the pressure increased from 0.7 to 0.9 MPa and was lower than that of phage Qβ. One possibility for this finding is that DNA-based viruses such as ΦX174 are more resistant to pressure because the DNA molecule may be single-stranded (ssDNA) in the form of a closed circle. This agrees with the earlier conclusion that the protein coats of ΦX174 are more permeable at higher pH (Yamamoto *et al.*, 1966). The inactivation characteristics of Qβ phage differed from those of ΦX174 phage owing to its structure, the complexity of the virus capsid and arrangement of amino acids, and the carbohydrate and lipid composition of the protein capsid. The results of this study indicate that Qβ phage is more sensitive to pressurized CO_2 than ΦX174 phage.

4.4.2. Inactivation of *E. coli* in response to treatments in various buffers and at various temperatures

Temperature and buffer were both found to influence the inactivation of *E. coli* by CO_2 in this study (Fig. 4.4). Increasing pressure to 0.7 MPa greatly enhanced the sterilization effect of CO_2 microbubbles against *E. coli*. Additionally, nearly 2 log reductions in 25 min were observed at 13.0±0.2°C, while 4.5 log reductions were observed at 19.7±0.3°C, and no *E. coli* survived after 20 min of treatment with pressurized CO_2 at 26.6±0.4°C. In another experiment, CO_2 treatment with buffer material (PBS) led to a 3.5 log reduction of *E. coli*, while under the same conditions of initial temperature, pressure and concentration, only the CO_2 treatment without buffer material induced a greater than 5.0 log reduction ratio (Fig. 4.4).

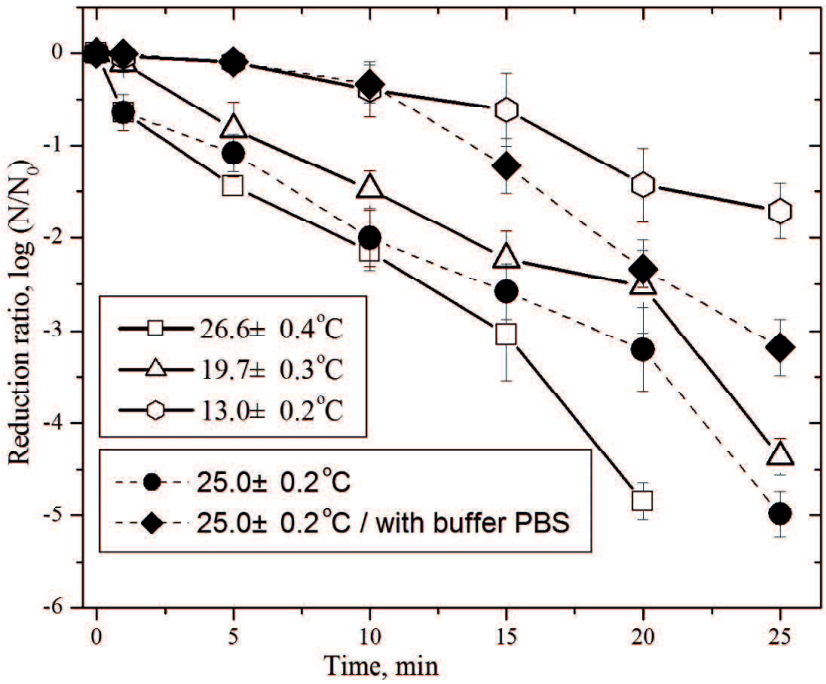


Figure 4. 4. Inactivation effect of pressurized CO_2 (0.7 MPa) against *E. coli* over time. Initial concentrations: 10^5–10^6 CFU/mL. Each line shows average measurements based on three replicates of the experiment. The error bars represent the standard deviation from the mean.

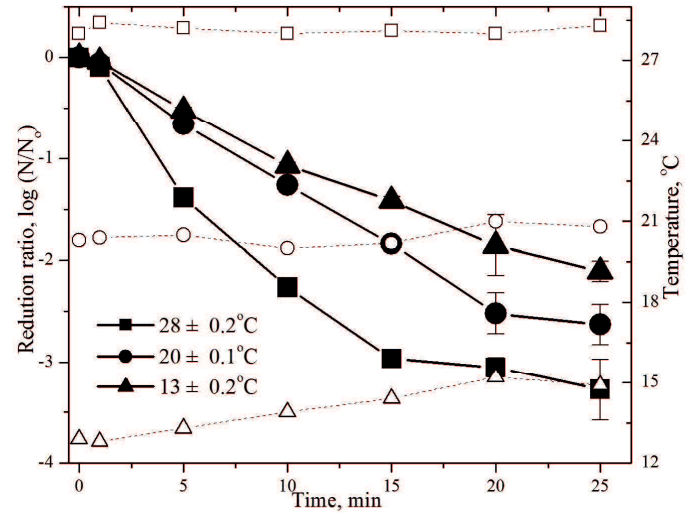
Although increasing temperatures led to decreased CO_2 solubility, these data suggest that higher temperatures enhanced the reduction of *E. coli*. The unique properties of CO_2, its lipo- and hydrophilic nature, and the supporting high temperature and pressure enable it to diffuse easily through the cell membrane of *E. coli* (Isenchmid et al, 1995; Hong and Pyun, 1999). Once the dissolved CO_2 accumulates in the cell wall and intracellular areas, a high volume of hydrogen ions is produced inside the cell, reducing the pH and destroying essential membrane domains (Pitchard, 1979). This explains why treatment with CO_2 alone resulted in a greater log reduction of *E. coli* than treatment with buffer material. Furthermore, high temperature modifies the fluidity of lipids in the membrane in a similar fashion as dissolved CO_2. Accordingly, the combined effects of temperature and CO_2 resulted in all *E. coli* being destroyed after 20 min (Fig. 4.4).

The effect of microorganism reduction with pressurized CO_2 followed the first-order kinetics of Chick's law, $\log (N/N_0)=-kt$, where N, N_0 are the microbial counts obtained at contact time t and t=0, respectively, and k is the inactivation rate constant (1/min). The value of inactivation constant depends on microorganism, pressure, temperature and

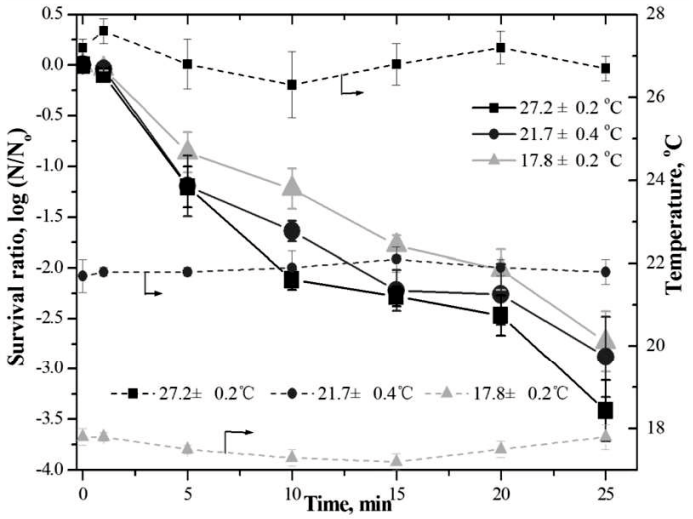
environmental water samples. Calculated by Microcal Origin software, the inactivation rate constants at 0.7 MPa and 25°C for *E. coli*, bacteriophage T4 and MS2 are -0.175 1/min, -0.163 1/min and -0.158 1/min, respectively. The correlation coefficient, R^2, was higher than 0.98 in all situations. These findings are possible to make predictions of inactivation effects under the identical conditions.

