DESTRUCTION AND INJURY OF *ESCHERICHIA COLI* UNDER VACUUM MICROWAVE: DEATH KINETICS AND TRANSCRIPTIONAL RESPONSES

by

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ABSTRACT

Rapid development in microwave applications in the home and industry along with the increase in the possibility of exposure to microwave radiation have raised concerns about the effect of microwaves on living cells. Although numerous studies have been conducted, microwave effects on living cells still are not fully understood. Some scientists believe that the effect is solely attributable to microwave heating while others suggested that additional effects, other than thermal, are required to explain various types of molecular formations and alterations in a target organism. The present work was designed to study the effect of 2450 MHz microwave radiation under vacuum (vacuum microwave or VM) on kinetic parameters and transcriptional response of mid-stationary *Escherichia coli* (ATCC 11775) cells and to search for possible non- thermal effects associated with VM. In addition, the *E. coli* transcriptome in late-log and mid-stationary phase of growth was studied.

In a preliminary study, the lethal effect of microwave radiation on the microorganisms naturally occurring on parsley during dehydration under vacuum was investigated. Fresh parsley leaves were dried with air-drying (AD) and vacuum microwave drying (VMD) at the same final temperature. This study showed that parsley leaves treated with VMD had lower microbial populations than AD samples at comparable water activity. In addition VMD was more effective against yeast and mould than against total aerobic populations. Since higher reduction in microbial population of fresh parsley leaves occurred not only in a shorter time but also at a lower final temperature as a result of VMD compared to AD, it can be concluded that VM drying was an effective method of reducing the number of naturally occurring microorganisms in parsley.

Death kinetics of *E. coli* in peptone water were determined in a continuous-flow vacuum system with a water bath or microwave as the heating source. Vacuum was used to control the boiling point of water and to maintain the bacterial suspensions at specified temperatures (49°C to 64°C). The z value in the water bath under vacuum was 9.0 °C whereas for VM treatments at 510W and 711W it was 6.0 °C and 5.9 °C respectively, suggesting that *E. coli* is more sensitive to temperature changes during microwave heating than conventional heat treatments. Based upon the Arrhenius calculation of the activation energy it is proposed that the mechanism of *E. coli* inactivation in VM treatment is different from the inactivation that occurs during conventional heat treatment. Thus the impact of temperature on *E. coli* destruction under vacuum was not the same when microwaves were the medium of heat transfer.

Further, a molecular biology approach, DNA microarray technology, was used to investigate *E. coli* transcriptional response to sub-lethal VM and water bath treatment at 50°C for 3 minutes. The results showed that the number of *E. coli* genes that their expression altered through water bath treatment was higher than during VM treatment. VM treatment had a larger effect on genes related to membrane structure and membrane transport systems suggesting that microwave destruction may follow the dielectric cell-membrane rupture theory. In addition VM affected the expression of genes encode for enzymes related to metabolism of carbohydrates, lipids and amino acids to a greater extent than the water bath treatment. Conversely the effect of conventional water bath treatment on ribosomal subunits was higher. Although both treatments were employed under vacuum and signs of anaerobic respiration would be expected, there was more evidence at the transcriptional level for the start of anaerobic respiration in water bath treated cells than in VM treated cells.

In the present work, the focus of the kinetic and gene expression studies was on stationary phase cells, while other gene expression studies have mostly worked with cells at the exponential phase of growth. To close the loop, another study was conducted to investigate the changes at the transcription level in *E. coli* cells between late-exponential and mid-stationary phase of growth. In mid-stationary phase, genes encoding for energy metabolism as well as amino acids and carbohydrate metabolism were down regulated. In addition *csg* genes, required for curli synthesis, were induced and 70.5% of genes involved in cell motility were down regulated or were not detected in mid-stationary cells indicating that in this stage cells may have been less mobile and had more tendency to clump or stick to surfaces. The transcription of *hupA*, *hupB*, *hlpA*, *himA* and *himD* genes previously reported to show up-regulation upon entry into stationary phase were down regulated in mid-stationary cells suggesting that the mechanisms involved in cell function are not only different between lag, log and stationary phase of growth but also may differ in early, mid and late stationary phases.

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LIST OF SYMBOLS & ABBREVIATIONS

%	Percent
°C	Degree(s) Celsius
°F	Degree(s) Fahrenheit
µg	Microgram(s)
µL	Microliter(s)
µM	Micro Molar
µm	Micrometer(s)
× g	Multiples of the Earth's gravitational field
16S rRNA	16 Svedberg unit ribosomal RNA
A	Absent, not detected
AD	Air drying
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
aw	Water activity
b no.	Blattner number
c	Velocity of electromagnetic wave
cDNA	complimentary DNA
CFU	Colony forming unit
cm	Centimeter(s)
DNA	Deoxyribonucleic acid
Dnase I	Deoxyribonuclease I
dNTP	Deoxyribonucleoside triphosphate
DTT	DL-Dithiothreitol
D-values	Decimal reduction time (min)
E. coli	*Escherichia coli*
e'	Dielectric constant
e"	Dielectric loss factor
E_a	Activation energy
EDTA	Ethylenediamine tetra-acetic acid
ELF	Extremely low frequency
Eq	Equation
EVOH	Ethylene vinyl alcohol copolymer
f	Frequency (s^{-1})
F_0	Sterilization value or accumulated lethality (min)
Fig	Figure
g	gram(s)
GHz	Gigahertz ($s^{-1} \times 10^9$)
HPLC	High Performance Liquid Chromatography
hsp	Heat shock protein
HTST	High temperature short time
Hz	Hertz (one cycle per second) or (s^{-1})

ID	Inside diameter
inHg	Inches of mercury
ISM	Industrial, Scientific and Medical
J/mole K	Joules per mole per degree Kelvin
kD	Kilo Dalton
kJ/mole	Kilo joules per mole
kW	Kilowatt(s)
log	Logarithm
LSD	Least significant difference
LTLT	Low temperature, long-time
M	Marginal
m	Meter(s)
m^3/s	Cubic meter per second
mbar	Millibar
MCWC	Microwave Circulated Water Combination system
MHz	Megahertz ($s^{-1} \times 10^6$)
min	Minute(s)
mL	Milliliter(s)
mM	milli Molar
mm	Millimeter(s)
MM	MisMatch
mmHg	Millimeters of mercury
MMLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase enzyme
MMLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
mT	Milli Tesla
MUG	4-Methylumbelliferyl-β-D-glucuronide
mW/cm^2	Milliwatts per square centimeter
NA	Nutrient Agar
NB	Nutrient Broth
nm	Nanometer(s) ($m \times 10^{-9}$)
OD	Outside diameter
orf	Open reading frame
P	Present, detected
PCA	Plate Count Agar
PCA-BS	Plate Count Agar + 1.5 g/L bile salts #3
PM	Perfect Match
pM	pico Molar
PVDC	Polyvinylidene chloride
r^2	Coefficient of determination
RNA	Ribonucleic acid
Rnase H	Ribonuclease H
rpm	Rotations per minute
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second

stdv	Standard deviation
T	Tesla (unit of magnetic flux density)
tan δ	Loss tangent
TDT	Thermal death time (min)
TE buffer	Tris/EDTA buffer
tRNA	Transfer RNA
U	Unit
USDA	United States Department of Agriculture
v/v	Volume per volume
VM	Vacuum microwave
VMD	Vacuum microwave drying
VM-U	Vacuum microwave at 711W compared to stationary phase sample
VM-W	Vacuum microwave at711W compared to water bath treatment
W	Watt(s) (J/s)
w/v	Weight per volume
W-U	Water bath treatment compared to stationary phase sample
z value	Temperature sensitivity value (C or K degrees)
λ	Wavelength (m)
σ^{32}	Sigma 32

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A WISH

I don't think that I like to dedicate my thesis to anyone in specific.
It already belongs to science. Hope it's worthy.

In the future, if this smoothes the bumpy road of research,
I would be content.
I would feel happy if I am around. My soul would lighten up when I am gone.

CHAPTER ONE

INTRODUCTION

1.1 General introduction

From 1945, the time that Dr. Percy LaBaron Spencer "father of the microwave oven" (Schiffmann 1997) discovered the specific heating properties of microwaves, till today, many microwave applications have been developed. Nowadays, microwave ovens can be found in almost every household and are used on a regular basis (Regier & Schubert 2001; Houšova et al. 1996). Some industrial microwave applications, such as microwave tempering units, microwave pasteurization plants and microwave driers (Houšova et al. 1996), have been adopted across the world.

The chance of exposure to microwave radiation (Saffer & Profenno 1989) has increased. Thus along with its development, concern about the effect of microwaves on living cells has been raised. Many studies have been conducted on microorganisms (Woo et al. 2000; Kakita et al. 1995), nematodes (Adams et al. 1999; Daniells et al. 1998), rats (Trošić et al. 1999) and human cells (Liu et al. 2002) to determine the effect of a wide range of frequency of electromagnetic radiation. There is evidence that microwaves cause different biological effects depending upon field strength, frequencies and duration of exposure (Banik et al. 2003).

It is clear that microwave heating is not identical to conventional heating, at the molecular level, but whether this difference could cause effects other than heat is not clearly understood. There are conflicting reports in the literature regarding the mechanism of microwaves effects. Some scientists believe that the effect is exclusively attributable to microwave heating and could be considered as a pure thermal effect (Yeo et al. 1999; Fujikawa et al. 1992) whilst others show evidence of changes in physicochemical characteristics of bacterial cells (Dreyfuss & Chipley, 1980) and suggested that additional effects other than

thermal are required to cause various types of molecular formations and alterations in target organisms (Banik et al. 2003; Woo et al. 2000; Papadopoulou et al. 1995).

The present research was initiated to investigate the effects of microwaves under vacuum (vacuum microwave) on a simple microorganism. A molecular biology approach, DNA microarray technology, was used to learn about microwave interactions with the bacteria. This technique provides a format for whole-genome expression profiling which enables global approaches to biological function in living cells (Blattner et al. 1997) give a better understanding of bacterial response to any factor, in this case microwave radiation. A simple bacterial model, *Escherichia coli* was chosen, as its genome map and physiology have been well studied (Blattner et al. 1997; Adams & Moss 2000) and it has been successfully used as a model for electromagnetic related studies (Nakasono & Saiki 2000; Saffer & Profenno 1989). In addition, if electromagnetic fields are found to affect the *E. coli* cells, a wide range of biochemical and molecular biology techniques can be applied, leading to deeper levels of understanding (Saffer & Profenno 1989).

1.2 Hypotheses

- The destructive effect of vacuum microwave on microorganisms is not purely due to the thermal effect. Other factor or factors are involved in the destruction process.
- Kinetic parameters of *E. coli* death under vacuum microwave heating condition are different from kinetic parameters of *E. coli* death during convective heating under vacuum.

- The transcriptional response of *E. coli* cells subjected to sub-lethal vacuum microwave heating is different from transcriptional response during sub-lethal convective heating for the same time and temperature.
- *E. coli* transcriptional response in late-exponential and mid-stationary phase of growth is different.

1.3 Overview of work plan

The aim of the present work was to study the effect of microwave radiation 2450 MHz on mid-stationary *E. coli* cells under vacuum. The first step was to determine the destruction effect of vacuum microwave on *E. coli*, followed by an investigation to search for non-thermal effect(s) through determination of kinetic parameters. The work continued with an examination of changes in transcriptional response of mid-stationary *E. coli* cells following vacuum microwave treatment. Since reported kinetic studies in the literature have been mostly carried out with stationary phase cells, and gene expression studies have focused on cells in exponential growth, another study was conducted to investigate the changes in *E. coli* cells at the transcription level between late-exponential and mid-stationary phase of growth.

The complete plan consisted of four phases:

Phase I involved a preliminary study of the destructive effect of vacuum microwave drying on naturally occurring microorganisms in parsley.

Phase II focused on the effects of 2450 MHz microwave radiation on survival and injury of *E. coli* under vacuum, along with determination of kinetic parameters of death as well as dielectric properties of the pellet of pure *E. coli* culture and heating medium.

Phase III was an investigation into changes in transcriptional response of mid-stationary phase *E. coli* cells subjected to sub-lethal treatment with vacuum microwave 2450 MHz and conventional heating.

Phase IV was a study of differences in *E. coli* gene expression at the transcription level in cells at late-exponential and mid-stationary phases of growth, to verify the changes in *E. coli* gene expression between these two stages of growth.

CHAPTER TWO

LITERATURE REVIEW

2.1 Electromagnetic radiation and the electromagnetic spectrum

The electromagnetic radiation is characterized by variations of electric and magnetic fields (Khalil 1987). In microwave and infrared frequencies, the electric field applies a force on charged particles. As a result they are impelled to migrate or rotate. Due to the movement of charged particles further polarization of polar particles may take place (Galema 1997). The electromagnetic spectrum includes several regions, that in order of increasing frequency and photon energy, consist of: radio waves, microwaves, infrared, visible light, ultraviolet, x-ray and gamma rays (Knutson et al. 1987; Copson 1975) (Figure 2.1).

2.1.1 Microwaves

Microwaves are located between the 300 MHz and 300 GHz bands in the electromagnetic spectrum. They travel at the speed of light (186,282 miles per second or 3×10^8 meters per second in vacuum) and their wavelength varies between 1 mm and 1 m (Equation 2.1).

$$\lambda = c/f \qquad \text{Eq (2.1)}$$

where λ = wavelength (m)

c = velocity of electromagnetic energy (m/s)

f = frequency (s^{-1})

For example the wavelength at 2450 MHz is 12.24 cm (Meredith 1998).

Only restricted microwave frequencies are allowed for heating purposes. Currently, in North America assigned frequencies for industrial, scientific and medical (ISM) applications are specified by the Federal Communications Commission (Knutson et al. 1987; Buffler 1993).

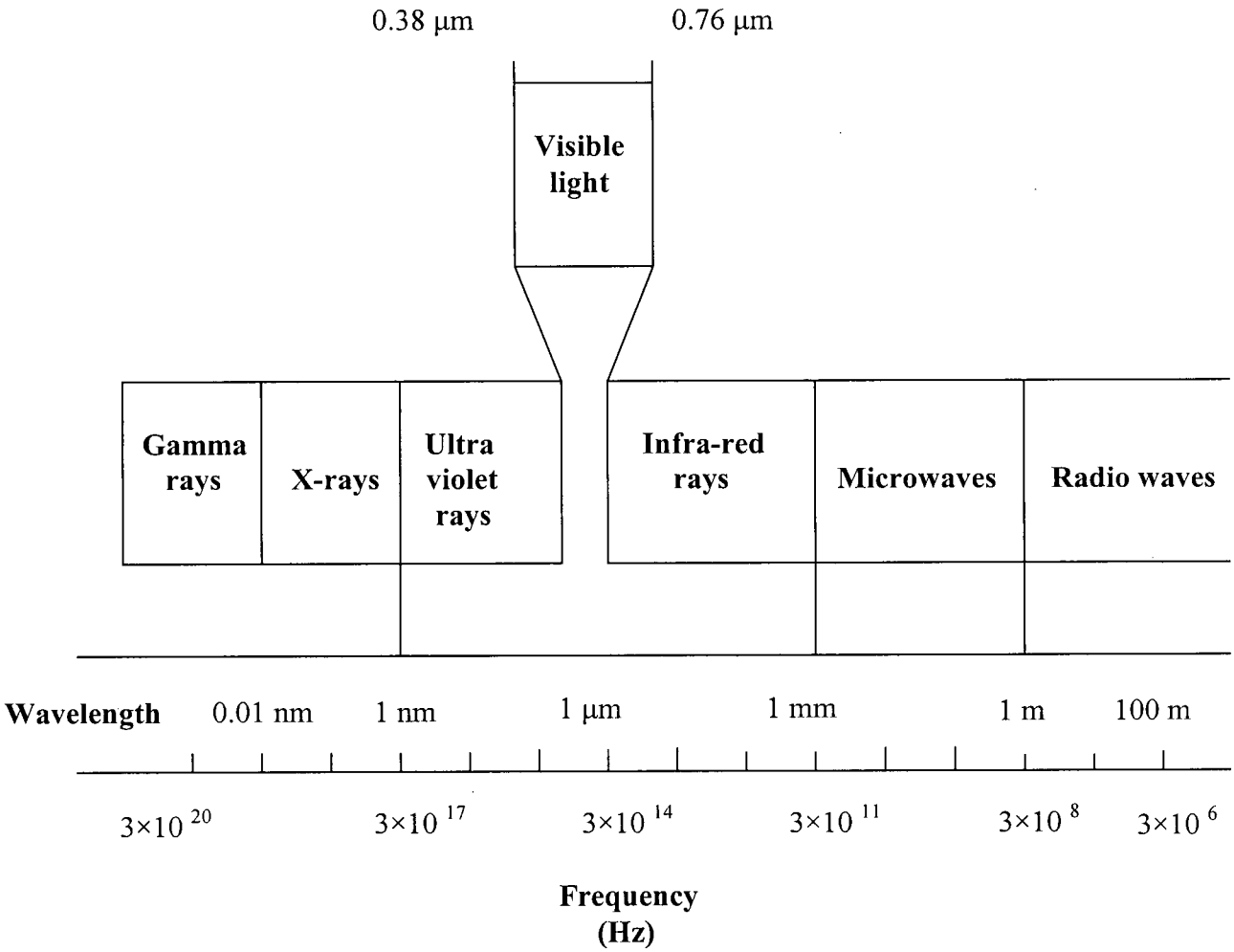

Figure 2.1. The electromagnetic spectrum

Those frequencies are: 915 ± 25 MHz, 2450 ± 50 MHz, 5800 ± 75 MHz, 22125 ± 125 MHz (Regier & Schubert 2001; Knutson et al. 1987; Khalil 1987). Other countries permit the use of these and/or additional frequencies. Household microwave ovens operate at 2450 MHz.