4.4.3. Effect of temperature on inactivation of bacteriophages MS2 and ΦX174

The temperature-reduction ratio relationship of phage MS2 and ΦX174 was similar to that of *E. coli* (Fig. 4.5). On Fig. 4.5a, we found a remarkable difference in the MS2 survival ratio from 13°C to 28°C. Specifically, log reductions of 2.0 were observed at 13°C, while approximately 2.7 log reductions were observed at 20°C and 3.5 log reductions at 28°C. Hence, the reduction ratio of all modeled bacteria and viruses was effective at approximately 20°C, with reductions of 4.8 log, 4.0 log and nearly 3.0 log being observed for *E. coli*, T4 and MS2, respectively. Increasing the pressure to 0.7 MPa greatly enhanced the sterilization effect of CO_2 microbubbles against ΦX174 phage. Additionally, nearly 2.8 log reductions in 25 min were observed at 17.8±0.2°C, while approximately 3.0 log reductions were observed at 21.7±0.4°C and there was a slight increase in the reduction ratio of ΦX174 at higher temperatures of 27.2±0.2°C. During the treatment time, operating temperature was controlled to be stable (Fig. 4.5). Although the temperature inside the device was slightly higher (27.2±0.2°C), this change was not considered to influence the experimental results. In Yamaguchi Prefecture, Japan, the weather changes seasonally, ranging from 16°C–26°C from April to November. Therefore, the results presented herein indicate that the best inactivation effect in this region will be observed during this period.



(a)



(b)

Figure 4. 5. Inactivation effect of pressurized CO_2 (0.7 MPa) against bacteriophage MS2 (a) and ΦX174 (b) at different temperatures after 25 min, initial concentrations: 10^7– 10^9 PFU/mL. The dotted lines indicate temperature change during the treatment time: (Δ) 13± 0.2°C (0 min), (O) 20± 0.1°C (0 min), (□) 28± 0.2 °C (0 min). Each of the three solid lines show average measurements based on three replicates of the experiment. The error bars represent the standard deviation from the mean.

Although increasing temperatures led to decreased CO_2 solubility, these data suggest that higher temperatures enhanced the reduction of ΦX174. Operation at high pressure and high contact efficiency between CO_2 bubbles in water augmented CO_2 solubility up to the saturated concentration. The unique properties of CO_2, its lipo- and hydrophilic nature, and the supporting high temperature and pressure enable it to diffuse easily through the

hydrophilic protein coats of ΦX174 (Hong and Pyun, 1999). Once the dissolved CO_2 accumulates in intracellular areas, a high volume of hydrogen ions is produced in the internal area, reducing the pH and destroying essential membrane domains. Furthermore, a high temperature modifies the fluidity of lipids in the membrane in a similar fashion as dissolved CO_2. Accordingly, the combined effects of temperature and CO_2 resulted in all ΦX174 phage being effectively destroyed (Fig. 4.5b).

4.4.4. Effect of pH on inactivation of bacteriophages MS2 and Qβ

To determine whether the reduction in pH induced by dissolved CO_2 is related to its inhibitory effects, inactivation curves of the four disinfectants were generated (Fig. 4.6). When HCl and pressurized N_2O were used to treat MS2, the log reductions were greater than 2.0 log, whereas treatment with pressurized CO_2 with and without buffer material induced reductions of over 1.7 log and approximately 2.6 log, respectively. For phage Qβ, when hydrochloric acid and pressurized N_2O were used to treat Qβ, the log reductions of the former was 2.0 log and the latter was 2.3 log, whereas treatment with pressurized CO_2 with and without buffer material induced reductions of over 1.6 log and approximately 3.4 log, respectively. A noteworthy finding in this study is that the inactivation effect of CO_2 after 20 min was greater than that of other disinfectants, even though pressurized N_2O, HCl, and CO_2/buffer were applied for 25 min (Fig. 4.7).

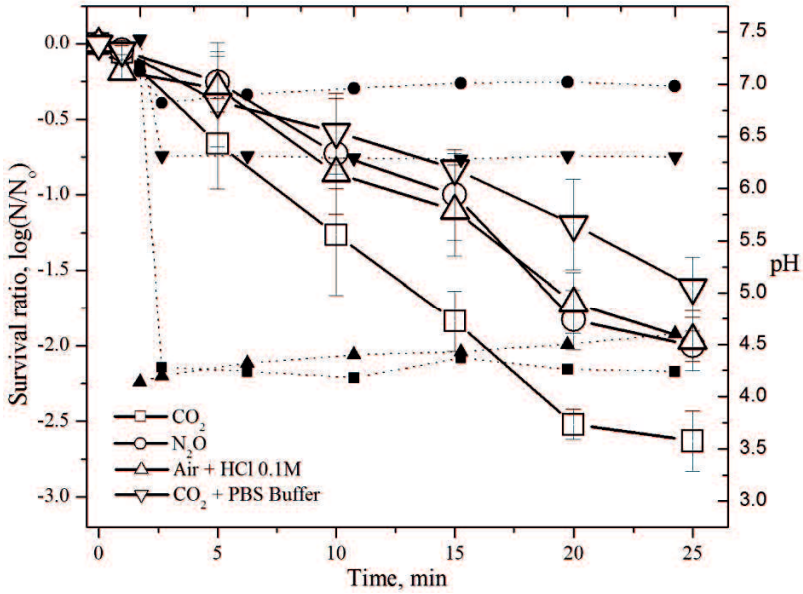


Figure 4. 6. Inactivation effect of CO_2, N_2O, the compressed air adjusted with 0.1M HCl and CO_2 with PBS buffer against bacteriophage MS2 at 0.7 MPa. Initial concentrations: 10^7–10^9 PFU/mL. Operating temperature: -22.0±0.2°C. The dotted lines demonstrate the pH

change: (■) CO_2, (▲) Air + HCl, (▼) CO_2 + Buffer, (●) N_2O. Each of the four solid lines show average measurements based on three replicates of the experiment. The error bars represent the standard deviation from the mean.