In industrial food processing 2450 MHz is commonly used in Europe, while 915 MHz dominates in North America and 896 MHz is used in the UK (Ryynänen 2002). In addition 433.92 MHz in Austria, Liechtenstein, Portugal, Switzerland and Yugoslavia (Metaxas & Meredith 1983) and 2375 MHz in some other countries (Datta & Davidson 2000) are used for heating purposes.

2.2 Microwave heating

Microwave heating is defined as the heating of a substance by electromagnetic energy (Buffler 1993). Heat is a secondary effect of an electromagnetic field interacting with matter. There are two mechanisms by which the microwave electric field is converted to heat within a material. The first, the ionic mechanism, comes from a linear acceleration of ions, usually from salts, within a non-metallic material. As the dissolved charged particles (ions) in a food or material, oscillate back and forth under the influence of the microwave field, they collide with their neighbour atoms or molecules. These collisions impart agitation or motion, which is defined as heat (Buffler 1993). The second mechanism is the molecular rotation of polar molecules, primarily water as the major constituent of nearly all food products, as well as weaker interactions with carbohydrates, proteins and fats. Polar molecules try to align themselves to the rapidly changing direction of the electric field (Buffler 1993). This alignment requires energy that is taken from the electric field. When the field changes direction, the molecules relax, and

the energy previously absorbed is dissipated to the surroundings directly inside the food (Ohlsson 2000).

2.2.1 Factors involved in microwave heating

Microwave heating is a complex phenomenon, which depends on several factors (Gunasekaran 1999; Prakash et al. 1997). The combination of these factors, influence the temperature development of material during microwave heating (Houšova et al. 1996). Factors include: volume, density, shape and dimension of material (Ohlsson 2000; Dorantes-Alvarez et al. 2000; Houšova et al. 1996; Ryynänen & Ohlssson 1996; Buffler 1993), packaging composition and geometry (Ohlsson 2000), specific heat capacity, thermal conductivity of heated material (Dorantes-Alvarez et al. 2000; Gunasekaran 1999; Buffler 1993), sample composition and dielectric properties (Ohlsson 2000; Dorantes-Alvarez et al. 2000; Gunasekaran 1999; Houšova et al. 1996; Ryynänen & Ohlssson 1996; Buffler 1993), initial temperature of the material (Dorantes-Alvarez et al. 2000; Gunasekaran 1999; Houšova et al. 1996; Buffler 1993) as well as process parameters, such as type of magnetron (Dorantes-Alvarez et al. 2000; Buffler 1993), microwave frequency, power supply (Ohlsson 2000; Dorantes-Alvarez et al. 2000; Houšova et al. 1996; Buffler 1993), field intensity (Gunasekaran 1999), load in the oven (Dorantes-Alvarez et al. 2000; Buffler 1993), and process time (Dorantes-Alvarez et al. 2000; Houšova et al. 1996; Buffler 1993).

2.2.1.1 Dielectric properties

Dielectric properties of a material are a measure of the dielectric charge movement inside that material in response to an external electric field (Kuang & Nelson 1998). When a sample is

placed in the path of microwaves, it will absorb energy from the waves, depending upon its dielectric properties (Engelder & Buffler 1991). Thus, the dielectric properties describe how non-magnetic materials interact with electromagnetic radiation. Dielectric constant (permittivity) is the ability of any material to absorb, transmit, and reflect energy from the electric portion of microwave fields (Engelder & Buffler 1991). In other word, the dielectric constant (e') shows the amount of energy absorbed by a specific material in a specific electric field while the loss factor (e") shows how much of this energy can be converted into heat. In addition, the loss tangent (tan δ= e"/e') defines the ability of a medium to convert electromagnetic energy into heat energy at a given frequency and temperature (Engelder & Buffler 1991; Galema 1997). Factors that affect the dielectric properties are the frequency of the electromagnetic waves, temperature, density, water content, salt content, percentage of solutes, and state (liquid, solid, or gas) of the material under examination (Yaghmaee & Durance 2001; Galema 1997).

Magnetic properties of microwaves must be accounted for when magnetic materials such as ferrites or metals are under study (Buffler 1993). In food science, only electrical interaction is considered, for no foods magnetically interact with microwaves (Buffler 1993).

2.3 Microwave applications

Microwaves were originally used for communication and radar (Coronel et al. 2003), but nowadays many of microwave applications involve the utilization of higher energy for direct interior heating (Ohlsson 2000). Researchers in many fields have conducted studies on various applications of microwaves. For example the use of microwave energy in chemistry for heterogeneous esterification (Chemat et al. 1998), analytical chemistry, synthesis of radio-

pharmaceuticals, inorganic complexes, intercalation reactions, polymer curing (Galema 1997), and decomposition reactions (Michael et al. 1991) have been reported. Other applications include use of microwaves in formation of an aerated cheese product (Jeffry 2003), microwave assisted digestion of seafood for analytical purposes (Li et al. 2003), use of microwaves to shorten PCR total reaction time (Fermer et al. 2003), microwave lysis (Menon & Nagendra 2001), microwave hydrolysis (Marconi et al. 2000), use of microwaves for eukaryotic DNA isolation (Goodwin & Lee 1993), ceramic processing (Michael et al. 1991), moisture determination (AOAC 16.239), removal of the feathers from poultry (Rosenberg & Bögl 1987b) and microwave rendering of fats (Decareau 1985).

The use of microwave energy in food processing can be classified into six main groups: heating and re-heating (Coronel et al. 2003; Heddleson et al. 1996), baking, cooking and pre-cooking (Regier & Schubert 2001; Knutson et al. 1987; Khalil 1987; Rosenberg & Bögl 1987a; Schiffmann 1986), tempering and thawing (Regier & Schubert 2001; Knutson et al. 1987; Edgar 1986) blanching (Dorantes-Alvarez et al. 2000; Knutson et al. 1987), dehydration (Kaensup et al. 2002; Ohlsson 2000; Mudgett 1989; Decareau 1985), pasteurization and sterilization (Regier & Schubert 2001; Knutson et al. 1987). Although microwave applications have a wide range of objectives, they are all established based on microwave heating properties and increase in temperature (Regier & Schubert 2001).

2.3.1 Microwave dehydration

The main rational for application of microwaves to dehydration is the shortened process time. In traditional air-drying methods process time is limited by low thermal conductivities (Regier & Schubert 2001; Fellows 2000; Garcia et al. 1988). Microwaves excite water and fat

molecules for some depth into the food. Moisture from the interior of the food can be expelled due to the increase in vapor pressure (Kaensup et al. 2002). Oxidation by atmospheric oxygen is minimized during microwave heating, since it is not necessary to heat large volumes of air (Fellows 2000). This can lead to rapid drying without overheating the atmosphere or creating surface damage (Kaensup et al. 2002). In addition more homogeneous drying, without large moisture gradients, improves moisture transfer during the later stages of drying and eliminates case hardening (Regier & Schubert 2001; Fellows 2000; Knutson et al. 1987). At the same time, unwanted changes in sensory attributes and nutrient loss due to long drying times or high surface temperatures, can be prevented (Regier & Schubert 2001). However the higher cost of microwaves and smaller scale of operation, compared with traditional methods of dehydration, has restricted microwave drying as a sole source of energy in dehydration (Fellows 2000). In most cases, microwaves are used in combination with conventional hot air drying for dehydration in pilot and industry levels (Ohlsson 2000). One of the earliest examples was drying of pasta (Decareau 1985) and the production of dried onions (Regier & Schubert 2001; Metaxas & Meredith 1983). Later, drying of vegetables and cereal products (Ohlsson 2000), agar gel and *Gelidium* (Garcia & Bueno 1998), spices, tomato paste, wild rice, snack foods and bacon pieces (Kaensup et al. 2002; Mudgett 1989) and final drying of potato chips (Kaensup et al. 2002; Knutson et al. 1987; Decareau 1985) were also reported.

2.3.2 Vacuum microwave

Microwave energy, by overcoming the low heat transfer rates of conduction, has led to higher drying rates and less shrinkage in the final product. Unfortunately the higher drying rates cause the loss of aromas (Regier & Schubert 2001; Decareau 1985). On the other hand vacuum

drying systems are normally used for sensitive materials that would be damaged or decomposed at high temperatures. During vacuum drying, high-energy water molecules rapidly diffuse to the surface and evaporate into the vacuum atmosphere (Gunasekaran 1999). Since vacuum drying takes place by evaporation at reduced boiling points in a low-pressure chamber, the product may be dried at a lower temperature at reduced pressure than at atmospheric pressure. Moreover, removal of air due to vacuum, during dehydration, diminishes oxidation reactions (Gunasekaran 1999). Conversely conduction or radiant heating that are normally used for vacuum drying maintain low drying rates because the moisture front is retracted and thermal conduction is slower (Kaensup et al. 2002; Gunasekaran 1999).

The use of vacuum along with microwaves has proved a good combination in production of high quality materials. While microwaves provide the fastest means available of transferring energy into the interior of biological solids (Durance & Wang, 2002), the reduced pressure keeps the product temperatures low, as long as a certain amount of free water is present. Therefore temperature sensitive substances like vitamins, colours, volatiles and flavours will be retained (Regier & Schubert 2001; Decareau 1985). Kim and colleagues (2000) reported retention of chicoric acid and caftaric acid in *Echinacea purpurea* flowers dried with vacuum microwave dryer. Kim and colleagues (1997) dried concentrated yogurt in a laboratory scale microwave vacuum dryer (10 mm Hg, 250 W, 2450 MHz) at 35°C and reported a substantial retention of lactic acid bacteria (*S. thermophilus* and *L. bulgaricus*). Decareau (1985) reported that retention of vitamin C and volatile compounds in orange juice powder was higher after vacuum microwave drying compared to other drying processes (Knutson et al. 1987). Sobiech (1980) also reported that microwave vacuum drying enhanced the flavour of dried sliced parsley root and retained the properties of fresh raw material.

Vacuum microwave drying has been used in dehydration of a wide range of products, starting from fruits and vegetables such as: banana slices (Mousa & Farid 2002, Mui et al. 2002); tomato slices (Durance & Wang 2002); chilli (Kaensup et al. 2002), parsley (Böhm et al. 2002), sweet basil (Yousif et al. 1999), carrot slices (Lin et al. 1998), potato chips (Durance & Liu 1996), cranberry (Yongsawatdiguul & Gunasekaran, 1996), and sliced parsley root (Sobiech 1980), to fruit juice (Regier & Schubert 2001) and tea powder (Schiffmann 1995) as well as grains (Decareau 1985), enzymes (Schiffmann 1995), pectin gel (Drouzas et al. 1999) and shrimp (Lin et al. 1999).

2.3.3 Microwave pasteurization & sterilization

The fast and effective heating with microwaves, short process times and the relatively low thermal exposure of the food material resulting in less changes in physical and chemical properties of the product, along with destruction effect of microwave on microorganisms, has made microwave radiation a promising candidate for pasteurization and sterilization purposes. Researchers have intensively studied the possibility of using microwaves in pasteurization and sterilization (Regier & Schubert 2001).

Some of the studies have focused on prolonging product shelf life. Cunningham (1980) studied the effect of microwave radiation (915 MHz) on total microbial counts of fresh cut chicken and suggested that minimal microwave radiation might be used to extend the refrigerated shelf life of fresh poultry. Wu and Gao (1996) reported that moon cake, breads and spring rolls treated at 850W of 2450 MHz microwaves had a 30 day shelf life, that was significantly longer than the 3-4 days of untreated samples. Herve and colleagues (1998) studied the effect of microwave treatment (2450 and 915 MHz) on inactivating surface spoilage

microorganisms in cottage cheese and suggested that microwave treatment decreased the number of psychrotrophs and would increase the shelf life of the cheese. Ohlsson (2000) pasteurized ready-made foods by microwave heating to 75°C to 80°C and prolonged their shelf life to approximately three to four weeks

Other scientists and researchers have focused on the calculation of process times or have checked the sterility of the contaminated product. Yang and co-workers (1947) reported that after pasteurizing wine at 140°F for 4 seconds with 26 to 34 MHz microwaves, no detectable microorganisms were found. Douglas and colleagues (1990) studied the effect of microwaves on sterilization of urinary catheters at home. Catheters were incubated for sixty minutes at 37°C in a phosphate buffer suspension containing sixteen species of microorganisms (10^4-10^6 cells/ml), isolated from patients with urinary tract infections including two strains of *E. coli*, *Klebsiella* sp., *Pseudomonas* sp., *Proteus* sp., *Enterobacter* sp., *Streptococcus* sp., *Staphylococcus* sp. and *Candida* sp. Catheters were removed from suspension and placed in a sterile plastic bag. They reported no live bacteria or yeast after 12 minutes exposure to microwaves (2450 MHZ, 650W). They concluded that microwave sterilization is a practical, efficient and cost-effective method of home catheter sterilization. Kudra and co-workers described a simple laboratory scale microwave system for the continuous heating of milk, using 700W, 2450 MHz (Kudra et al. 1991). Diaz-Cino & Martinelli (1991) studied the effect of microwave (700W, 2450 MHz) on *Aspergillus nidulans*, *Escherichia coli*, *Bacillus subtilis* and *Bacteriophage T4*. They reported complete sterilization at 85°C, 30 min for all tested microorganisms except for *B. subtilis*. They concluded that the microwave method was faster, but while it can be used for pasteurization, is not suitable for sterilization process, since viable spores remained (Diaz-Cino & Martinelli 1991). Odani and colleagues (1995) performed a study on microwave pasteurization and

reported that bacteria in frozen shrimp (4.1×10^3 CFU/g), refrigerated thick custard (1.2×10^2 CFU/g) and frozen pilaf (1.2×10^2 CFU/g) were killed by microwave radiation after 40 seconds, 60 seconds and 4 minutes, respectively. They also exposed cultures of *E. coli* (4.2×10^3 CFU/ml), *S. aureus* (7.0×10^3 CFU/ml) and *B. cereus* (1.6×10^5 CFU/ml) diluted in saline to microwave and reported a pasteurization time of 30 seconds at 50°C for *E. coli* and *S. aureus* cultures and 90 seconds at 100°C for *B. cereus*. They observed that spores of *B. cereus* survived even after 30 minutes at 100°C. Lau and Tang (2002) pasteurized pickled asparagus using 915 MHz microwave and reported better heating uniformity, shorter process time and marked reduction in thermal degradation of asparagus compared to the conventional method. Hiti and colleagues (2001) used microwave (2450 MHz, 600W) to sterilize contact lens cases inoculated with *Acanthamoeba* (*A. comandoni, A. castellanii, A. hatchetti*) and their cysts and stated that Trophozoites as well as cysts, were effectively killed by microwave treatment in 3 minutes, regardless of the type of lens case used. Guan and colleagues (2003) conducted a study on macaroni and cheese inoculated with *Clostridium sporogenes* (PA 3679) spores packed in trays flushed with nitrogen, heat sealed and sterilized in a 915 MHz Microwave-Circulated Water Combination (MCWC) system. They reported that microbial destruction by MCWC system matched with calculated F_0 values of sterilization.

Microwave pasteurization and sterilization of many other products have been described. For example microwave pasteurization of ready made food and packed food (Ohlsson 2000), shell eggs (Sullivan & Padua 1999), fruit juices (Copson 1975), apple juice, apple cider, pineapple juice (Kozempel et al. 1998), raw cow's milk and goat's milk (Mann 1997; Villamiel et al. 1996), yogurt and pouch-packed meals (Regier & Schubert 2001; Decareau 1985), pasta meals, soft bakery goods and peeled potatoes, fruits in syrup (Fellows 2000), baby foods,

puddings, custard, sauces, soups, pharmaceuticals and gelatines (Armfield 2001) have been reported. In addition microwave sterilization of reusable pharmaceutical glass vials, tissue culture plates, culture media, contact lenses, dental instruments, baby bottles, and decontamination of clinical specimens containing bacterial pathogens has also been studied (Douglas et al. 1990).

2.3.3.1 Microwave pasteurization and sterilization systems

Although using microwaves in pasteurization and sterilization has been investigated for many years, introduction on a commercial level has only happened in the past few years (Ohlsson 2000). For both processes it is very important to be able to properly control heating uniformity within the product to ensure microbial destruction and the microbiological safety of the processed foods (Guan et al. 2003; Regier & Schubert 2001; Ohlsson 2000; Prakash et al. 1997). The existence of hot and cold spots in microwave ovens due to uneven microwave distribution (Tassinari & Landgraf 1997; Sieber et al. 1996; Sigman-Grant et al. 1992; Knutson et al. 1987), is the main reason that up to now microwave pasteurization and sterilization has been mostly utilized for batch sterilization operations (Regier & Schubert 2001).

In general, techniques that have been used to improve heat uniformity include rotating, oscillating and moving of samples, using cooling medium immediately after or simultaneous with microwave exposure, surrounding samples with a medium of higher dielectric constant, applying microwave in cycles (Datta & Davidson 2000), or applying microwaves along with high pressure. Researchers and scientists have used either one or a mixture of these techniques to design their pasteurizer or sterilizer systems.

Some of the most common approaches to achieve uniform heating are: microwave pasteurizers with conveyor belt systems (Knutson et al. 1987); microwave sterilizers with conveyor belt and heating chamber with sliding door (Kumeta 1997); inserting a stainless steel cooling tube with cold water flow inside the plastic process tube within the microwave to maintain the temperature of the liquid (Kozempel et al. 1998); conveyor tunnel with a combination of microwaves and hot air (Fellows 2000); continuous hydrostatic microwave sterilizer for laminated microwave transparent pouches made from polypropylene /EVOH or PVDC/polypropylene while they are submerged in a medium with higher dielectric constant than the product (Fellows 2000); pressurized HTST sterilization system with water immersion technique (Tang et al. 2001; Ohlsson 1991); continuous fluid pasteurization and sterilization systems with tubes intersecting waveguides or small resonators, in such a way that heating is accomplished across the tube cross section (Regier & Schubert 2001; Ohlsson 2000; Decareau 1985); microwave heaters with sliding doors and special compression and decompression systems (Regier & Schubert 2001); UHT/HTST microwave pasteurizer with water cooling (Armfield Limited, Ringwood, England 2001) and Microwave-Circulated Water Combination (MCWC) system, consisting of microwave generator, pressurized microwave heating vessel and a water circulation heating and cooling system (Guan et al. 2003).

Continuous microwave pasteurization systems are commercially used in Belgium (Tops Foods, Belgium), Japan (Otsuka Chemical Co., Osaka, Japan), and England (Armfield Limited, Ringwood, England) but to the knowledge of the author do not exist in North America (Tang et al. 2001; Ohlsson 2000).

2.4 Effect of microwaves on microorganisms

There have been conflicting reports in the literature regarding the effects of microwaves on microorganisms (Vasavada 1986) along with an ongoing debate for over fifty years on the existence of effects other than heat associated with electromagnetic energy (Kozempel et al. 2000). Since the mid 1920s, numerous studies have been carried out at various microwave frequencies in an attempt to solve the debate (Mertens & Knorr 1992). Some researchers attribute the destruction of microorganisms subjected to microwave energy solely to thermal effects, whereas others have indicated injury to cells regardless of temperature (Datta & Davidson 2000; Vasavada 1986; Dreyfuss & Chipley 1980).

Those researchers believing in a microwave effect either have reported greater lethality with microwave treatment than conventional heating (Tajchakavit & Ramaswamy 1995; Khalil & Villota 1988) or have found smaller D-values for the microwave-treated cells compared to conventional destruction method (Tajchavit et al. 1998) or showed evidence of characteristic biological changes such as changes in metabolic function of the microorganism under study (Woo et al. 2000; Dreyfuss & Chipley 1980).

Olsen (1965) treated loaves of bread, inoculated with cultures of *Aspergillus niger*, *Penicillium sp.* and *Rhizopus nigricans*, with microwave radiation (5 kW, 2450 MHz) and reported that the numbers of viable spores were greatly reduced. He concluded that since the destruction happened at a temperature lower than the thermal death point of these microorganisms, the results of microwave treatment were probably not due to conventional thermal kill. Culkin & Fung (1975) studied the pattern of *E. coli* and *Salmonella typhimurium* destruction with microwave (915 MHz) in cooked soups while measuring temperatures with temperature sensitive strips. They found that although the top portion of the soup was the

coolest region, it showed the greater decrease in microbial survival for any given exposure time. They stated that if the lethal action of microwaves on microorganisms were solely due to the heat generated by the waves, then samples from the warmest region of the soups would show the lowest survival values. Dreyfuss & Chipley (1980) studied the effect of sublethal microwave radiation (2450 MHz) on enzymatic activity of *Staphylococcus aureus* compared to conventional heat treatment for 10, 20, 30 and 40 seconds, while internal temperatures of microwave treated flasks did not exceed 46°C. They reported some changes in physicochemical characteristics in microwave-treated cells, such as higher activity of malate dehydrogenase, α-ketoglutarate dehydrogenase, cytochrome oxidase, and cytoplasmic ATPase. They also observed that the activity of glucose-6-phosphate dehydrogenase was decreased by microwave radiation but increased by conventional heat treatment. In general they concluded that the effect of microwave radiation on the metabolic activity of *S. aureus* can not be explained by thermal effects alone.

Khalil & Villota (1988) also studied the effect of microwave radiation on *S. aureus* metabolic function and reported that microwave-treated cells regained their ability to produce enterotoxin A at a slower rate, and did not reach the amount produced by untreated cells after 72 hours of recovery, while conventionally treated cells regained production levels almost identical to the unheated cells. They concluded that microwaves have intrinsic injurious effects on biological systems, other than those brought on by heat (Khalil & Villota, 1988). Reznik & Knipper (1994) studied the microwave pasteurization of liquid egg and found a higher degree of microbial kill with microwaves compared to a conventional pasteurizer. They also reported less re-growth of bacteria even when the egg was maintained at room temperature (Kozempel et al. 2000). Woo and colleagues (2000) performed a study on the effect of microwave radiation on

E. coli and *B. subtilis* cells and reported a higher amount of nucleic acid leakage, rough and swollen cell surfaces, along with the presence of dark spots in the cytoplasm of microwave treated cells.

Odani and colleagues (1995) investigated the presence of protein in the cell-free supernatant of *E. coli* cells exposed to microwave radiation for 0-12 seconds at 15°C. The result of acrylamide gel electrophoresis with silver staining showed release of proteins from microwave treated cells compared to untreated samples. They suggested that the mechanisms of killing of bacteria depends not only on temperature, but also on other effects of microwave irradiation. Other authors reporting results which appeared to indicate nonthermal effects are Papadopoulou et al. (1995), Rosaspina et al. (1994), Galuska et al. (1988) and Webb & Dodds (1968).

At the same time there are several scientists who reported that there is no non-thermal effect associated with microwaves (Fujikawa et al. 1992). This group have found no difference between microwave and conventional heat destruction or reported no destruction with microwaves at lower temperature. They argued that reported nonthermal effects are due to the lack of precise measurements of the time-temperature history (Datta & Davidson 2000).

Goldblith & Wang (1967) heated suspensions of *E. coli* and *Bacillus subtilis* with microwave (2450MHz) and conventional heating. They reported an identical degree of deactivation for both treatments. Hamrick & Butler (1973) exposed *E. coli* and *Pseudomonas aeruginosa* cultures to microwave radiation (2450 MHz, 60mW/cm2) and maintained the temperature at 37°C for 12 hours. They plotted the growth curve of microorganisms and detected no deviation of cell replication rate. Vela & Wu (1979) studied the effect of 2450 MHz microwaves on various bacteria, Actinomycetes, fungi, and bacteriophages in the presence and

absence of water. They reported that dry or lyophilized organisms were not affected even by extended exposure time. The authors stated that microorganisms were killed by a thermal effect (Dreyfuss & Chipley 1980; Vela & Wu 1979). Welt and colleagues (1994) found no difference between conventional and microwave inactivation of *Clostridium sporogenes* PA3679 spores at 90, 100 and 110°C. In their study they continuously cooled suspensions and reported no detectable inactivation (Datta & Davidson 2000). Yeo and colleagues (1999) studied the effect of microwave radiation (2450MHz, 800W) on *Staphylococcus aureus* on stainless steel discs and reported that destruction of microorganisms was mainly due to heat transfer from the stainless steel substrate and very little direct energy was absorbed from the microwaves.

A third group of researchers has suggested that electromagnetic energy acts in a way to magnify the thermal effect (Kozempel et al. 2000; Brunkhorst et al. 2000; Reznik & Knipper 1994; Ramaswamy & Tajchakavit 1993). Mittenzwey and co-workers (1996) reported that extremely low-frequency magnetic fields (2-50 Hz, 1-10 mT) act on *Photobacterium phosphoreum* only as a co-stressing factor which activates processes or reactions already initiated by other stresses. Tajchakavit and co-workers (1998) reported rapid destruction of *S. cervisiae* and *L. plantarum* at temperatures of 60-65°C, which were 10-15°C lower than those used for thermal destruction. Their data for *S. cervisiae* showed a D-value of 25.1 s for thermal destruction and 2.08 s for microwave destruction at an intermediate temperature of 55°C. For *L. plantarum*, at an intermediate temperature of 60°C, the D-values were 21.9 and 3.83 s, under thermal and microwave heating modes respectively. They concluded that microwave heating was more efficient than conventional heating, and indicated the possibility of some non-thermal or enhanced thermal effects associated with microwave heating. Kozempel and co-workers (1998) studied microwave destruction of *Pediococcus sp.* in water, 10% glucose solution, apple

juice, tomato juice and apple cider and beer using a continuous microwave system with water flow as a cooling medium to keep the temperature of the hot spot at 40°C. Bacterial counts were more readily reduced in water, glucose solution and apple juice than in apple cider, tomato or pineapple juice, and none were killed in skim milk. They suggested that microorganisms become more susceptible to stresses like acidic pH when the temperature goes up. Kozempel and co-workers (2000) described a continuous steady state microwave process (7kW, 2450 MHz) while simultaneously removing the thermal energy to maintain low temperature. They tested *E. coli, Pediococcus sp., Listeria innocua, Enterobacter aerogenes* and yeast in water, beer, whole egg, egg white, apple juice and tomato juice in the temperature range of 26-45°C for 2-9.7 minutes. Microwave energy in the absence of other stresses such as heat, pH or anti-microbial did not destroy microorganisms at low temperature. The authors added that it is possible that microwave energy may complement or magnify thermal effects. Khalil & Villota (1988) indicated that added lethality of microwaves lies in their ability to distribute the thermal energy instantaneously to the heat sensitive subcelluar components. Accordingly, higher amounts of thermal energy would be generated within the cellular suspension, thus more heat-induced injury to the cellular components would occur. Mittenzwey and colleagues (1996) studied the effect of extremely low-frequency electromagnetic fields (magnetic strength 1 to 8 mT and frequency of 2-50 Hz) on different *Escherichia coli, Proteus vulgaris, Photobacterium phosphoreum, Photobacterium fisheri* at temperatures ranging from 25 to 37°C. They reported that extremely low-frequency electromagnetic fields might act on bacteria as a co-stressing factor by activating a process or reaction already initiated by other stresses like heat.

2.4.1 Microwave kinetic parameters

The lethal effect of heat on bacteria is a function of time, temperature, bacterial population and bacterial thermal resistance. The D-value, the decimal reduction time, is a means of characterizing the death rate or lethal effect of a temperature at different times. It is the length of time necessary to produce a "one-log" reduction in microbial population at a specific temperature. The z is a measure of the sensitivity of an organism to changing lethal temperatures. Calculating microbial destruction for a microwave heating process is more complicated than for a conventional thermal process, because of the difficulty in keeping precise constant temperatures inside the microwave oven (Heddleson & Doores 1994).

Fujikawa and colleagues (1992) exposed *E. coli* culture to microwaves in a container placed on a rotary plate inside a microwave oven. They found that the profile of destruction of bacteria by microwave radiation was approximated by a set of three linear relationships and was difficult to understand. Fujikawa & Ohta (1994) also reported that the survival curves of *E. coli* and *Staphylococcus aureus* exposed to microwave radiation at 200, 300 and 500 W approximated a set of three linear phases. The destruction profile of *Bacillus cereus* spores in saline showed two linear phases at 200 W and was approximated by a single linear function at 300 or 500 W. When Kakita and colleagues (1995) plotted the relative survival populations of bacteriophage PL-1 as a function of time on a semi-logarithmic graph, they reported that the bacteriophage were inactivated by microwave irradiation according to almost first-order kinetics with some lag period at the beginning. Odani and colleagues (1995) reported two linear survival curve for *E. coli, S. aureus* and *B. cereus* in saline after microwave irradiation. Tajchakavit and co-workers (1998) studied the destruction kinetics of *Saccharomyces cervisiae* and *Lactobacillus plantarum* in apple juice and reported that the inactivation profiles in continuous microwave

system followed a first-order kinetic model. They also reported $D_{52.5}$ of 4.8 s, D_{55} of 2.1 s and $D_{57.5}$ of 1.1 s for *S. cervisiae* and $D_{57.5}$ of 14 s, D_{60} of 3.8 s, and $D_{62.5}$ of 0.79 s for *L. plantarum* with corresponding z-values of 7 and 4.5°C. D-values under conventional heating were 58, 25 and 10 s at 50, 55 and 60°C for *S. cervisiae* and 52, 22 and 8,4 s at 55, 60 and 70°C for *L. plantarum* with corresponding z-values of 13.4 and 15.9°C. Rosenberg & Sinell (1990) studied the effect of microwave (2450 MHz), on *Staphylococcus aureus, Salmonella typhimurium* and *E. coli* and reported D_{55} of 11.6, 2.3 and 2.9 min respectively while in water bath treated cells D_{55} was 17.8, 2.4 and 3.0 min. They also reported corresponding z-values of 11.6, 4.7 and 24.4 °C for *Staphylococcus aureus, Salmonella typhimurium* and *E. coli* treated with microwave while reported z for water bath treated cells was 6.5, 4.6 and 13.6 °C respectively.

2.4.2 Mechanism of thermal destruction

Many factors affect the heat resistance of an organism, including type of organism, inherent resistance (the differences among species and strains within the same species, spores and vegetative cells), number of cells, age of cells, stage of growth, growth condition (growth temperature, growth medium), and environmental condition during the time of heating (pH, water activity, type of medium, salts and other organic and inorganic compounds).

The preservative effect of heat processing is due to the irreversible heat denaturation of proteins, nucleic acids, enzymes or other vital components of microorganisms (Datta & Davidson 2000; Fellows 2000; Heddleson & Doores 1994). Denaturation stops enzyme activity and as a result metabolic functions related to that specific enzyme will be stopped and cell death will occur (Fellows 2000). Some of the enzymatic activities reported to be affected by heat are:

glucose phosphate isomerase, fructose diphosphate aldolase and lactose dehydrogenase (Khalil & Villota, 1988; Bluhm & Ordal 1969).

Another reported cellular effect of heat is perturbation of the integrity of DNA including DNA damage and loss of negative superhelicity (Champomier-Verges et al. 2002; Delaney 1989). In addition, membrane damage or disruption of membranes has been observed as a result of thermal treatment (Champomier-Verges et al. 2002; Datta & Davidson 2000; Heddleson & Doores 1994; Khalil & Villota, 1988). Metabolites and cofactors crucial to cellular function may leak through damaged membrane and cause cellular death (Heddleson & Doores 1994). The presence of intra cellular compounds such as ninhydrin positive material, purines, pyrimidines and ribonucleotides in the medium indicated damage to the cell at the membrane level (Khalil & Villota, 1988).