Even though N_2O and CO_2 have analogous properties (molecular weight, solubility in water, critical temperature and critical pressure), treatment with N_2O had a lesser inhibitory effect than treatment with CO_2. N_2O differs from CO_2 in the sense that its application to water does not lead to acidification (Fig. 4.6 & 4.7). These findings confirm that the reduction in pH caused by CO_2 plays an important role in its sterilization effects. Many hydrogen ions are produced by pressurized CO_2, after which they easily penetrate the protein coats of bacteriophages, dissolve the phospholipids, and modify the physiological features of proteins. Once an abundant amount of CO_2 molecules have accumulated inside, they reduce the intracellular pH to levels exceeding the buffering capacity of the cell and lead to metabolic disorder (Garcia-Gonzalez *et al.*, 2007; Spilimbergo and Mantoan, 2006). In another examination, a sample treated with CO_2 was compared with one treated with HCl at the same pressure. Although similar pHs were achieved in both samples (4.3–4.5), CO_2 resulted in a greater reduction of Qβ. These findings correspond with those of previous studies, and likely occurred because normal acids cannot penetrate the protein coat of phage Qβ as effectively as pressurized acids (Hong and Pyun, 1999; Wei *et al.*, 1991).

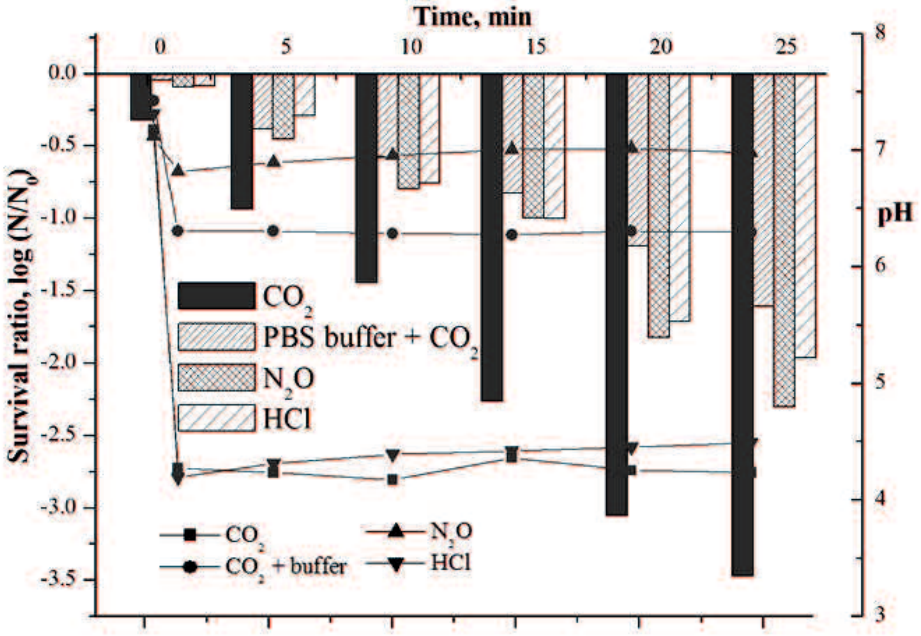


Figure 4. 7. Inactivation effect of pressurized CO_2 and N_2O (0.7 MPa) with buffer solution and HCl (0.1M) against bacteriophage Qβ. Initial concentrations: 10^7–10^9 PFU/mL) at 22.0±0.2°C. The dotted lines demonstrate the pH change: (■) CO_2, (▼) HCl, (●)CO_2 + Buffer, (▲) N_2O.

4.5. CONCLUSIONS

The results from this study open a number of new avenues for further research investigating the application of CO_2 to water and wastewater disinfection. Furthermore, the data presented here provide a greater understanding of the correlation of the effects of changes in pH caused by pressurized CO_2 and those of other acidic disinfectants on microbial inhibition. A pressure of 0.7 MPa was found be effective for the inactivation of *E. coli*, T4, MS2, ΦX174 and Qβ in distilled water at 20°C–25°C. However, these findings are limited by the use of distilled water samples spiked with microorganisms. Future trials should assess selective environmental samples including effluent wastewater, groundwater, and river water. The results of this study provide important information that can be used in future studies to replace conventional disinfectants that cause undesired disinfection by-products during water treatment.

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CONNECTING TEXT: CHAPTER 4 → CHAPTER 5

Findings from chapter 3 and chapter 4 support the idea of using pressurized CO_2 for target disinfection of pathogens in some environmental wastewater samples as well the feasibility of CO_2 application in water treatment. Chapter 5 describes the potential application of pressurized CO_2 treatment and shows the inactivation rates to different microorganisms.

CHAPTER V
APPLICATION OF PRESSURIZED CARBON DIOXIDE FOR AGRICULTURAL IRRIGATION WATER DISINFECTION

5.1. ABSTRACT

Irrigation water and recycled water used for farm gardens can be a potential source of contamination of microbial pathogens that cause harmful illness. This study investigated the use of pressurized carbon dioxide to inhibit pathogens in water sources. An apparatus producing microbubbles was operated with pressure up to 0.7 MPa, room temperature and a common period for disinfection, 25 minutes. Target environmental water samples, including distilled water, artificial ground water and effluent wastewater, were subjected to microbial contamination with desired concentrations of *Escherichia coli* (ATCC 11303 and ATCC 13965) and bacteriophages. Under identical conditions, approximately 4.0 – 5.0 log of *E. coli* were inactivated in water samples, whereas the reduction ratio of bacteriophages are nearly 3.0 – 4.0 log. The chemical nature of CO_2 molecule (acidification, diffusivity and solubility in water) was indicated to be the main factors causing the microorganism deaths. Besides that, high pressure, depressurization rate, characteristics of microbubbles and pumping cycle contributed to microorganism inhibition. These findings in this investigation may be considered to use carbon dioxide as a novel disinfectant to water treatment in agricultural irrigation. Moreover, carbon dioxide treatment produces no disinfection by-products and excessive pressure after disinfection can be an advantage to enhance irrigating to plants.

Keywords: irrigation water, carbon dioxide, inactivation effect, water disinfection, microbubbles.

5.2. INTRODUCTION

Water resources used for various targets in agricultural irrigation require preliminary treatment to be safe to use. Water disinfection is an important treatment to control the microorganism growth in the irrigation water system and minimize the diseases related to the waterborne pathogens, e.g., bacteria, viruses, fungi, cysts…. Agricultural reuse of wastewater becomes the potential irrigation water in the big cities and urban. The water resources from secondary treatment contain the residual viruses and pathogens, which can

persist to varying degrees after release to the environment (Rose et al. 1991). Hence, the effluent wastewater is required special care before irrigating the crops for direct human consumption (WHO. 1973). Especially, agricultural food crops, such as vegetable fields (barley, avocado, cabbage, lettuce, strawberry…), orchards and vineyards, nurseries (flowers)…are required secondary treatment and disinfection for irrigated water (Asano et al. 2007). Irrigation water can be disinfected using non-chemical methods (heat, Ultraviolet radiation and filtration), or chemical methods (chlorine, chlorobromide, ozone, chlorine dioxide…). UV disinfection is effective and environmentally friendly treatment. However, this requires the water to be free of suspended particles and UV-absorbing substances which exist abundantly in agricultural irrigation water. Chlorination is the most widely used disinfectant in water treatment. Recently, many potential problems have arising due to the reaction of residual chlorine with natural organic matter (NOM) in water causing health effect in humans. Whereas ozone, chlorine dioxide and hydrogen peroxide react with water contaminants are transferred to a series of free-radicals to oxygen as the end reaction product. These reactions cause harmful to the plant and reduce its growth rate. Using high pressure carbon dioxide (CO_2) to inhibit the microorganism growth is considered as a novel disinfectant for water treatment without forming disinfection by-products (DBPs).