2.4.3 Mechanism of microwave destruction

Several theories have been advanced to explain how microwave energy kills microorganisms (Brunkhorst et al. 2000) such as: breakage of hydrogen bonds and secondary linkages (Kalant 1959), release of bound water (Ballario et al. 1975), electron tunneling (Cope 1976), pearl chain formation (Khalil & Villota, 1988; Lambert 1980), particle orientation and molecular resonance (Lambert 1980), change in the charged nucleus surface (Shckorbatov et al. 1998), interference with cell signaling pathways (de Pomerai et al. 2000), and changes in secondary and tertiary structure of proteins (Banik et al. 2003). In general there are four predominant theories with supporting evidence that have been focused on in literature: electroporation, dielectric cell membrane rupture, magnetic field coupling, and selective heating (Kozempel et al. 1998).

The electroporation theory states that the electrical potential across the cell membrane causes pore formation in the weakened membrane of the microorganisms, resulting in leakage of cellular material and cell lysis (Datta & Davidson 2000; Kozempel et al. 1998). Rosaspina and co-workers (1994) examined microwave treated *Mycobacterium bovis* and reported a series of progressive changes in bacterial morphology. These changes consisted of formation of pits, which penetrated deeply into the bacterial cell until they passed through the entire width. Liquefaction appeared to occur, so that individual cells could no longer be distinguished. They added that these phenomena increased progressively with increasing exposure time until nearly total disintegration of the cells was achieved and the remaining fragments appeared to be a shadow of the destroyed cellular body. With the application of dry or moist heat, the changes were less extensive and complete cellular disintegration was never observed (Rosaspina et al. 1994). Woo and colleagues (2000) studied destruction of *E. coli* and *B. subtilis* exposed to microwave radiation (2450 MHz). They reported that most of the microwave treated cells were ghost cells from which intracellular materials were released into the cell suspension. At the same time they did not find any decrease in cell optical density at 600 nm in spite of a significant reduction in the viable count. Therefore they suggested that this might be due to the fact that microwave-treated cells were not completely lysed. They also found that the surface of microwave treated *E. coli* cells were damaged and had become rough and swollen, while no damage to surface structure was observed for *B. subtilis*. Considering that both microorganisms were inactivated by microwave irradiation, they suggested that the damage to the surface structure of microorganisms might not be the main reason for inactivation by microwave heating.

In the dielectric cell-membrane rupture theory, an external electric field is thought to induce an additional trans-membrane electric potential, which is larger than the normal potential

of the cell. This drop of voltage across the cell membrane may be sufficient for membrane rupture (Datta & Davidson 2000; Kozempel et al. 1998; Zimmermann et al. 1974). It also may result in pore formation, increased permeability, and lost of cell integrity (Brunkhorst et al. 2000, Kozempel et al. 2000). Ke and co-workers (1978) reported positive correlation of peroxide value in fresh mackerel fillets with the length of exposure to microwave energy at 2450 MHz and suggested that the energy from microwaves might disrupt the membrane and/or subcellular structure, thus releasing the lipids.

In the magnetic field coupling theory, cell lysis was explained by a coupling of the electromagnetic energy with critical molecules within the cells, such as protein or DNA. Disrupting the internal components of the cells may cause them to die (Kozempel et al. 1998). Mertens and Knorr (1992) suggested that the oscillating magnetic field couples energy into the magneto-active parts of large biological molecules with several oscillations. When a large number of magnetic dipoles are present in one molecule, enough energy can be transferred to the molecule to break a covalent bond. Therefore certain critical molecules in a microorganism, like DNA, or proteins, could be broken, hence death of microorganisms or at least reproductive inactivation will occur (Brunkhorst et al. 2000; Kozempel et al. 2000). On the other hand Heddleson & Doores (1994) reported that the quantum energy of microwave is 1.2×10^{-5} eV, whereas the energy needed to break hydrogen bonds is 5.2 eV, thus microwaves are unable to break the hydrogen bonds. Woo and colleagues (2000) observed several dark spots in the cytoplasm of microwave treated cells, examined by scanning electron microscopy, while no dark spots were observed in the untreated cells. They suggested that the dark spots could be aggregated proteins caused by microwave heating. Kakita and co-workers (1995) conducted a study on the effect of microwave radiation on the survival of bacteriophage PL-1. When phage

particles were exposed to microwave radiation (2450 MHz, 500W) for 60 s with the maximum temperature of 75°C, DNA molecules within the phage particles were randomly broken into small fragments, whereas DNA treated with conventional heating (70°C or 80°C for 75 s) or untreated phages remained intact.

In the fourth proposed theory, the selective heating theory, the microorganisms are thought to selectively absorb the electromagnetic energy. The solid microorganisms are thought to heat faster than the surrounding fluid and reach lethal temperatures while the surrounding fluid remains below lethal temperatures (Kozempel et al. 1998). Nelson and Charity (1972) conducted a study on energy absorption of winter wheat *Triticum aestivum* and adults of the rice weevil *Sitophilus oryzae* and found the degree of selective heating depends upon the relative values of the dielectric properties and the loss factor between insects and grain. They observed a better selective heating of insect at the frequency of 40 MHz than at 2450 MHz (Kozempel et al. 2000; Nelson & Charity 1972). Wang and colleagues (2003) also studied the effect of microwave radiation (27 and 915 MHz) on in-shell walnuts and gellan gel as a model for coldling moth larvae and reported 1.4 to 1.7 times greater heating of insects than walnuts at 27 MHz while no detectable preferential heating was observed at 915 MHz.

2.4.4 Injured microorganisms

Microorganisms may be injured by sublethal environmental stresses such as heat, freezing, ionizing or non-ionizing radiation (Kang & Siragusa 1999; Aktas & Özilgen 1992). The injured bacteria may escape detection by common food microbiology techniques as used by the food industry and regulatory agencies (Vasavada 1986). Injured or stressed microorganisms are characterized by their inability to form colonies and multiply in a medium that contains a

selective agent, which has no inhibitory effect on unstressed cells (Kang & Siragusa 1999). The differential in counts between selective and nonselective media is a means to determine the sub-lethally injured population (Kang & Siragusa 1999).

The occurrence of injured or stressed organisms in thermally processed food is a matter of concern in the food industry (Vasavada 1986). Following heat treatment, sub-lethally injured food-borne pathogens could be assumed to be dead while they are alive and potentially as dangerous as their uninjured counterparts (Kang & Siragusa 1999). The stressed organisms can undergo repair and produce toxins thus cause public health hazard (Vasavada 1986).

Although the existence and the extent of injury in bacteria resulting from microwave irradiation have been stressed by some researchers, very little information is available on injured organisms with respect to microwave application (Vasavada 1986). Khalil & Villota (1988) studied effects of microwave radiation at 50°C for 6 hours on destruction and injury of *S. aureus* in phosphate buffer compared to conventional heat treatment and reported greater injury in microwave-treated cells. They also observed that the stationary lag phase, which often indicates a repair and adaptation period, was approximately twice as long for the microwave-injured cells compared to conventionally injured cells (Khalil & Villota 1988). *S. aureus* injured by microwave treatment often displays minimal metabolic capacity and an inhibition of enterotoxin synthesis (Khalil & Villota 1988; Bluhm & Ordal 1969). Aktas & Özilgen (1992) studied injury and death of *E. coli* by microwaves in a tubular pasteurization flow reactor and reported that generally 15 to 25% of the surviving microorganisms were injured, but this ratio increased drastically near total sterilization conditions. Shin & Pyun (1997) exposed suspensions of *Lactobacillus plantarum* cells to conventional heating, continuous microwave or pulsed microwave irradiation at 50°C for 30 minutes. They reported a higher injury in cells treated with

pulsed microwave irradiation followed by continuous microwave and conventional heating. They also observed that lactic acid production in injured cells was restored during recovery and acid production at a detectable level for conventionally and both microwave treatment was started after 10 and 20 hours respectively.

2.5 Biological effects of microwaves

Although the concern about the biological effects of non-ionizing radiation on humans and other eukaryotes began years ago, very little information about the effect of microwaves at frequencies of 2450 and 915 MHz is available. Most of the studies have focused either on very low electromagnetic frequencies (de Pomerai et al. 2000; Mittenzwey et al. 1996) or very high frequency range (Banik et al. 2003; Pakhomov et al. 1998; Lambert 1980). Thus these results can not be applied to the whole electromagnetic spectrum.

Evidence indicates that alternating electromagnetic fields interfere with the functioning of DNA and RNA, stimulate the activity in certain biochemical systems linked with cancer growth, affect molecules that are essential for the functioning of the nervous system and may disturb the normal function of the cell membrane (Mertens & Knorr 1992). The human body begins to significantly absorb electromagnetic radiation when the frequency exceeds about 15 MHz and the absorption varies for different body parts (Banik et al. 2003).

A number of studies indicated that microwaves could affect the fine chromosome structure and function of cells, cell tolerance to standard mutagens, and lesion repairs (Banik et al. 2003). In the 1960s and 70s researchers showed that protein, RNA and DNA could absorb microwaves at the frequency of 65-75 GHz, and that microwaves were able to interfere with

repair mechanisms or even to induce gene mutation in bacteria (Banik et al. 2003; Pakhomov et al. 1998).

Lambert (1980) comprehensively reviewed the studies on the biological effects of microwaves for the period of 1940-1980 (Knutson et al. 1987). In his review, he stated that the result of various studies, on blood cells, macromolecules, organs and organ systems, bone marrow, cell membrane, testes and blood forming systems, showed that microwave radiation at higher frequencies and higher power densities, caused biological responses that were adverse to living organisms. For example at frequency of 10000 MHz, skin heats with sensation of warmth. Lens of the eye and testicles are susceptible at frequencies between 3300 to 10000 MHz. Frequencies of 150 to 1200 MHz could damage the internal organs by overheating. The body is transparent for frequencies less than 150 MHz, which have a wavelength over 200cm (Lambert 1980). Smialowicz and co-workers (1980) showed that exposure of male albino rats, injected with bacterial endotoxin, to continuous-wave microwave radiation (2450 MHz) was associated with significant elevation of body temperature directly related to the power density (10 mW/cm^2> 5mW/cm^2> 1 mW/cm^2).

Some researchers studied the effects of microwave exposure over time on people who are in daily contact with microwave radiation such as welders, television and radio transmitter technicians, particle accelerator workers and steel factory workers engaged in tempering steel. They reported a predominance of fatigue in some of the exposed groups as well as a reduction in alertness (Baranski & Czerski 1976). But to the knowledge of the author there have been no confirmed cases of people being seriously injured from exposure to microwaves.

Yao (1978) reported that the exposure of corneal epithelium of Chinese hamsters to microwaves (2450 MHz, 100 mW/cm^2) produced an abnormal configuration in the animal's

chromosomes. Liburdy and his group (1985) found that exposure of rabbit erythrocytes to microwaves (2450 MHz, 100 mW/g) increased sodium passive transport only at membrane phase transition. In animals and humans, local microwave exposure stimulated tissue repair and regeneration, alleviated stress reactions and facilitated recovery in a wide range of diseases such as gastric, duodenal ulcers, tuberculosis, cardiovascular and skin diseases (Banik et al. 2003; Pakhomov et al. 1998). Ortner and colleagues (1983) reported that continuous exposure to 2450 MHz microwave radiation had no effect on microtubular polymerization or depolymerization, or on the secondary structure of purified tubulin *in vitro*.

Galvin and colleagues (1984) exposed the whole body of pregnant mice to 2450 MHz microwave radiation at a power density of 30 mW/cm^2 for two, four hour periods per day in total for 6 or 15 days. They found no effect on peripheral blood morphology (no change in lymphocytes, neutrophils or monocytes number). Trošić and co-workers (1999) exposed male Wistar rats (13 week old) to 2450 MHz microwave at 5-15 mW/cm^2, 2 hours per day, maximum 5 days a week for the period of 1,8,16 and 30 days. The result of peripheral blood cell response showed a decreasing tendency in total leukocyte count as well as lymphocyte percentage in the treated rats. They also reported an increase in the percentage of granulocytes while the absolute erythrocyte count was increased over the first eight days, and kept falling afterwards, yet still remained within the physiological range.

2.6 *Escherichia coli*

E. coli is the abbreviated name for the bacterium *Escherichia* (Genus) *coli* (Species) (Adams & Moss 2000) a member of *Enterobacteriaceae* family. The name *Escherichia* comes from the name of Theodor Escherich, who in 1885 isolated and characterized this bacterium for

the first time. This enteric bacterium is gram negative, non-spore forming, rod shaped and facultative anaerobe, which is an almost universal inhabitant of the lower intestinal tract of humans, warm blooded animals, and birds (Adams & Moss 2000; Neidhardt 1987). *E. coli* is a typical mesophile and its optimum growth temperature is around 37-39°C, with the maximum limit of 48-50°C and the minimum border of 7-10°C (Madigan et al. 2003; Adams & Moss 2000). A near-neutral pH is optimal for their growth but they also can grow at pH level as low as 4.4 (Adams & Moss 2000). This bacterium can grow in media with glucose as a sole source of energy, and carbon and ammonium salt as sole source of nitrogen (Magasanik 2000) and metabolically can transform glucose into all the necessary macromolecular components (Madigan et al. 2003). *E. coli* is a catalase-positive, oxidase negative, fermentative bacterium (Adams & Moss 2000).

Physiologically, *E. coli* is flexible and can adapt to the characteristic of its environment (Bell & Kyriakides 1998). It inhabits in the lower gut of animals and survives when released to the natural environment, allowing widespread distribution to new hosts (Bell & Kyriakides 1998; Blattner et al. 1997). It is well known that pathogenic *E. coli* strains are responsible for infections of the enteric, urinary, pulmonary, and nervous systems (Madigan et al. 2003; Blattner et al. 1997). Initially *E. coli* were used as indicators of direct or indirect fecal contamination and possible presence of enteric pathogens in food. Their presence in heat-processed food causes great concern and is a sign of incomplete processing or post-process contamination.

E. coli can respond to environmental signals such as chemicals, pH, osmolarity (Ramaswamy et al. 2003), heat (Ramaswamy et al. 2003; Arsène et al. 2000; Delaney 1989), acetate and propionate (Polen et al. 2002), peroxides and superoxides (Lindquist 1992), ethanol, ultraviolet light, nalidixic acid, coumermycin (Delaney 1989: Neidhardt & VanBogelen 1987),

and hydrogen peroxide (Zheng et al. 2001). The mechanisms of *Escherichia coli* stress response is well studied (Nakasono & Saiki 2000).

In addition, *Escherichia coli* has been used as a model system in many studies. Nakasono & Saiki (2000) used this bacterium to determine whether extremely low frequency magnetic fields (5-100 Hz) can be considered as a general stress factor. *E. coli* containing the plasmid pUC8 also has been used as model to detect athermal effects of non-ionizing electromagnetic radiation through assessment of β-galactosidase activity (Saffer & Profenno 1989).

Because of its unique position as a preferred model in biochemical genetics, molecular biology, and biotechnology, *E. coli* K-12 was the earliest organism to be suggested as a candidate for whole genome sequencing (Blattner et al. 1997). Today *Escherichia coli* is probably one of the best understood living organisms in terms of genome map and physiology (Nakasono & Saiki 2000; Adams & Moss 2000).

2.7 Stress response and stress proteins

Living organisms respond at the cellular level to stressful conditions by a rapid and temporary acceleration in the expression rate of stress genes (Morimoto et al. 1990). It is well established that a general stress response is universal among prokaryotes and eukaryotes (Champomier-Verges et al. 2002; Nakasono & Saiki 2000; Goodman et al. 1994; Delaney 1989). Overall, the stress response represents a general mechanism for coping with increased protein damage while cells or organisms are under stressful conditions. Protein damage appears to be the common signal that elicits the activation of most stress-inducible genes (Daniells et al. 1998). In addition other stress conditions such as oxidative and acid stress can affect the gene

expression (Teixeira-Gomes et al. 2000). The product of these genes is commonly referred to as stress proteins or heat shock proteins (Morimoto et al. 1990). In the literature, the abbreviation "hsp" is used for the whole stress protein family.

Stress proteins are induced by a large variety of stress conditions such as heat (Zhang & Griffiths 2003), cold (Gualerzi et al. 2003), toxic chemicals, reactive oxygen species (Shallom et al. 2002), ethanol, anoxia, electron transport inhibitors, amino acid analogs, virus infections (Weigl et al. 1999), arsenite and cadmium, starvation (Lindquist 1992), complex metabolic processes (Arsène et al. 2000) and low frequency magnetic field (Mittenzwey et al. 1996). Their induction is often accompanied by tolerance to these stresses (Lindquist 1992).

Stress proteins can be clustered in two main groups: general stress proteins and specific stress proteins. General stress proteins are the most studied in all kinds of stress and probably all kinds of bacteria. They are induced non-specifically by several stimuli and are involved in DNA or protein repair including chaperons DnaK, GroEL, GroES, or proteases such as Clp proteases. The specific stress proteins are induced as a result of a given specific stress such as cold shock or acid shock. In addition there is another group that some researchers consider as stress proteins. Proteins in this group normally belong to general metabolism but they can be affected by some specific stresses, for example, the proteins of the glycolytic pathway (Champomier-Verges et al. 2002).