The sterilizing technique by high pressure CO_2 has been successfully implemented in the preservation of food and concluded to be effective for inactivation of variety pathogens (Dillow et al. 1999; Enomoto et al. 1997; Haas et al. 1989; Hoang and Pyun. 1999; Lin et al. 1994; Nakamura et al. 1994; Wei et al. 1991). A recent study by Kobayashi et al. (2009) involved to apply high pressure carbon dioxide for water disinfection. His group found that Escherichia coli (*E. coli*) were inactivated up to 6 log at the pressure of 2 MPa around 40^{o}C after 60 minutes. Our preliminary investigation indicated that CO_2 microbubbles at 0.7 MPa significantly inhibited *E. coli* cells in distilled water to approximately 5.0 log reduction (Vo et al. 2013). However, no attempt was investigated to inactivate various microorganisms and environmental waters by high pressure CO_2.

In order to assess inactivation effect of CO_2 in many different microorganisms and in environmental water resource, the experiments in this study were run using three kinds of *E. coli* (ATCC 11303, ATCC 23631, ATCC 13706) and three kinds of bacteriophages (T4, Qβ, ΦX174) and tested water samples includes effluent treated wastewater, artificial ground

water and distilled water. By using different water samples, this study aims to apply pressurized carbon dioxide for garden irrigation water disinfection with small scale.

5.3. MATERIALS AND METHODS

5.3.1. Preparation of microorganisms

Three kinds of *E. coli* cells and bacteriophages were used as target pathogens for disinfection. *Escherichia coli* ATCC 11303, ATCC 23631 and ATCC 13706 from stock cultures (American Type Culture Collection, Manassas, VA, USA) were respectively propagated in flasks containing 100 ml Luria-Bertani (LB) broth media (Wako Chemical Co. Ltd., Osaka, Japan) and incubated at 37°C with continuous shaking for 16–18 h at 150 rpm. Whereas, bacteriophage suspensions were prepared from T4 (ATCC 11303-B4TM), Qβ (ATCC 23631-B1TM) and ΦX174 (ATCC 13706 -B1TM) and grown to high titers by overnight incubation at 37°C in *E. coli* hosts ATCC 11303, 23631 & 13706, respectively. The remaining cells and cell debris were eliminated by centrifugation at 2,000×g for 10 min. The supernatant, including the phage, was then filtered through a membrane filter with a pore size of 0.20 μm (Millipore, Carrigtwohill, County Cork, Ireland). Cells and virus suspensions with initial concentrations of 10^7–10^9 PFU/mL were stored in 20% glycerol. For storage, samples were initially refrigerated at -20°C for 24 h, and then reduced to -80°C to prevent temperature shock.

5.3.2. Microbial enumerated tests

5.3.2.1. Bacteria enumeration

The cell concentration was determined by spreading aliquots on LB agar plates (Wako), incubating the samples overnight at 37°C, and then determining the number of colony-forming units (CFU) from plates containing 25–300 colonies. The initial concentration was estimated to be approximately 10^7-10^9 CFU/mL. For each experiment, 100 mL of *E. coli* stock inoculated LB was incubated at 37°C and 150 rpm for 12–18 h.

5.3.2.2. Bacteriophage titer

Surviving infectious T4, Qβ and ΦX174 were enumerated by forming lawns of sensitive strains of *E. coli* hosts and then conducting plaque phage assays using double layers of agar on the plates. Initially, 0.1 ml phage suspension was mixed with 0.2 ml *E. coli* host culture and incubated at 37°C (50 rpm) for 30 min. This mixture was then blended directly in a test tube containing 5 ml of top layer of liquefied Tryptic Soy Agar (TSA) 0.7%

[wt/vol] (Wako) and poured rapidly onto a Petri dish containing TSA at 1.5% [wt/vol]. Plaque-Forming Units (PFU) were determined after overnight incubation at 37°C based on plates containing 30–300 PFU.

5.3.3. Preparation of water samples

Microorganism suspensions and distilled water, artificial ground water and effluent wastewater before disinfection (Ube wastewater treatment plant, Yamaguchi, Japan) were intermingled to attain the desired concentration at room temperature as the wastewater samples. The artificial groundwater was made from $CaCl_2$ 0.125mM; $MgCl_2$ 0.05mM; KCl 0.103 mM; $NaHCO_3$ 1.5 mM (Wako) and autoclaved in 15 min at 121°C before using (You et al. 2005). Whereas, the components of effluent treated wastewater were pH (7.1), COD (9.6 mg/L), BOD (6.2 mg/L), SS (4.0 mg/L), N (17.9 mg/L), P (1.24 mg/L) (Ube city environment department).

5.3.4. Apparatus and procedure for disinfection

The disinfection device was tested based on the high contacted efficacy between CO_2 and water (Fig. 5.1). Highly dissolved CO_2 in water was distributed thoroughly inside due to high pressure and pump cycle. Initial temperature was remained unchanged from 20-25°C.

At the beginning, 7000 mL of wastewater contaminated microorganisms was pumped into and operated during treatment time, 25 minutes at flow rate of 13-15 l·min-1. The working pressure indicated from optimum condition from previous study (Vo et al. 2013) was 0.7 MPa. Blowdown valve was used to take the samples.

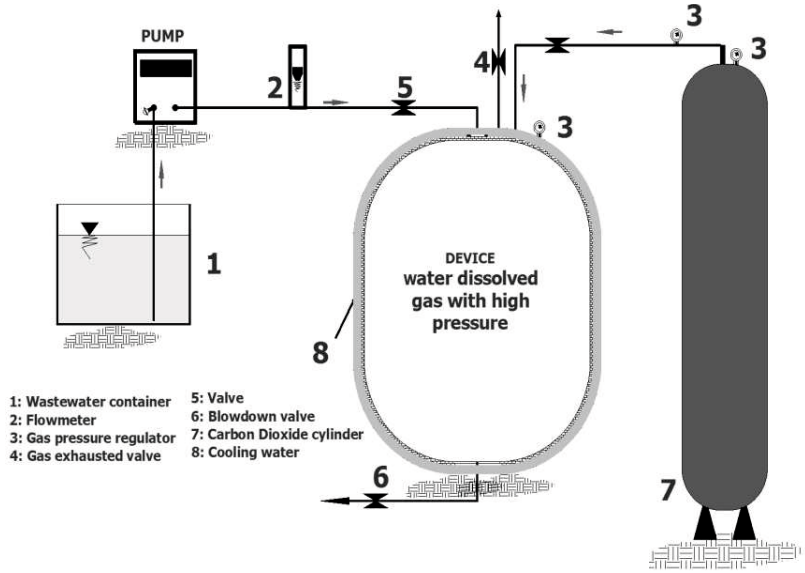

Figure 5. 1. Schematic of experimental apparatus.

Inactivation effect was assessed via the inactivation results at various microorganisms and environmental water resources.

5.3.5. Inactivation rate

The calculation of inactivation rate was based on slope of the linear relationship between log (N/N_0) and time t, where N and N_0 are the final and initial plate count numbers per milliliter (PFU/mL) and t represents time in minute.

5.4. RESULTS AND DISCUSSION

5.4.1. Inactivation effect to different bacteria.

The first set of analysis examined the impact of carbon dioxide disinfection to variety bacteria. Reduction ratios of all three kinds of *E. coli* over the time change similarly. After 20 minutes of inactivation, *E. coli* ATCC 11303 was inhibited a nearly 4.2 log reduction, while *E. coli* ATCC 23631 and 13706 were inactivated for 3.9 log and 3.8 log, respectively (Fig. 5.2). Interestingly, inactivation effect reached the same reduction ratio for all bacteria, approximately 4.5 log after 25 min.

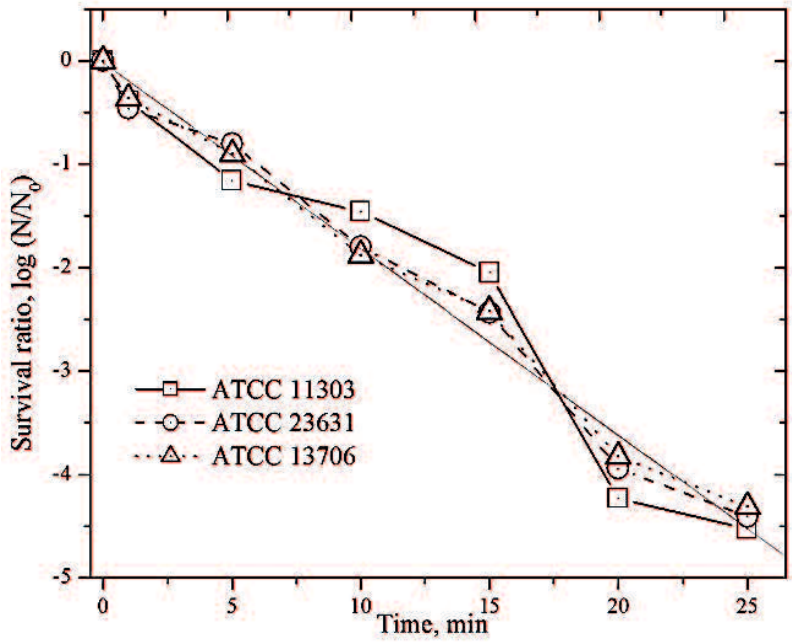

Figure 5. 2. Inactivation effect of pressurized CO_2 (0.7 MPa) against different bacteria. Environmental waters were distilled water. Initial concentration: 10^7-10^9 CFU/mL. Room temperature (22°C).

As showed on Fig. 5.2, inactivation rates increased slightly in the first 15 minute (2.0-2.5 log/15 min), but then grew significantly after that. This agrees with the earlier result by Vo et al. (2013) that CO_2 treatment with 20 min at 25^oC, *E. coli* was completely inactivated with the initial concentration of around 10^5-10^6 CFU/mL. In general, inactivation rate of pressurized carbon dioxide against *E. coli* follows the first-order kinetics and was indicated to be 0.18 log /min (R^2>0.945). This finding has important implications for predicting the inactivation process of *E. coli* by CO_2 in water. CO_2 microbubbles under high pressure were considered to be effective to diffuse and disintegrate *E. coli* cells. They permeate through cell wall membranes, disorder cell components and exceed intracellular pH change (Hong and Pyun. 1999; Haas et al. 1989; Lin et al. 1994).

5.4.2. Inactivation effect to different bacteriophages.

In another experiment, bacteriophages were used as virus indicators. Phage T4 (double stranded DNA) and ΦX174 (single stranded DNA) representative for DNA viruses, phage Qβ (single stranded RNA) is as RNA virus. Under the same conditions of *E. coli* inactivation tests, approximately 4.0 log of phage T4 was inhibited by CO_2 treatment, whereas the reduction ratios of phage Qβ and ΦX174 were nearly 3.4 log and 2.9 log, respectively. During the treatment time by CO_2, pH decreased to approximately 4.0 from the first minute for all experiment. Demonstrated on Fig. 5.3, during the first 15 minutes the inactivation rates to all phages are familiar. However, after that the inactivation rates are different. The reduction rate of phage T4 increased significantly, 0.16 log/min (R^2>0.999), while the inactivation rate of phage Qβ increased slightly, 0.13 log/min (R^2>0.995) and the survival ratio of phage ΦX174 was highest with the only decrease of 0.11 log/min (R^2>0.96).

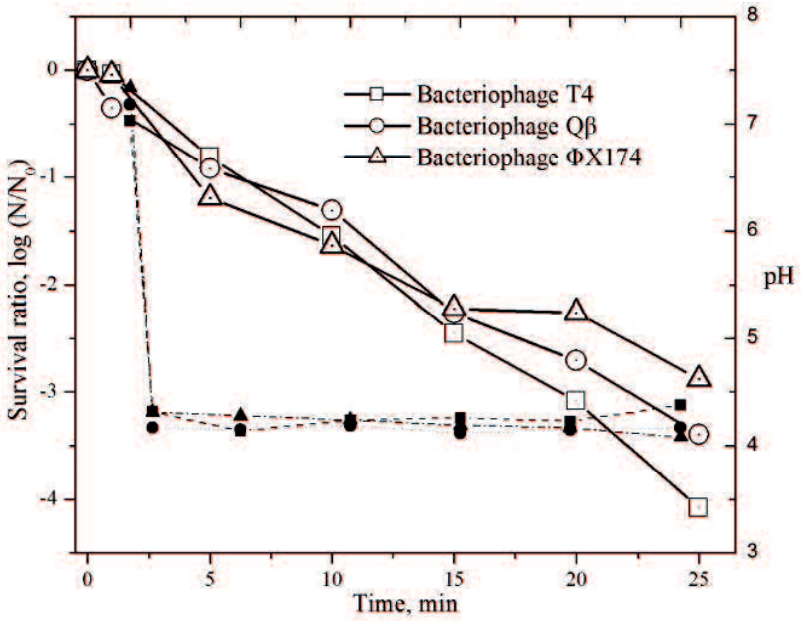

Figure 5. 3. Inactivation effect of pressurized CO_2 (0.7 MPa) against different bacteriophages. Wastewater were distilled water contaminated by bacteriophages. Initial concentration: 10^7-10^9 PFU/mL. Room temperature (22°C). Dotted lines illustrate pH change over the time: (■) T4, (●) Qβ, (▲) ΦX174.