2.7.1 Function of stress proteins

The primary function of stress proteins is to protect cells or organisms from environmental conditions, allowing them to recover and continue their normal metabolic processes (Morimoto et al. 1990; Delaney 1989). Some are required for growth at high

temperatures while others are required for long-term survival at temperatures just beyond the normal growth range, and yet others are specialized to provide protection against extremes (Lindquist 1992). Several hsps are also present at normal conditions and play vital roles in cell growth as well as in stress tolerance (Lindquist 1992; Morimoto et al. 1990).

Hsps are generally directly or indirectly involved in protein degradation (Heitzer et al. 1992). They promote the folding and unfolding of other proteins, the assembly and disassembly of proteins in oligomeric structures and the degradation of proteins that are improperly assembled or denatured (Lindquist 1992). Hsps, as molecular chaperones, help other proteins to assemble with their proper partners (Weigl et al. 1999; Lindquist 1992). They also bind to unfolded polypeptides during their movement in the cell, enabling the transport of these polypeptides through membranes or their integration into cell organelles (Weigl et al. 1999).

Hsp involvement in synthesis of various macromolecule such as bacteriophage development, chromosomal and plasmid DNA replication, RNA synthesis and protein synthesis have been reported (Heitzer et al. 1992). Their function in the immune response of organisms have also been studied (Weigl et al. 1999).

2.7.2 Heat shock response in *Escherichia coli*

The cellular response of an organism to heat shock was first described when a brief pulse of heat induced puffs in specific locations on the polytene chromosomes in the salivary glands of *Drosophila buskii* (Delaney 1989). Since then heat shock response has been studied in a wide range of organisms: for example, *Mycobacterium tuberculosis* (Stewart et al. 2002), *Brucella melitensis* (Teixeira-Gomes et al. 2000), *Haloferax volcanii* (Kuo et al. 1997), Archaea (de Macario & Macario 1994), maize seedlings (Greyson et al. 1996), soybean seedlings (Krishnan

& Pueppke 1987), mammalian cells (Landry et al. 1982), and Chinese hamster fibroblasts (Laszlo 1988). In *E. coli*, temperature increase from 30 to 42°C causes a rapid increase up to 15-fold induction of more than 20 heat shock proteins, followed by an adaptation period where the rate of hsp synthesis decreases to reach a new steady-state level (Arsène et al. 2000).

The conditions that induce expression of heat shock proteins in *E. coli* and the effect of heat shock protein on *E. coli* resistibility to stress factors has been studied extensively. Yamamori & Yura (1980) stated that up-shift of temperature by 3°C above 34°C in batch cultures of log phase *E. coli* result in induction of heat shock proteins. Seyer and co-workers (2003) studied the production of DnaK in exponentially growing *E.* coli (ATCC 25922) culture immersed in a shaking water bath at 50 or 55°C for 3 and 5 minutes. The heated *E. coli* were cooled to 37°C in an ice-water bath. They observed higher DnaK in heat-treated cells at 50 and 55°C compared to cells grown at 37°C. Heitzer and colleagues (1992) heated *E. coli* cultures grown at 37°C to 42°C for 2 or over 60 minutes and reported that *htpG* was induced faster in the 2 minute treatment. They also observed a strong correlation between temperature increase and expression pattern of *htpG* gene.

Pagan & Mackey (2000) studied the effect of heat shock on the resistance of *E. coli* H1071 to pressure. *E. coli* cells were harvested at 4°C and pellets were re-suspended in a 45°C pre-heated phosphate buffered saline (pH 7.0) for 45 minutes. Pagan & Mackey (2000) observed an increase in *E. coli* resistant to pressures between 200 and 400 MPa due to induction of heat shock proteins. Yamamori & Yura (1982) reported that *E. coli* cells grown at 30°C, then shifted directly to a lethal temperature (50°C) were rapidly killed. However, if the cells were first pre-heated by growth at 42°C for 30 minutes, the rate of killing upon shift to lethal temperature was dramatically decreased. Chow & Tung (1998) showed that recovery rate of early log phase *E.*

coli after 24 hours frozen storage at –80°C was ten times higher in cells that were exposed to heat shock of 42°C for 30 min, prior to freeze treatment compared to unheated control cells. They also added that this higher recovery was related to the accumulation of heat shock proteins induced before frozen storage.

2.7.2.1 Regulation of heat shock response in *E. coli*

The regulation of heat shock response for different organisms and even different cell types within an organism varies. For instance, heat shock response in yeast is controlled at the transcriptional level while in *Drosophila* both transcriptional and translational regulations are involved (Delaney 1989).

The *E. coli* heat shock response is positively controlled at the transcriptional level by the product of the *rpoH* gene. At first, the transcription of *rpoH* gene is increased through four different promoters. Three promoters are σ^{70}-dependent and one promoter requires the σ^{E} factor (Arsène et al. 2000). Each of these promoters is responsible for expression of some genes at various metabolic and environmental conditions (Kallipolitis & Valentin-Hansen 1998).

The second step is an increase in translation of *rpoH* mRNA and results in an increase in σ^{32} level (Arsène et al. 2000). *E. coli* strains lacking the heat shock transcription factor (σ^{32}) cannot grow above 20°C and they are unable to induce hsps at higher temperatures (Lindquist 1992). The σ^{32} along with RNA-polymerase induce the expression of heat shock genes and heat

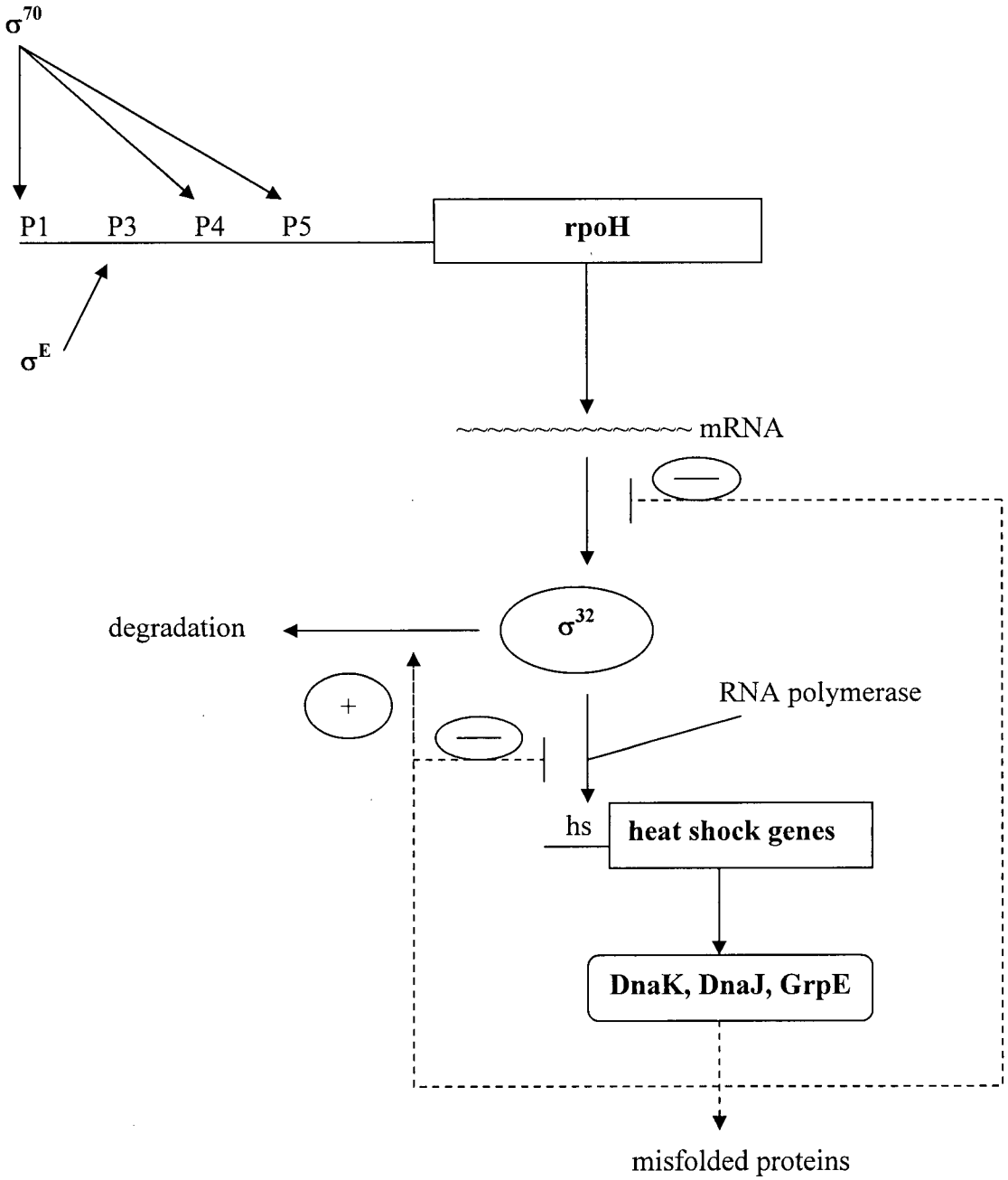

Figure 2.2. The *E. coli* heat shock regulon (Arsène et al. 2000).

shock proteins. The heat shock proteins start the task of refolding or degrading the misfolded proteins. This induces a signal to shut off the heat shock response. During the shut off phase, the DnaK, DnaJ and GrpE heat shock proteins act as negative modulators by repressing the translation of *rpoH* mRNA, causing efficient degradation of σ^{32}, and repression of σ^{32} activity (Arsène et al. 2000) (Figure 2.2).

2.7.3 Microwaves and stress response

Extremely low frequency electromagnetic fields at frequencies of 50 and 60 Hz have been shown to induce a classic stress response in cells, enhanced induction of stress proteins and altering cellular metabolism in a number of models including cell cultures, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Escherichia coli* (Shallom et al. 2002; Daniells et al. 1998). Goodman and co-workers (1994) exposed human HL-60 cells to 60 Hz electromagnetic field for 20 minutes and reported that the transcript level of hsp70 was increased. Nakasono & Saiki (2000) also reported that yeast and HL60 human leukemia cells responded to magnetic fields by the synthesis of hsp 70 or the transcription factor σ^{32}, which is similar to the general environmental stress response. de Pomerai and co-workers (2000) exposed soil nematode *Caenorhabditis elegans* to microwave radiation at 750 MHz, 0.5 W for 18 hours and reported the induction of hsps.

Shallom and co-workers (2002) investigated the effect of microwaves (915 MHz, 3.5 or 5 W) on chick embryo and showed that microwave exposure increased the induction of hsp 70 by 20 to 60% while the temperature rise was not enough for activation of heat shock pathway. They also reported that after 30 minutes, chicks exposed to microwaves had significantly higher survival rates than controls.

Induction of stress protein in *E. coli* cells after exposure to electromagnetic fields (50 Hz) was reported by Chow & Tung (2000). Nakasono & Saiki (2000) found no reproducible change in the level of protein synthesis after exposing *E. coli* to frequencies 5-100 Hz. Cleary and colleagues (1997) also saw no significant induction in hsps levels following exposure of HeLa cells to microwaves (2450 MHz, 25 W/kg, 2 hours) compared to mild heat stress (40°C, 2 hours).

2.8 DNA microarray technology

In the past, to define stress-related, global regulatory responses researchers have often relied upon either separation of protein fractions from stressed cultures or the use of transgenic test organisms carrying a stress gene whose product could be easily detected (such as beta-galactosidase from the *Escherichia coli* lac Z gene) (Daniells et al. 1998). However, traditional methods usually work with one gene in one experiment, which is time consuming and unable to show an overview of the organism's response.

In the last several years, a new technology, called DNA microarray has become available and received a great deal of attention (Schulze & Downward 2001). This technology has increased the speed of the investigation of gene regulation and has provided a system for the simultaneous measurement of the expression level of thousands of genes in a single hybridization assay with the possibility for further understanding of the total organism response to a specific condition.

The most commonly used microarray systems, classified according to the arrayed material are: complementary DNA (cDNA) and oligonucleotide microarrays. Probes for cDNA arrays are usually products of the polymerase chain reaction generated from cDNA libraries or

clone collections. In oligonucleotide arrays, short 20-25mers are synthesized *in situ* (Schulze & Downward 2001) or by conventional synthesis.

In general, a microarray consists of a series of DNAs target sequences (primarily PCR products or oligonucleotides) spotted on to a carrier (glass slide, nylon filter, silica "chip", or membrane), in an orderly manner. Subsequently, labeled RNA or cDNA probes synthesized from mRNA isolated from a sample are hybridized on to the array (Snijders et al. 2000). Hybridization intensities for each DNA sequence are determined using an automated process and converted to a quantitative read-out of relative gene expression levels (Harrington et al. 2000), which provides a measure of the expression of thousands of genes in a single experiment (Snijders et al. 2000). The data can be further analyzed to identify expression patterns and variation correlated with cellular development, physiology and function (Harrington et al. 2000).

2.8.1 DNA microarray applications

One of the most significant applications of this technique was the gene expression profiling of the whole genome. Genomic-wide expression levels of *Saccharomyces cervisiae* (Wodicka et al. 1997) and *Escherichia coli* (Richmond et al. 1999) have been monitored with microarray technology (Oh & Liao 2000). An attempt to monitor the genomic-wide expression of *Caenorhabditis elegans* (Kim et al. 2001) has been reported.

Gene expression patterns can be used to assign functions to unknown genes, improve understanding of cellular function, identify potential drug targets, generate genome-wide snapshots of transcriptional activity in response to any stimulus (Harrington et al. 2000), resolve the changes in gene expression that accompany adjustments to cellular physiology, identify genes differentially expressed in response to changes in environmental parameters, define

developmental programs or to evaluate mutations in regulatory and metabolic pathways (Conway & Schoolnik 2003). In addition, gene expression patterns of tumors can often tell the oncologist in advance whether a patient will respond to certain chemotherapeutical or hormonal agents (Snijders et al. 2000). At the same time for many researchers, the ultimate goal is to investigate the transcriptome of bacteria growing within infected tissues thus to study the host-adapted transcriptional responses (Conway & Schoolnik 2003).

2.8.2 DNA microarray limitations

Like any other technique, some limitations are associated with DNA microarray technology including, the high costs, the need for specialized technical expertise, the need for collaboration between different disciplines, difficulty in coping with very large amounts of data, and uncertainty about the biological meaning and clinical relevance of the results (Snijders et al. 2000). In addition this technique shows the expression of genes in transcription, which does not necessarily matche the translational changes. The expression of a specific gene is not an indicator for the presence of related protein or enzyme. Thus any regulatory change at post-transcriptional level can not be detected. Nevertheless, arrays have proved to be quite successful in describing trends in gene expression patterns that reflect operon, regulon and stimulon organization (Conway & Schoolnik 2003).

CHAPTER THREE

PRELIMINARY STUDY:

EFFECT OF VACUUM MICROWAVE DRYING ON NATURALLY OCCURING MICROORGANISMS OF PARSLEY

3.1 Introduction

Parsley, a member of the Umbelliferae family, has been cultivated since the days of the Romans (Sobiech 1980). The fresh leaves are employed for garnishing, seasoning and flavoring. They also were used as medicine by the ancient Greeks and Romans (Small 1997). Fresh parsley is a good source of vitamin C (133 mg/100g fresh), β-carotene (5054 μg/100g fresh) and vitamin K (phylloquinone 1640 μg/100g fresh) (USDA database). It is the most popular of all garnishing herbs in the West and many other parts of the world (Small 1997).

Microbial contamination of vegetables and herbs may occur in the field through irrigation, harvesting and handling. Generally, harvested fresh parsley is chilled with cold water and carried on ice to the market. Fresh parsley can be stored for a month at 0°C and relative humidity of 90-95% (Small 1997). Due to high water content and high number of naturally occurring bacteria and fungi, parsley was the source of several food poisoning outbreaks in 1998 (Crowe et al. 1999).

Drying, as the most commonly applied method of increasing shelf life, inhibits the growth of microorganisms and delays the onset of some biochemical reactions (Böhm et al. 2002). The common method for drying parsley is hot air blast drying. Hot air causes heat and oxidative damage to the plant tissue and changes the physical and chemical characteristics of the product (Böhm et al. 2002). In the market, quality of dried herbs mainly depends on their colour, aroma, and absence of off-flavour defects (Böhm et al. 2002).