The high inhibition of phage T4 was indicated to be sensitive to pressurized CO_2 microbubbles. One possibility is that the large size of phage T4, 90 nm wide and 200 nm long, linked by a long tail and head is easy to be broken under pressurized CO_2 molecules (Miller et al. 2003). Phage Qβ and ΦX174 has much smaller sizes, only 25–30 nm that will be difficult for CO_2 microbubbles to diffuse effectively as phage T4 shapes. Phage Qβ was found that it survived better in an alkaline environment than in the water containing a lot of hydrogen ions. In this study, phage Qβ was inhibited more effectively than phage ΦX174, this agrees with the previous investigation (Feng et al. 2003). Inactivation mechanism of pressurized CO_2 against bacteriophages is similar to one of *E. coli* cells. Molecular CO_2 with high pressure can also penetrate through protein coat of coliphage. Once accumulated excessively, they will change the order loss of the lipid chains and destruct the domains. In addition, a strongly decrease intracellular pH denaturing DNA and RNA characteristics leads to the inhibition of coliphage.

5.4.3. The influence of environmental water to inactivation effect.

The environmental water samples contaminated by *E. coli* were compared in order to assess inactivation effect of CO_2. The results obtained from the preliminary disinfection of

the wastewater made by distilled water, the artificial groundwater and the real effluent wastewater are presented in Fig 5.4. The reduction ratio of *E. coli* in the effluent treated wastewater only 3.5 log. And this reduction is also lower over the time than others. Whereas, both distilled water and the artificial groundwater had the similarly high inactivation ratios, approximately 4.5 log (Fig. 5.4). Compared to pH change in water on Fig. 5.3, the pH change of three samples in this case had a slight difference. One possibility is that buffering capacity of chemical components in the artificial groundwater and the effluent wastewater are higher. pH after the first-minute treatment reached nearly 5.0, while pH of the distilled water attained around 4.0.

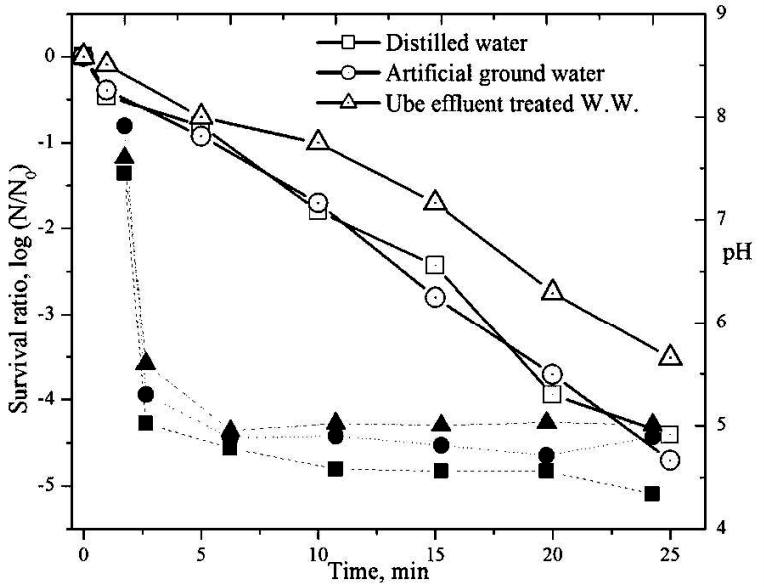

Figure 5. 4. Inactivation effect of pressurized CO_2 (0.7 MPa) against *E. coli* ATCC 11303 in different environment waters. Initial concentration: 10^7-10^9 CFU/mL. Room temperature (22°C). Dotted lines illustrate pH change over the time: (■) Distilled water, (●) Artificial ground water, (▲) Ube effluent treated wastewater.

The suspended solids (SS=4.0 mg/L) in the effluent treated wastewater as the particles of turbidity provide shelter for *E. coli* cells and reduce their exposure to CO_2 microbubbles. For this reason, the inactivation rate against *E. coli* in the effluent treated wastewater is 0.14 log reduction/min (R^2>0.990), slower than in the distilled water (0.18 log/min, R^2>0.992) and the artificial groundwater (0.184 log/min, R^2>0.998). SS factor may explain the relative good correlation between the effectiveness of disinfection process and water quality. This

finding, while preliminary, suggests that the inactivation effect of pressurized CO_2 reaches the higher rate in the raw water with lower turbidity.

Depressurization rate after discharging treated water also concerns to cell deaths (Enomoto et al. 1997). The change of pressure as shear force makes physiological characteristics adapt suddenly and breaks cell walls and viral coat proteins. Moreover, long exposure time with continuous pumping cycle (25 min) causes to microorganism inhibition.

5.5. CONCLUSIONS

The present study was designed to determine the inactivation effect of pressurized CO_2 microbubbles against pathogen indicators. Under identical pressure condition (0.7 MPa) and around room temperature (22^{o}C), approximately 4.5 log of *E. coli* cells and nearly 3.0–4.0 log of bacteriophages (T4, Qβ, ΦX174) were inhibited by CO_2 microbubbles. The evidence from this study suggests that the irrigating water quality with low turbidity has higher inactivation effect. Moreover, the excessive pressure after treatment remains high and a good condition to utilize for irrigating to plants at far distance. This research will serve as a base for future studies and potential application of pressurized CO_2 for the agricultural irrigating water and wastewater disinfection. However, with a small scale and the batch model, caution must be applied, as the further inactivation effect has not deeply investigated to continuous model.

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CONNECTING TEXT: CHAPTER 5 → CHAPTER 6

Next chapter summarizes all obtained results in this dissertation and gives the conclusions and a further outlook trend.

Chapter VI

CONCLUSIONS AND FUTURE WORK

6.1. SUMMARY

In this investigation, the inactivation effects of pressurized CO_2 microbubbles on disinfection efficiency against microorganisms (including bacteria and viruses) and other related aspects of the pH role by dissolved CO_2 in inactivation mechanism, temperature, pressure and environmental water samples were investigated. The significant findings can be categorized into five following major groups and on **Table. 6.1**.

- **Bactericidal & virucidal effect of pressurized CO_2**: the most obvious finding to emerge from this study is that CO_2 microbubble is the more effective to inhibit *E. coli* cells than N_2O and N_2. For example, under identical treatment conditions at 0.7 MPa and room temperature, a greater than 5.0-log reduction in *E. coli* was achieved by CO_2, while 3.3 log and 2.4 log reductions were observed when N_2O and N_2 were used, respectively. Moreover, high pressure CO_2 was found to be the high viricidal effect. For phage T4 and phage Qβ, a reduction of nearly 4.0 log in the former and more than 3.3 log in the latter were achieved by CO_2 at 0.7 MPa, while approximately 3.0 log reduction was observed for phage MS2 and phage ΦX174.

- **The lowered pH caused by pressurized CO_2**: a comparison of the inactivation effects of pressurized CO_2, N_2O, air/a common acid and CO_2/buffer solution against bacteriophages (Qβ and MS2) revealed that the change in pH caused by CO_2 plays an important role in their virucidal effects. Treatment with other systems had a lesser inhibitory effect than treatment with CO_2. Namely, air/HCl and pressurized N_2O were used to treat MS2, the log reductions were greater than 2.0 log, whereas treatment with pressurized CO_2 with and without buffer material induced reductions of over 1.7 log and approximately 2.6 log, respectively.

- **Intracellular release and cell damage**: the high levels of nucleic acids and proteins was measured based on the absorbance of samples at 260 nm and 280 nm within the first 10 minutes. Under SEM observation, no cells could be identified after treatment with CO_2, while no or only a few cells appeared broken with treatment of other gases.

- **Pressure/temperature**: a pressure range from 0.6 MPa to 1.0 MPa and temperature of 13°C to 28°C were investigated for inactivating *E. coli* and bacteriophages. Strong

evidence of high inactivation effect of CO_2 on microorganisms were found when the pressure and temperature increase. The performance of CO_2 inactivation against E. coli reached 4.7-5.2 log at 0.7-0.9 MPa. Whereas, the reduction log from 3.5-4.5 for T4 and 2.6-3.7 for MS2 were observed at 0.6-0.8 MPa, respectively. Both of these phages were strongly inhibited at 0.9 MPa, approximately 5.5 log. There was a ligh difference for inactivating ΦX174 and Qβ when only 2.7 log (Qβ) and 2.4 log (ΦX174) were attained at 0.6 MPa, but both were highly inactivated at 07-0.9 MPa, for example, 2.8-3.2 log for ΦX174 and 3.6-4.2 log for Qβ.

Table 6. 1: Summary

Objectives	Outcome
Comparison of inactivation effect of different disinfecting gases	$CO_2 > N_2O > N_2$
Bactericidal effect	A over 5-log reduction of *E. coli* was achieved by pressure CO_2 at 0.7 MPa/20 min.
Virucidal effect	At 0.7 MPa, a reduction of nearly 4 log for T4, over 3.3 log for Qβ and approximately 3.0 log for MS2 and ΦX174
Release of intracellular substances	Nucleic acids and protein were leaked under CO_2 treatment
Membrane damage	By SEM observation, *E. coli* cells could not be identified
Effect of pH caused by dissolved CO_2	$\|CO_2\| > \|N_2O\| > \|Air/HCl\| > \|CO_2/buffer\|$
Pressure effect	Increased pressure accelerated the inactivation effect of CO_2. Pressure of 0.7 MPa was found to be suitable for water treatment system.
Temperature effect	Increasing temperature lead to increase inactivation effect. The temperature range from 20°C to 25°C was indicated to be effective for inactivation.
Environmental water effect	Distilled water/ artificial ground water > effluent treated wastewater Inactivation effect may depend on the characteristics of real wastewater (SS, turbidity)

The correlation of inactivation effect is related to temperature. At low temperature (13°C), only 2.0 log of *E. coli* and MS2 reduction were conducted at pressure of 0.7 MPa. This effect changed at higher temperature, the reduction log of 4.5 for *E. coli*, 2.7 log for MS2 at nearly 20°C and 2.8-3.0 log for ΦX174 at 18-22°C. Especially, no survival cell of *E.*

coli was observed after 20 min at 27°C and around nearly 3.5 log of MS2 and ΦX174 was inhibited at 28°C.

- **Environmental waters**: the inactivation effect of pressurized CO_2 on *E. coli* in different water samples still reaches at high reduction level. While 3.5 log of *E. coli* inactivation was achieved in effluent treated wastewater, nearly 4.5 of reduction log happened in the distilled water and artificial ground water. This change is due to the turbidity and suspended solids in wastewater sample.

6.2. CONCLUSIONS

It was also shown that the following conclusions can be drawn from this study:

- These present results confirm previous findings in the field of food preservation and contribute additional evidence that suggests pressurized CO_2 may be applied in water treatment.
- The decrease of pH in water and high diffusivity and high solubility induced by treatment with CO_2 is considered to be the most effective factor leading to its microbicidal effects. In addition, the physical factors (high pressure, pumping cycle) support and accelerate to microbial inhibition.
- *E. coli* cells, T4 and Qβ are strongly inactivated by pressure CO_2 microbubbles, while MS2 and ΦX174 may be lesser sensitive to CO_2.
- Increasing pressure and temperature leads to the adjustment of CO_2 state and have a strongly effect on the microbicidal efficiency. However, the suitable operating conditions to inactivate above target microorganisms found in this study are the pressure of 0.7 MPa and a temperature range from 20 °C to 25 °C.
- A little difference of inactivation effect between the real wastewater and laboratory wastewater (distilled water and artificial wastewater) revealed that this method has the potential application for water treatment. A secondary disinfectant such as chlorine, chloramines or chlorine dioxide may be used with pressurized CO_2 for a complete disinfection system.

6.3. CONTRIBUTIONS TO KNOWLEDGE

High inactivation effect of pressurized CO_2 that this investigation has identified therefore assists in our understanding of the new role of CO_2 in water treatment. These findings were originally inherited from the discoveries of using high pressure CO_2 to

inactivate pathogens in food industry. Carbon dioxide on the other hand is safe to handle (it becomes active only when dissolved in water, no special alloy or plastic distribution piping is required for CO_2 system, CO_2 leaks dissipate safely into atmosphere) easy to apply, efficient, relatively low toxicity and naturally abundant. Once CO_2 can be withdrawn from the environment, applied in water treatment and returned to the environment, this method is considered to be ecologically safe.

Whilst the present disinfecting methods are facing to the problems with disinfection by-products, use of pressurized CO_2 for the target inactivation of pathogens does partially substantiate no forming the residual toxicity.

6.4. LIMITATIONS OF THE CURRENT RESEARCH

A number of caveats need to be noted regarding the present investigation. The current research was not specifically designed to evaluate factor related to intracellular pH of inactivated cells. This, if done, can give a convince explanation for the deaths of microorganism by high dissolved CO_2. In addition, experiments of CO_2 treatment have been performed under the only batch system and raw water samples that almost of distilled water. Thirdly, the study did not specifically evaluate for the inactivation mechanism of virus.

6.5. FUTURE WORK

The issue of successful inactivation by CO_2 treatment in this study is an intriguing one which could be usefully explored in further research. It is recommended that further research be undertaken in the following areas:

- Further investigation and experimentation into intracellular pH is strongly recommended.
- Considerably more work will need to be done to determine the inactivation effect of pressurized CO_2 on the real wastewater as well as more trials to better understand the inactivation mechanism of virus.
- A future study investigating on a continuous system to inactivate by pressurized CO_2 microbubbles would be very interesting.
- This information can be used to develop targeted interventions aimed at bio solids, a kind of biological sludge containing pathogens with high water content.