The food industry is always searching for improvements in dehydration process to preserve product quality while using less heat, mechanical shear, and additives. The use of vacuum along with microwave has proved a good combination in production of high quality materials. While microwaves provide the fastest means available of transferring energy into the

interior of foods (Durance & Wang 2002), the reduced pressure keeps the product temperatures low, as long as a certain amount of free water is present. Thus temperature sensitive substances like vitamins, colours and flavours will be retained (Regier & Schubert 2001; Decareau 1985).

Vacuum microwave drying has been employed to dehydrate a wide range of products including banana slices (Mousa & Farid 2002, Mui et al. 2002), chilli (Kaensup et al. 2002), pectin gel (Drouzas et al. 1999), shrimp (Lin et al. 1999), sweet basil (Yousif et al. 1999), carrot slices (Lin et al. 1998), potato chips (Durance & Liu 1996) and cranberry (Yongsawatdiguul & Gunasekaran, 1996). Böhm and co-workers (2002) reported that the retained colour and odour of parsley dried under vacuum microwave (642 W, pulsed microwave, 40 mbar vacuum) was better than with the air drying method (75°C, 50-70 min). Sobiech (1980) also reported that microwave vacuum drying (2450 MHz, 2.0 kW) enhanced the flavour of the dried sliced parsley root and retained the properties of fresh raw material.

Although destruction of microorganisms under microwave radiation has been studied for years (Dreyfuss & Chipley 1980; Welt et al. 1994; Kozempel et al. 2000), very little information on the effect of microwave drying or vacuum microwave on microbial reduction can be found. Daglioglu and colleagues (2002) dried tarhana dough (fermented product of yogurt-cereal mixture) inoculated with *Staphylococcus aureus* (10^4 CFU/g) with a hot air oven at 55 +/-2°C for 36 hours or in microwave oven (1500 W, 2450 MHz, 30% power level) for 10 minutes. They reported that *S. aureus* was completely eliminated after microwave drying. They recommended microwave drying as a more efficient way to decrease the microbial population. Kim and colleagues (1997) dried concentrated yogurt in a laboratory scale microwave vacuum dryer (10 mmHg, 250 W, 2450 MHz) at 35°C and reported a great survival of lactic acid bacteria (*S. thermophilus* and *L. bulgaricus*).

The main objective of this study was to gain knowledge of the lethal effect of microwave radiation on the naturally occurring microorganisms of parsley during dehydration under vacuum as part of a long-term objective to investigate the response of microorganisms to vacuum microwave. In addition we wished to determine the impact of the drying method on microbial reduction.

3.2 Materials & Methods

3.2.1 Plant source

Parsley, *Petroselinum crispum*, was purchased from a local herb cultivator in Vancouver, BC, then washed with tap water and stems were removed. Only the leaves of the freshly harvested plant were used for all experiments

3.2.2. Drying

Parsley leaves were dried with two different methods.

3.2.2.1 Air drying (AD)

Five hundred g fresh parsley leaves were air-dried in duplicate using a commercial air-drier (Vers-A-Belt, Wal-Dor Industries Ltd. New Hamburg, Ontario, Canada) for 35, 60, 70 and 105 minutes. Air flow rate was 0.9 m^3/s with initial relative humidity of 10%. The dryer temperature was set at 60-73°C and samples were placed on conveyor belt exposed to hot air.

3.2.2.2 Vacuum microwave drying (VMD)

Three hundred to five hundred grams of fresh parsley leaves from the same batch were placed in a perforated cylindrical high density polyethylene drying basket (0.26 m radius and 0.23 m length) (Figure 3.2-d). The basket was rotated on its horizontal axis in a vacuum microwave drier (2450 MHz, 4KW, ENWAVE Corp, Vancouver, BC, Canada) at a rate of 3 rotations per minute. Samples were dried in duplicate at 1.5 KW power, 26-28 in Hg vacuum for 9, 17, 19 and 20 minutes (Figure 3.1, 3.2). The final temperature of samples was measured using an infrared thermometer (Model 39650-04, Cole-Parmer Instruments, Co. USA).

All the dried samples were stored in polyethylene vegetable bags (Reggie Veggie, Richmond, BC), heat sealed and stored at 4°C for 48 hours before further analysis.

3.2.3 Temperature measurement of parsley during vacuum microwave drying

Three hundred to five hundred grams fresh parsley at room temperature (20-25 °C) were placed in the VM drier under the same drying condition (1.5 kW power, 26-28 in Hg vacuum, 3 rpm, 17-20 min). The drying process was stopped at timed intervals and the temperature of the sample was measured using the infrared thermometer immediately after opening the drier door. Temperature measurements were performed in triplicate.

3.2.4 Determination of moisture content

To determine the moisture content, duplicate samples (2-4 g) were placed in aluminum dishes and dried in an air-drying oven (Blue M, Blue M-electric Company, Illinois, USA) at 103°C for 4-6 hours (AOAC method 6.004) or until constant weight.

3.2.5 Water activity measurement

Water activity of the samples was measured in duplicate using a water activity meter (Rotronic-Hygroskop DT, Rotronic Instrument corp. Huntington, NY). The instrument was calibrated before measurements using humidity standards (Rotronic, Rotronic Instrument Corp. Huntington, NY).

3.2.6 Microbiological Analysis

3.2.6.1 Microbiological sampling

Twenty five grams of dried sample or 50g fresh parsley leaves were transferred aseptically into a stomacher bag, then 225 ml or 450 ml sterile peptone water (0.1% w/v) was added to dried and fresh samples respectively. Samples were soaked 15 minutes at room temperature before homogenizing in stomacher unit (Seward - Stomacher 400- Lab System Seward Stomacher England) for 4 minutes at medium speed. Serial dilutions of 10^{-1} to 10^{-4} were prepared.

3.2.6.2 Total microbial count

One ml aliquot from each dilution was pour plated in duplicate using Plate Count Agar (Difco) for total microbial count and incubated at 35 ± 1°C for 48 hours.

3.2.6.3 Yeast & mould counts

A 1 ml aliquot of each dilution was pour-plated in Potato Dextrose Agar (Difco) + 12 ml/L sterile tartaric acid (1:10 w/v), final pH of 3.5 (Beuchat & Nail 1985). Plates were completely wrapped in aluminum foil and incubated at room temperature 20–25°C for 5 days.

Plates with 25-250 colonies were selected for calculation. The average number of colonies per plate was multiplied by the corresponding dilution factor. Final values for dry and fresh samples were reported as colony forming units (CFU) per gram of sample (dry weight) and calculated as following:

$$w_d = \frac{100-m}{100} \qquad \text{Eq (3.1)}$$

where w_d= percent dry matter

m= moisture content (wb)

$$\frac{\text{CFU/g sample}}{w_d} = \text{CFU/ g sample (dry weight)} \qquad \text{Eq (3.2)}$$

where:

CFU/g sample= colony forming unit per gram sample (dry weight)

3.2.7 Statistical Analysis

Analysis of Variance: Estimate Model (SYSTAT 8.0, 1998) was used to determine the significant among treatments. LSD (SYSTAT 8.0, 1998) was used to compare the treatment means ($p< 0.05$).

3.3 Results

The initial moisture content of the fresh parsley leaves was 83.9 +/- 0.9 % on a wet basis. The average initial microbial population of fresh parsley was 9.3×10^6 and 1.8×10^5 CFU per gram dry sample for total microbial counts, and yeast and mould counts, respectively.

Unfortunately, due to basket rotation which would damage the fiber optic probe, it was not possible to measure the temperature of the product during the VMD process. Thus the final

temperature of product was considered as a temperature indicator instead of the actual temperature of the sample. The results of batch temperature measurements of parsley during VMD showed good reproducibility and the average coefficient of variation among replicates was 4.17% (Figure 3.3).

To check the effect of drying level on microbial population, samples were divided into three groups according to their post-drying water activity values: i) equal or greater than 0.9 ii) from 0.5 to 0.7, and iii) from 0.2 to 0.5 (Table 3.1). Total microbial counts, for air dried samples with water activity 0.964 were 3.2×10^5 CFU/g dry sample while for VM dried parsley leaves with water activity 0.952 were 4.9×10^4 CFU/g dry sample. Although VM dried samples showed one log more reduction in microbial population compared to AD, no significant difference in microbial population between drying method and fresh sample was observed. This could be explained by variation among replicates. In the second group, dried samples with water activity values from 0.5 to 0.7, a significant difference in both total aerobic and yeast and mould counts between VMD and AD samples was observed ($p<0.05$). For samples with water activity values between 0.2 to 0.5, there was a significant difference for total microbial counts and yeast and moulds between VMD and AD and fresh samples. There was no significant difference in total microbial counts and yeast and mould population of dried parsley between treatments in the 0.2 to 0.5 water activity range (Table 3.1).

3.4 Discussion

Actively growing microorganisms may contain more than 80% water. The process of dehydration removes water from the bacterial environment and cells, thus multiplication stops. Partial drying is less effective than total drying, although for some microorganisms, partial

drying as in concentration may be sufficient to arrest bacterial growth. Bacteria and yeasts generally require more moisture for growth than molds (Potter & Hotchkiss 1995). Other factors influencing microbial survival in dried samples are drying temperature, drying time and water activity of final product. Nutrient transportation into microbial cells is affected by the reduction in water activity. Cells can only adapt to environmental conditions within a limited individual range (Rödel 2001), beyond which they are no longer capable of reproduction. Almost all microbial activities are inhibited below a water activity of 0.6 (Fellows 2000). The minimum water activity for multiplication of bacteria is 0.75; for yeast and moulds it is 0.62 and 0.61 respectively (Rödel 2001). In the present study the total microbial count for AD samples was not significantly different from fresh parsley leaves for samples in a_w ranging from 0.2 to 0.5. One possible explanation for higher population on AD samples in water activity of 0.2-0.5 is that the batch of fresh sample used had a higher microbial population compared to the other two batches and it might have contained spores and microorganisms with higher heat resistance. Thus exposure to drying condition of 65°C was not enough for the reduction of total aerobic population. While in VMD the presence of vacuum along with higher final temperature 75°C may have increased the reduction in microbial population.

One possible explanation for higher microbial population in samples with $a_w \geq 0.9$ is that these samples were exposed to heat and/or microwave for a shorter period of time compared to drier samples, 9 minutes compared to 20 min for VM dried and 34-35 min compared to 105 min for AD dried samples. Therefore, although there was a reduction in the number of microorganisms compared to fresh parsley, it was not enough to show a significant difference.

The results of this experiment indicated that VMD was more effective than AD for reduction in total microbial population and yeast and mould population in the water activity

range of 0.5 –0.7. VMD could be an efficient method for yeast and mould reduction considering that fungal spoilage of foods occurs more often than bacterial spoilage at a_w 0.61-0.85 (Beuchat 1983). Hamid and colleagues (2001) exposed inoculated air to microwave 2450 MHz for a total of 35 min exposure time (with a 2.5 min on and 5 min off cycle). They detected no fungi after 10 min microwave radiation and recommended using microwave in the cheese packaging section of a dairy plant to eliminate fungi. Legnani and co-workers (2001), in a study on the effect of microwave treatment (100°C for 15 minutes) on black pepper, red chili, oregano, rosemary and sage, reported that microwave heating had little effect on spore forming bacteria but was effective on the moulds and bacteria that were indicators of fecal contamination.

VM dried parsley samples in the present study showed 1.04 to 3.04 log reduction in total microbial count and a 1.85 to 2.97 log reduction in yeast and mould counts while only 0.22 to 1.20 log reduction in total microbial count and a 0.23 to 1.3 log decrease in yeast and moulds population was detected for air dried samples. These results are in agreement with the data presented by Daglioglu and colleagues (2002) who reported total mesophile aerobic bacteria counts decreased approximately 2 log in conventionally dried tarhana dough (fermented product of yogurt-cereal mixture) in an air oven at 55 +/-2°C for 36 hours and 4 log in microwave dried samples in microwave oven (1500 W, 2450 MHz, 30% power level) for 10 minutes. They observed 3 log reduction after conventional drying and about 5 log reduction after microwave drying for yeast and moulds.

The batch measurement of the temperature of parsley throughout VMD showed that temperature gradually rose in the process and that parsley samples were not at the maximal final temperature for longer than 2 to 3 minutes compared to a total drying time of 17-20 minutes (Figure 3.3). Thus times at a specific temperature is shorter with VMD, 17-20 minutes

compared to 60-105 minutes in AD. In addition, the average temperature needed to reduce microbial population in VMD was less than in AD.

3.5 Conclusion

This study showed that parsley leaves treated with VMD had microbial populations less than AD samples at comparable water activity. VM dried parsley samples in the present study showed 1.04 to 3.04 log reduction in total microbial count and a 1.85 to 2.97 log reduction in yeast and mould counts while only 0.22 to 1.20 log reduction in total microbial count and a 0.23 to 1.3 log decrease in yeast and moulds population was detected for air dried samples. In addition VMD was more effective against yeast and mould than total aerobic population. Since higher reduction in microbial population of fresh parsley leaves occurred not only in a shorter time but also at a lower final temperature in VMD compared to AD, it can be concluded that VM drying was an effective method of reducing the number of naturally occurring microorganisms in parsley.

These data support the hypothesis of existence of lethal factor(s) other than heat associated with VM. However, there were limitations in the preliminary data. No attempt was made to identify the microorganisms in the microbial population. In addition batch temperature measurements during VMD were not as accurate as measuring temperature continuously over the drying process. Therefore to define differences between VM heating and conventional heating, more precise and meticulous measurements of time and temperature on specific microbial population were needed.

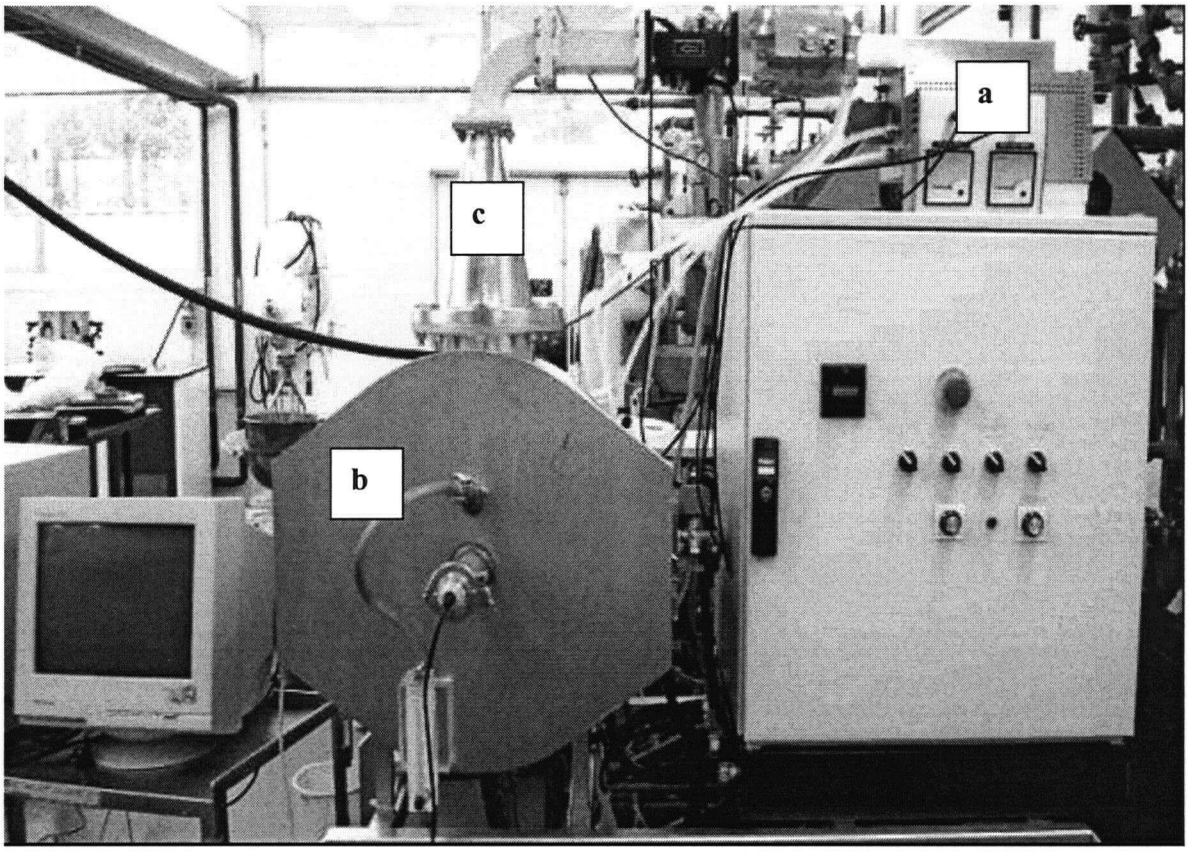

Figure 3.1. Vacuum microwave drier- door closed: a) microwave generator, b) vacuum chamber, c) waveguide.