APPENDIX

List of scientific conferences, list of publications, awards

A- LIST OF SCIENTIFIC CONFERENCES

PART OF THE THESIS HAS BEEN PRESENTED AT SCIENTIFIC CONFERENCES

1. **Huy Thanh Vo**, Tsuyoshi Imai, Truc Thanh Ho, Singo Kokado, Takaya Higuchi, Ariyo Kanno, Koichi Yamamoto, Masahiko Sekine. Potential application of high pressure carbon dioxide in wastewater and water disinfection: recent overview and further trends. WET 2014 Water and Environment Technology conference. Tokyo, Japan. June 28-29, 2014.
2. **Huy Thanh Vo**, Tsuyoshi Imai, Truc Thanh Ho, Tuan Van Le. Inactivation kinetics of pressurized carbon dioxide on microbial contaminated wastewater. *The 9th young scientist seminar in Asian Core program*. Yamaguchi, **Japan**. 18-19 November, **2013**.
3. Tuan Van Le, Tsuyoshi Imai, Takaya Higuchi, Daisuke Ayukawa and **Huy Thanh Vo**. Separation of oil-in-water emulsions by microbubble technology and a potential application for the treatment of real palm oil mill effluent. *The 9th young scientist seminar in Asian Core program*. Yamaguchi, **Japan**. 18-19 November, **2013**. (CO-AUTHOR)
4. **H.T. Vo**, T. Imai, H. Yamamoto and S. Kokado. Inactivation effect of pressurized carbon dioxide on bacteriophage Qβ and ΦX174 as a novel disinfectant for water treatment. *The 5th IWA- Aspire conference*. Daejeon, **Korea**. 8 –12 September **2013**.
5. **Huy Thanh Vo**, Tsuyoshi Imai, Hidenori Yamamoto, Singo Kokado and Tuan Van Le. Potential application of pressurized carbon dioxide for agricultural irrigation water disinfection. *The 5th international conference on fermentation technology for value added agricultural products*. Khon Kaen, **Thailand**. 21st -23rd August **2013**.
6. **Huy Thanh Vo,** Tsuyoshi Imai, Hidenori Yamamoto, Tuan Van Le, Takaya Higuchi, Ariyo Kanno, Koichi Yamamoto, Masahiko Sekine. Disinfection using pressurized carbon dioxide microbubbles to inactivate *Escherichia coli*, bacteriophage MS2 and T4. *WET 2013 Water and Environment Technology conference*. Tokyo, **Japan**. June 15-16, **2013**.
7. Tuan Van Le, Tsuyoshi Imai, Takaya Higuchi, Daisuke Ayukawa, **Huy Thanh Vo**. Potential application of cyclone microbubbles in the treatment of palm oil mill

effluent under batch and continuous operations. *WET 2013 Water and Environment Technology conference*. Tokyo, **Japan**. June 15-16, **2013**. (CO-AUTHOR)

8. **Huy Thanh Vo**, Tsuyoshi Imai, Jantima Teeka, Tuan Van Le, Kanthima Phummala, Takaya Higuchi, Ariyo Kanno, Masahiko Sekine. Comparison of pressurized gas bubbles CO_2, N_2O and N_2 on *Escherichia coli* disinfection. *The 4th IWA young water professional Conference (APYWP2012)*. Miraikan, Tokyo, **Japan**. 7-10 December **2012**. (Poster)
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10. **Huy Thanh Vo**, Tsuyoshi Imai, Jantima Teeka, Tuan Van Le, Kanthima Phummala, Takaya Higuchi, Ariyo Kanno, Masahiko Sekine. Comparison of bactericidal effect on *Escherichia coli* by pressurized gases of CO_2, N_2O, and N_2. *The 6th young scientist seminar in Asian Core program*. Yamaguchi, **Japan**. 9-10 September **2012**.
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B- LIST OF PUBLICATIONS

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1. **Huy Thanh Vo**, Tsuyoshi Imai, Truc Thanh Ho, Loc Thi Thanh Dang, Takaya Higuchi, Ariyo Kanno, Koichi Yamamoto, and Masahiko Sekine. Potential application of high pressure carbon dioxide in wastewater and water disinfection: recent overview and further trends. *Journal of Water and Environment Technology*. *(under revision)*
2. **Huy Thanh Vo**, Tsuyoshi Imai, Truc Thanh Ho, Masahiko Sekine, Ariyo Kanno, Takaya Higuchi, Koichi Yamamoto, Hidenori Yamamoto. 2014. Inactivation effect of pressurized carbon dioxide on bacteriophage Qβ and ΦX174 as a novel disinfectant for water treatment. *Journal of Environmental Sciences*, Vol. 26(6), **1301-1306**.
3. **Huy Thanh Vo**, Tsuyoshi Imai, Tuan Van Le, Kanthima Phummala, Takaya Higuchi, Ariyo Kanno, Masahiko Sekine, Koichi Yamamoto. 2014. Potential application of pressurized carbon dioxide for agricultural irrigation water disinfection. *KKU Research Journal*, Vol. 19, xx-xx. (*In Press*)
4. **Huy Thanh Vo**, Tsuyoshi IMAI, Hidenori YAMAMOTO, Tuan Van LE, Takaya HIGUCHI, Ariyo KANNO, Koichi YAMAMOTO, Masahiko SEKINE. (**2013**). Disinfection using pressurized carbon dioxide microbubbles to inactivate *Escherichia coli*, bacteriophage MS2 and T4. *Journal of Water and Environment Technology*, Vol. 16(6), **497-505**.
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6. **Huy Thanh Vo**, Tsuyoshi IMAI, Singo KOKADO, Tuan Van LE, Hidenori YAMAMOTO, Takaya HIGUCHI, Koichi YAMAMOTO, Ariyo KANNO, and Masahiko SEKINE. (**2013**). Potential application of pressurized carbon dioxide for agricultural irrigation water disinfection. The 5th international conference on Fermentation Technology for Value Added Agricultural Products. Khon Kaen, Thailand, August 21st-23rd, 2013, **p. 148-154**. (FULL PAPER)

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7. Tuan Van Le, Tsuyoshi Imai, Daisuke Ayukawa, Hiroaki Fujinaga, **Huy Thanh Vo**, Takaya Higuchi, Thanh-Loc Thi Dang and Yatnanta Padma Devia. **(2014)** Application of tiny microbubbles ozonation enhanced by coarse bubbles on treatment of fine oil-in-water emulsions presented humid acid. *IWA specialist conference*: *Advances in particle science and separation: from mm to nm scale and beyond*. Sapporo, Japan. June 15-18, 2014. **p. 371-378**.

8. Tuan Van Le, Tsuyoshi Imai, Takaya Higuchi, Koichi Yamamoto, Masahiko Sekine, Ryosuke Doi, **Huy Thanh Vo**, Jie Wei. (**2013**). Performance of tiny microbubbles enhanced with "normal cyclone bubbles" in separation of fine oil-in-water emulsions. *Chemical Engineering Science*, Vol 94, **1-6**.

C- AWARDS

1. "**WET excellent research award**" is issued by Japan Society of Water Environment, JSWE (2013).