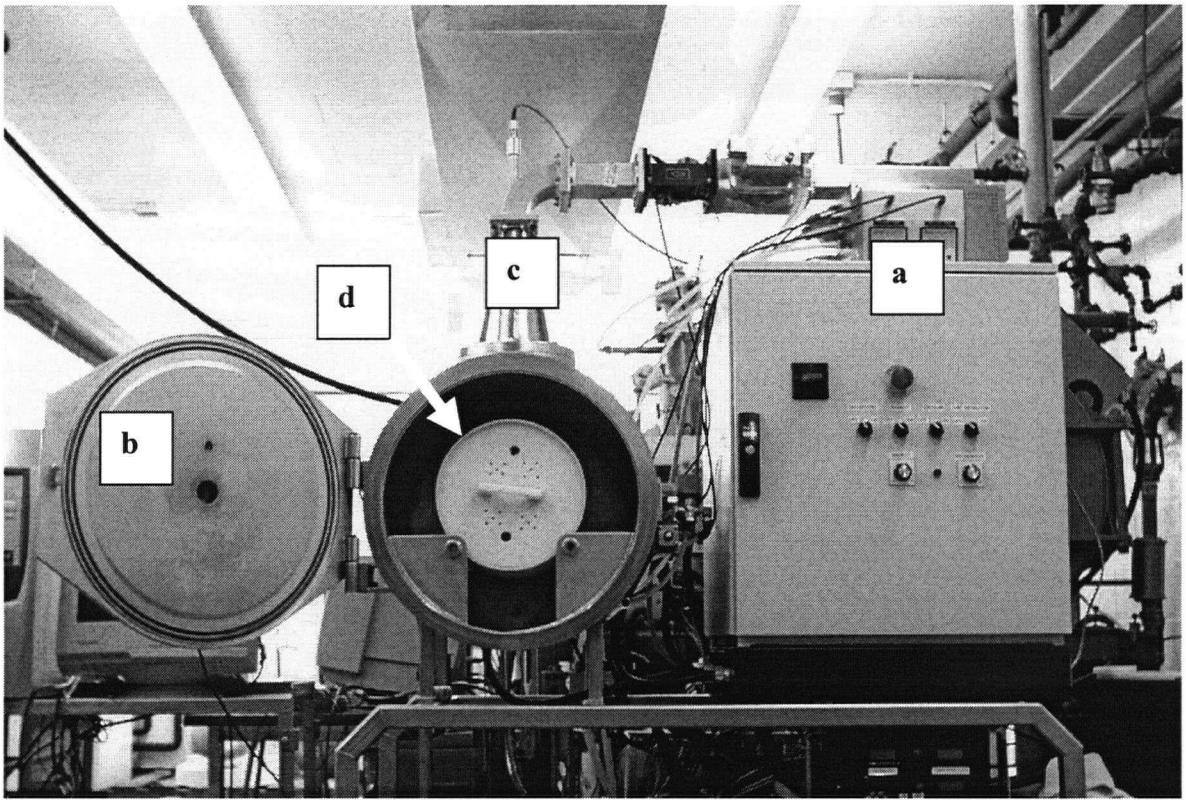

Figure 3.2. Vacuum microwave drier- door open: a) microwave generator, b) vacuum chamber door, c) waveguide, d) drying basket

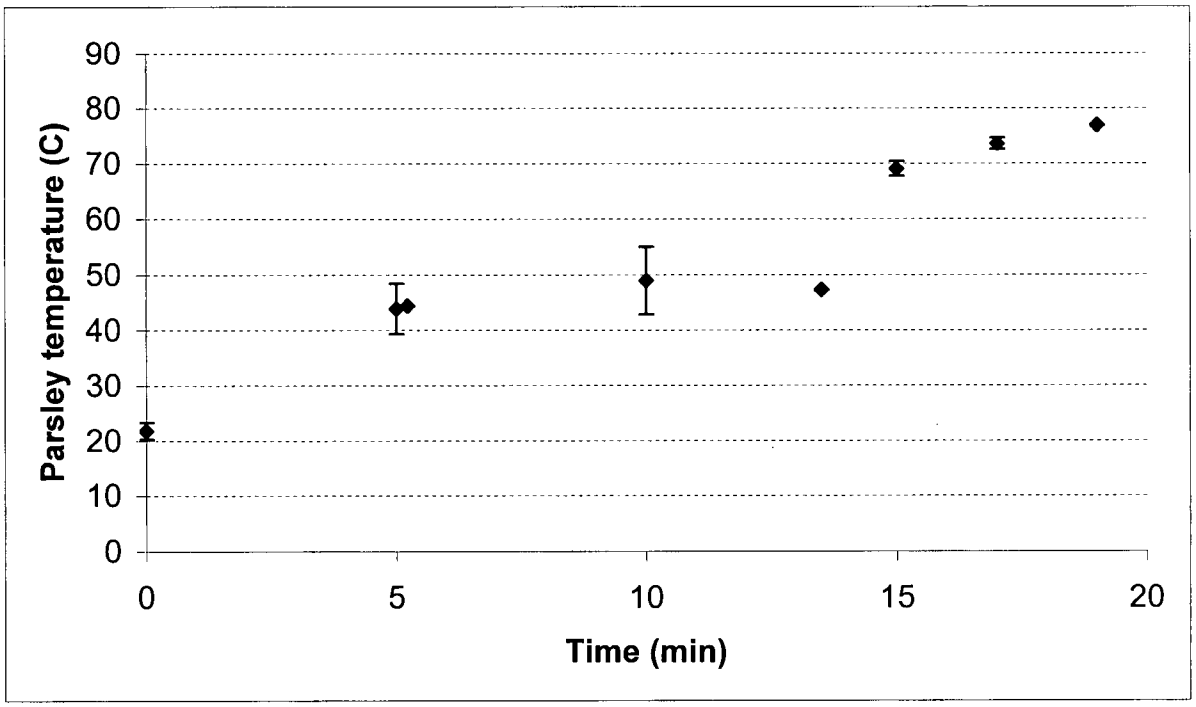

Figure 3.3. Time-temperature profile of fresh parsley leaves during vacuum microwave drying process: 2450 MHz, 1.5 kW, 26-28 in Hg vacuum with basket rotating at the speed of 3 rpm. Each value is average of 3 readings ± standard deviation.

Table 3.1. Total microbial and yeast & mould counts for fresh, air dried and vacuum microwave dried parsley.

	a_w	Total microbial count CFU/g dry weight*	log reduction	Yeast & Moulds count CFU/g dry weight*	log reduction	Final temperature of parsley
$a_w \geq 0.9$						
Fresh parsley	0.97 ± 0.02	$5.4 \times 10^{5\,a}$		$5.6 \times 10^{4\,a}$		----------
Air dried	0.964	$3.2 \times 10^{5\,a}$	0.22	$1.8 \times 10^{4\,a}$	0.49	73.0°C
VM dried	0.952	$4.9 \times 10^{4\,a}$	1.04	$7.9 \times 10^{2\,a}$	1.85	42.1°C
$a_w = 0.5 - 0.7$						
Fresh parsley	0.923	$3.7 \times 10^{6\,a}$		$2.2 \times 10^{5\,a}$		----------
Air dried	0.67 ± 0.01	$2.4 \times 10^{5\,b}$	1.18	$1.1 \times 10^{4\,b}$	1.30	65.0°C
VM dried	0.66 ± 0.03	$5.0 \times 10^{4\,c}$	1.86	$6.7 \times 10^{2\,c}$	2.51	(47.9 – 62.7)°C
$a_w = 0.2 - 0.5$						
Fresh parsley	0.929	$3.0 \times 10^{7\,a}$		$1.7 \times 10^{5\,a}$		----------
Air dried	0.496	$1.6 \times 10^{7\,a}$	0.27	$9.9 \times 10^{3\,b}$	0.23	(64.0 – 65.0)°C
VM dried	0.323 ± 0.13	$2.7 \times 10^{4\,b}$	3.04	$1.8 \times 10^{2\,b}$	2.97	(66.5 – 75.0)°C

* Each reported value is the average of two samples.

[a,b,c] Values within the same column for a given water activity which are not sharing the same superscript letter are significantly different ($p < 0.05$) from each other.

CHAPTER FOUR

EFFECT OF VACUUM MICROWAVE ON *ESCHERICHIA COLI*: A STUDY OF DEATH KINETIC PARAMETERS AND DIELECTRIC PROPERTIES

4.1 Introduction

Special attributes such as faster heating rate and greater penetration depth have made microwaves a unique tool for many industrial applications such as tempering, thawing, blanching, cooking, dehydration, sterilization, and pasteurization (Knutson et al. 1987; Rosenberg & Bögl 1987a, b). Attempts to use microwaves to destroy microorganisms had begun before the microwave oven was built (Fleming 1944). One of the earliest studies applied microwave energy to extend the shelf life of bread (Olsen 1965). That study was successful in reducing the number of viable spores of *Aspergillus niger*, *Penicillium sp.* and *Rhizopus nigricans* by exposure to microwave energy (5 kW, 2450 MHz) at a temperature lower than their thermal death point. Goldblith and Wang (1967) exposed *E. coli* cultures suspended in a phosphate buffer/ice mixture to 2450 MHz microwaves. They observed no change in microbial population in the bacterial suspensions after 100 s of microwave radiation with the final temperature of 51.5 °C, and concluded that inactivation of *E. coli* was due solely to the thermal effect. Kakita and colleagues (1999) showed that the complete sterilization of a piece of cloth, experimentally contaminated with bacteria, could be achieved quite rapidly by microwave irradiation before the cloth was dried to the water content of clothes usually worn (about 2.4 %). Papadopoulou and co-workers (1995) studied the bactericidal effect of microwaves on certain pathogenic enterobacteria and first reported the possibility of differences between thermal and electromagnetic lethal effects.

The mechanism of destruction of microorganisms by microwaves is controversial. Some have stated that inactivation of microorganisms by microwaves is entirely by heat, through the same mechanisms as other biophysical processes induced by heat, such as denaturation of proteins, nucleic acids, or other vital components, as well as disruption of membranes (Datta &

Davidson 2000; Heddleson & Doores 1994). Others have linked destruction to nonthermal effects, since a lower final temperature may be needed to kill microorganisms. Woo and colleagues (2000) studied the effect of microwave radiation on *E. coli* and *B. subtilis* and reported protein and DNA leakage, severe damage on the surface of cells and cell walls, and appearance of dark spots in bacterial cells, as a result of microwave treatment. They also indicated that most of the microwave- treated cells were "ghost cells" from which intracellular materials had been released into the cell suspension. Kakita and co-workers (1995) studied the effect of microwave radiation on the survival of bacteriophage PL-1 and observed that most of the particles turned out to be the ghost particles (with empty heads). They reported microwave radiation broke the DNA located deep in the phages core, whereas heating the phage particles from the outside did not.

Although there is a controversy about the mechanisms of microwave-induced death of microorganisms, there is no doubt about the destructive effect of microwaves. Microwave destruction of many bacteria (*Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Enterococcus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Salmonella sofia, Proteus mirabilis,* and *Pseudomonas aeruginosa*) has been reported (Chipley 1980; Datta & Davidson 2000; Heddleson & Doores 1994; Knutson et al. 1987; Papadopoulou et al. 1995; Rosenberg & Bögl 1987b).

Uneven temperature distribution is one of the biggest limitations of microwave applications. Non-uniform heat distribution in products can result in survival of unwanted microorganisms and incomplete pasteurization. Knutson et al (1988) attempted to simulate high-temperature, short time (HTST) and low temperature, long-time (LTLT) milk pasteurization processes using microwave heating, and found that the non-uniform heat distribution in the milk

resulted in recovery of viable *Salmonella typhimurium*, initially inoculated a population of 10^3-10^4 CFU/ml.

With respect to studies of microwave destruction of bacteria, four main experimental difficulties are evident in the literature: i) lack of convenient technology for on-line temperature measurement in a microwave field, ii) uneven heating due to inconsistent microwave field distributions, and the physical and electrical nature of the sample, iii) inability to control the temperature of microwave-heated samples at a specific level, iv) uncontrolled concentration of solutes due to evaporative losses from the sample during heating (Welt et al. 1994).

Since microwave absorption continuously generates heat, temperatures tend to rise throughout the microwave process. To keep the temperature consistent, either the microwave power needs to be turned on and off during the process or some cooling medium needs to be applied. In this section, vacuum was used to control the boiling point of water and thus maintain temperatures in microwave-treated bacterial suspensions at specific levels while the microwave power remained constant. In such a system, once the boiling point of water at a specific pressure is achieved, the temperature of the medium remains constant as long as the pressure remains unchanged.

Thermal destruction of organisms follows classical first order destruction kinetics of time versus absolute temperature, known as the Arrhenius relationship (Fellows 2000; Stumbo 1965). If microwave lethality is entirely thermal, the Arrhenius activation energies should be equivalent whether the heat is supplied by thermal conduction or generated by microwave. The aim of this study was to investigate the lethal effect of microwave irradiation under vacuum on pure cultures of *E. coli* compared to conventional heating in a water bath under vacuum and to search for possible non-thermal effects of the vacuum microwave treatment. In addition we wished to

determine if the level of microwave power had an impact on *E. coli* destruction independent of temperature.

4.2 Materials and methods

4.2.1 Bacterial Strain

Escherichia coli (ATCC 11775) isolated from human urine was purchased as a freeze dried sample from American Type Culture Collection, Rockville, USA.

4.2.2 Stock culture and inoculum preparation

Lyophilized *Escherichia coli* culture was transferred to 50 ml of Nutrient Broth (Difco) and incubated at 37°C. Some microbiological tests were initially carried out to ascertain the purity of the culture (Appendix I, Table 8.1). Static culture was maintained by daily transfer of incubated culture to fresh Nutrient Broth. After propagating the microorganism, 1 ml of 18-hour culture was transferred into a 1.5 ml flat top sterilized micro-centrifuge tube (Siliconized Flat Top Microtubes, Fisher Scientific, Pittsburgh, PA, USA) containing 1ml sterilized glycerol and mixed. All the micro-centrifuge tubes were kept at –80 °C as stock culture.

To prepare inocula, one micro-centrifuge tube was removed from –80°C every two weeks and kept at 4°C for two to three hours to completely thaw. Next it was transferred to room temperature (20-25 °C) for 2 hours to avoid any sudden temperature change. Finally the whole liquid was aseptically poured into 50 ml of room temperature Nutrient Broth and incubated at 37 °C for 24 hours. A sub-culture was then prepared by transferring 1ml of bacteria suspension into 50 ml Nutrient Broth after 16-18 hours. Each sample was sub-cultured three times before being used in an experiment.

4.2.3 Growth index

The turbidity of Nutrient Broth containing *E. coli* was measured at 580 nm with a spectrophotometer (UNICAM UV/VIS Spectrometer UV2 ATI UNICAM, Cambridge, UK) and simultaneously the same broth was spread plated in duplicate on Plate Count Agar (PCA) to count the population of microorganisms. A_{580} nm was taken as an index of the population of the microorganism in the liquid medium.

4.2.4 Sample preparation

Stationary phase *E. coli* (10^7 to 10^8 CFU/ml) were separated from Nutrient Broth by centrifuging at 1310×g for 4 minutes at 4°C using a Micro Centrifuge (Micro Centrifuge 5415C, Brinkmann Instruments, Inc. N.Y., USA). The supernatant was discarded, 1 ml room temperature 0.1 % peptone water (Bacto peptone, Difco) was mixed with the culture and it was centrifuged again at 1310 × g for 4 minutes at 4°C to wash the microorganisms and remove the nutrient broth residues. After discarding the supernatant, cells were suspended in 1 ml 0.1 % (w/v) peptone water for water bath treatment and vacuum microwave treatments respectively. The microorganisms and peptone water were mixed using a sterile syringe by passing them through the syringe several times just before the experiment.

4.2.5 Microwave power determination

Microwave power was determined using IMPI2-Liter test (Buffler 1993). Two 1-liter Pyrex beakers of distilled water (20 ± 2°C) were placed in the center of the microwave chamber and heated for 122 s, then stirred and temperature was measured immediately using mercury in

glass thermometer (Fisherbrand, Fisher Scientific co. USA). The power output was calculated by multiplying 70 by the average of temperature rise of two beakers. Measurement of power output was repeated three times for each power and the average and standard deviation were reported (Section 9, Appendix III).

4.2.6 Continuous flow vacuum system

Microwave and water bath treatments were conducted in a continuous-vacuum system (Figure 4.1). More detailed information about the continuous-vacuum system can ba found in Appendix II (Figure 9.1-9.5). A glass container (glass vacuum chamber) with three long side-arms was built and placed in the heating environment. For microwave treatments, three holes were made in the back of a microwave oven (General Electric- JE435, Mississauga, Canada), and a copper tube (OD = 45 mm. ID = 41 mm, 89mm long) placed in each hole. The glass vacuum chamber was placed in a microwave oven with the side arms projecting through apertures in the oven wall. Apertures were sized so as not to propagate microwaves and microwave leakage was monitored with a microwave radiation detector (HI-3520, Holaday Industries, Inc., Eden Prairie, MN, USA). The two lower side arms were connected to stainless steel tubing and a pump (pressure-loaded compact low-flow pump head without canister, Micropump, Inc. Vancouver, WA, USA) for liquid circulation at a flow rate of 410 - 645 ml/min. The upper side arm was connected to the water-ring vacuum pump (SIHI pumps Ltd. Guelph, Canada) to reduce pressure in the chamber (Figure 4.2, 4.3). The temperature was monitored with a data logger (DATA TAKER, FIELD LOGGER, DT 100F, Data electronics (Aust.) Pty, Ltd. Australia) through a union Tee-connector outside the microwave oven with a 1 mm diameter needle thermocouple tip connected to an 11 mm diameter copper-constantan

thermocouple. Thermocouples were previously calibrated with an ASTM mercury-in-glass thermometer (ASTM 1c, -20/150 CP, VWRbrand, VWR). The data logger collected temperatures every 20 s. Samples were taken with a syringe through another union Tee with septum (SEPTA 11.5 CS, SGE Analytical Science, Austin, TX, USA) (Fig. 4.1., F). In water bath treatments under vacuum, the glass vacuum chamber was placed in a water bath (Versa-Bath, Fisher Scientific Co. USA) and the bath level was higher than liquid level in the container.

4.2.7 Determination of temperature consistency inside the glass vacuum chamber

To ensure the recorded temperature using thermocouple during the treatment is the true representative of temperature inside the glass vacuum chamber, a fiber optic probe (Luxtron Fluoroptic temperature probe MSA, Luxtron corporation, Santa Clara, CA, USA) was placed inside a glass beaker containing 1000 ml distilled water. Time temperature profile of water at 22.5 in Hg vacuum was monitored (Figure 4.4). Although start temperature varied by 5°C, final temperature was constant (±0.3 °C) once equilibrium was established.

4.2.8 Determination of residence time distribution

To determine the time needed for *E. coli* cells to distribute evenly in the system, a simple residence Time Distribution Study was performed. *E. coli* cells were used as the tracer material. The circulation system with no heating source was run at 410 and 580 ml/min under the highest vacuum (26 inHg). Stationary phase *E. coli* cells (10^7 to 10^8 CFU/ml) were introduced at the inlet and samples were taken every 5 s. After serial dilution they were spread plated on PCA in duplicate and incubated at 37 °C for 24 hours before enumeration.

4.2.9 Sanitizing

To prevent any contamination before and after each experiment the micro pump and tubing were autoclaved (121 °C, 20 minutes) and the whole system was sanitized with 500 ml 70 % (v/v) ethanol for 10 - 15 minutes at room temperature (20-25 °C), then rinsed with 500 ml sterile distilled water for 10 - 15 minutes. To check asepsis, before injecting the culture for each treatment, 0.5 - 1 ml peptone water was taken from the equilibrated liquid system and spread plated directly on PCA.

4.2.10 Vacuum microwave (VM) and water bath under vacuum (control) treatments

The glass chamber was filled with 700 - 900 ml 0.1 % (w/v) peptone water. The lid was sealed with vacuum grease (Dow Corning Corporation, Midland, Mich, USA) and the vacuum pump and microwave were turned on. Vacuum pressure was controlled with an adjustable aperture (as a screw-clamp on a tygon tube) connected to the vacuum trap to vary the boiling point of water inside the glass chamber to set points between 49 °C and 65 °C. After the desired temperature equilibrium was achieved, 1ml of suspended *E. coli* (10^7 to 10^8 CFU/ml) was injected into the liquid system. At time intervals of 35 - 40 seconds, 0.5 ml samples were taken with a sterile syringe (Latex free syringe, Becton, Dickinson, USA) and needle (Precision Glide needle 20G 1 ½" Becton Dickinson, USA) and injected into 9.5 ml room temperature 0.1 % (w/v) peptone water. Microwave experiments were conducted at two microwave powers; 711 ± 20 W and 510 ± 5 W. Control treatment followed the same protocol except the glass vacuum chamber was immersed in a water bath with the side-arms and pump projecting out of the water.

For temperature less than 53 °C, treatment continued until a 2 log reduction in bacterial population was achieved (except for 49°C at 510 W) and for temperature greater than 53°C treatment was continued until no microorganisms survived (5 - 6 log reduction).

4.2.11 Enumeration of surviving and injured *E. coli*

Immediately after each experiment, dilutions of 10^{-1} to 10^{-4} were prepared by adding 1ml samples to 9 ml 0.1 % (w/v) peptone water. Serial dilutions were spread plated in duplicate on PCA, and PCA to which Difco bile salts #3 had been added at a final concentration of 1.5 g/L (PCA-BS) (Facon & Skura 1996). All plates were incubated at 37 °C for 24 hours. Total number of surviving microorganisms was determined on PCA and injured microorganisms were calculated by subtracting the number of bacteria growing on PCA-BS from those that growing on PCA.

4.2.12 Correction for loss of heating medium during experiments

As the liquid in the continuous-flow vacuum system was boiling, the volume of liquid bacterial suspension continuously decreased. Therefore the volume of peptone water in the glass vacuum chamber at the time of sample injection and after taking the last sample was recorded. The evaporation rate was calculated:

$$\text{evaporation rate} = (v_i - v)/t \qquad \text{Eq (4.1)}$$

where v_i = initial volume

v = final volume

t = process time (min)

Then the survival population at each sampling time was corrected using the following equations:

$$v_i - (\text{Evaporation rate} \times \Delta t) = v_c \qquad \text{Eq (4.2)}$$

where v_i = initial volume

Δt = sampling time

v_c = corrected volume

$$(\text{dilution factor} \times \text{CFU} \times v_c)/ v_i = \text{CFU}_c \qquad \text{Eq (4.3)}$$

where v_i = initial volume

v_c = corrected volume

CFU = bacterial population enumerated by plating

CFU_c = corrected CFU

4.2.13 Check for microorganism loss and possible bio-film formation in the chamber

Two swab samples using sterile calcium alginate swabs (Puritan, Calgiswab type 3, Hardwood Products Co., Guilford, Maine, USA) were taken from the internal surface of the lid (307 cm^2) and inside wall (764 cm^2) of glass chamber after each treatment. The swabs were soaked in 9 ml 0.1 % (w/v) peptone water for 15 minutes. Then 1 ml sodium hexamethaphosphate (10 %) (Fisher Scientific) was added to completely dissolve the calcium alginate swabs. Then 0.1 ml of each suspension was spread plated in duplicate on PCA followed by incubation at 37°C for 24 hours.

4.2.14 Calculation of kinetic parameters

The survival curves were prepared by plotting the log surviving population versus time for each experiment. The decimal reduction time (D-value) was computed for each temperature as the negative reciprocal regression slope of these curves. Two methods were used, Arrhenius and Thermal Death Time (TDT), to define temperature sensitivity (Lund 1975). In TDT technique the D-values on logarithmic scale were plotted against the temperature and z, as the negative reciprocal regression slope of the log D versus temperature, was calculated (using Microsoft Excel 1998). The reaction constant (k) in the Arrhenius technique, was calculated with equation (4.4):

$$k = 2.303/D \qquad \text{Eq (4.4)}$$

where D = the D-value for that specific temperature

Then ln k was plotted against 1 / T (T is the treatment temperature in degree Kelvin) and the slope of the regression line was calculated. Activation energy (E_a) was calculated using equation (4.5):

$$\text{Slope} = -E_a/R \qquad \text{Eq (4.5)}$$

where R is the gas constant = 8.3144 (J/mole K)

E_a= activation energy (J/mole).

4.2.15 Dielectric measurement of pure culture

Four litres of Nutrient Broth containing a 16 – 18 hours *Escherichia coli* culture (stationary phase) were centrifuged at 10 – 15 °C (GSA rotor, SORVALL RC 5B, Dupont,

Newtown, CT, USA) at 27300 × g for 30 minutes. The supernatant was discarded; the pellets were pooled and resuspended in nutrient broth and centrifuged again at the same condition. This was repeated four times till the pellet was hardened and a grayish paste was formed. Dielectric properties, apparent dielectric constant ε' and apparent dielectric loss factor ε" at 2450 MHz, of paste and sterile peptone water were measured in triplicate with an Open-ended coaxial Probe and network analyzer (HP 8752C, HP 85070B, Hewlett-Packard Company, Fullerton, CA, USA).

The probe was calibrated with air, a metal fitting that provided an electrical short, and de-ionized distilled water before each set of experiments. The probe was washed with water, 75% (v/v) ethanol and rinsed with deionized distilled water and dried after each measurement.

4.2.16 Statistical analysis

The differences between slopes of temperature sensitivity curves and activation energies were evaluated by homogeneity of regression test (Steel & Torrie 1980). Regression test- Linear regression (SYSTAT 1998) was used to determine correlation between temperature and population of injured microorganisms in different treatments ($p < 0.05$).

4.3 Results

Liquid inside the chamber under vacuum was in turbulent flow and well mixed in all treatments. The temperature variation during this study did not exceed ±1.5 °C, and in most cases was within ± 1 °C. The result of Residence Time distribution showed that *E. coli* cells were distributed evenly in the liquid system after 25 - 30 seconds (Fig. 4.4), and had reached the

expected population density of 2×10^6 CFU/ml, given the volume of the system and the number of bacteria introduced.

4.3.1 Monitoring *E. coli* growth

The growth of *E. coli* after transferring into 50 ml Nutrient broth was monitored to determine the time required to reach to stationary phase of growth. The population density of 1.9×10^8 CFU/ml was achieved after 18 hours incubation at 37°C (Figure 6.1).

4.3.2 D-value

The regression equations for survival curves of *E. coli* in VM at 711 W, 510 W and control treatment are shown in Tables 4.1, 4.2 and 4.3 respectively. Log CFU versus time data from all the experiments formed straight lines on semi-log plots, indicating that regardless of the type of treatment, the inactivation profiles were approximated by first-order kinetic models in this range of temperatures. The destruction rate increased with increasing temperature for all the treatments. These results are in agreement with the data reported by Tajchakavit and co-workers (1998).

4.3.3 z value

The temperature sensitivity curves of *E. coli* (z value) for VM 711 W and 510 W and control treatments are shown in Fig. 4.5 a, b and c. The regression equations of the temperature sensitivity curves for all treatments are shown in Table 4.4. Since the relationship between log D and temperature is linear, z values can be used to predict the impact of changing temperature on D-values. As can be observed in Fig. 4.5-d, for temperatures less than 53 °C in VM at 711 W

and temperature less than 56 °C in VM at 510 W, microbial destruction in the control treatment was faster than in VM treatments. For example $D_{52\,°C}$ calculated from the regression equation for the water bath under vacuum treatment was 3.65 minutes while for VM 711 W and VM at 510 W it was 4.2 and 6.0 minutes respectively. At the same time for temperatures higher than 53°C for VM at 711 W and higher than 56°C in VM at 510 W, the D-values obtained for VM treatments were considerably shorter than those obtained for water bath under vacuum treatments. For instance $D_{57\,°C}$ for control treatment was 1.0 minute while for VM at 711 W and 510 W it was 0.6 and 0.9 minutes.

At the same time, as can be seen in Table 4.4, the value for z in the control treatment was 9.0 °C while for VM at 510 W and 711 W it was 6.0 °C and 5.9 °C, respectively. This indicates that *E. coli* was more sensitive to temperature changes during VM treatments than water bath under vacuum treatments. Statistical analysis showed that there was no significant difference in z values for *E. coli* exposed to 510 W and 711 W microwave power levels. In other words, microwave power level did not affect temperature sensitivity of *E. coli* in this temperature range. Similar results have been reported for *E. coli* exposed to microwaves (2450 MHz) in apple juice at 720W and 900W (Cañumir et al. 2002).

4.3.4 Activation energy

Activation energy for *E. coli* destruction in water bath under vacuum treatment was 232 kJ/mole while for VM at 510 W and 711 W it was 338 and 372 kJ/mole respectively (Table 4.5). Statistical analysis showed that between VM treatments and the control there was a significant difference in activation energy, while there was no significant difference between the values for VM 510 W and VM 711 W. Therefore, VM treatments needed higher levels of energy to initiate

destruction, relative to the control. Activation energies have not often been reported for *E. coli* but can be calculated from reported z values according to the following equation

$$z = 2.303RTT_0/ E_a \qquad \text{Eq (4.6)}$$

where z = temperature sensitivity (z value)

R= gas constant (8.3144 J/mole K)

T & T_0 = minimum and maximum temperature for calculation of z value (°K)

For example Huang & Juneja (2003) reported a z value of 7.6 °C, equivalent to 279.3 KJ/mole for *E. coli* O157:H7 in 93% lean beef during thermal processing in the temperature range of 55-65 °C. Dock and colleagues (2000) reported a z value of 6 °C equivalent to 332.9 KJ/mole for *E. coli* O157:H7 in apple cider while reported value for another strain of *E. coli* in apple cider by Splittstoesser and colleagues (1996) was 435.5 KJ/mole which is equivalent to z value of 4.8 °C.

4.3.5 Injured microorganisms

Differential plate counts on PCA and PCA-BS for all the treatments showed the number of injured microorganisms. Survival curves of *E. coli* plated on PCA and PCA-BS for VM 711W, 510W and water bath treatment at 50 and 58°C are shown in Appendix VI, Figures 9.8 to 9.13. Correlation coefficient tests indicated that there was no correlation between temperature and injured microorganisms population indicating that temperature variation did not have a significant effect on microbial injury in this experiment. Statistical analysis of the number of injured microorganisms among different treatments showed no significant difference. Likewise the type of heat treatment, whether microwave or water bath, had no significant effect on the

population of injured microorganisms. The present data is in contrast with the study of Shin & Pyun (1997) on *Lactobacillus plantarum* cells in MRS broth exposed to microwave and conventional heat at 50°C for 30 min. Shin & Pyun (1997) reported a greater injury in cells treated with microwave radiation. Khalil & Villota (1988) also exposed *S. aureus* in phosphate buffer to microwave at 50°C for 30 min under aerobic and anaerobic conditions. Although they reported a greater injury in microwave treated cells compared to water bath treated cells for both conditions, the difference in the number of recovered *S. aureus* was less in anaerobic conditions. They observed that while the percent of recovery for water bath treated *S. aureus* was not affected by the absence of oxygen, anaerobic conditions enhanced the recovery of microwave treated *S. aureus*.

4.3.6 Dielectric properties

The final weight of the *E. coli* pellet for dielectric measurements was (5.01 ± 1.62 g). Dielectric constant, loss factor and loss tangent of sterile peptone water and of the pellet of *E. coli* culture at room temperature are shown in Table 4.6. Dielectric properties of the *E. coli* pellet and peptone water were significantly different.

4.4 Discussion

The range of temperatures employed in this experiment was restricted due to very high rate of *E. coli* destruction at temperatures above 64 °C. Furthermore, our vacuum microwave equipment was not capable of maintaining constant temperatures below 49 °C. Goldblith & Wang (1967) also reported that because of the sensitivity of *E. coli* to heat, they could not obtain accurate inactivation studies at temperatures greater than 60 °C.

The present study showed that vacuum microwave treatment inactivated *E. coli* cells. The larger D-values for VM treatments at lower temperatures and small D-values at higher temperatures compared to conventional heat treatments provides evidence that there is a factor(s) in VM, different from water bath treatment, which delays *E. coli* destruction at lower temperatures and increases destruction rates at higher temperatures. One possible explanation for lower destruction rates at lower temperatures is that direct heating of microorganisms with microwave enhances the production of heat shock proteins, thereby increasing their resistance compared to the control. Heat resistance of some bacteria increases upon exposure to temperatures slightly higher than their optimum (Foster & Spector 1995; Kaur et al. 1998). Another possible explanation is the difference between heating rates of bacteria in the microwave treatment compared to water bath under vacuum treatments. Kaur and colleagues (1998) also studied the effect of heating rate on the survival of *E. coli* at 60 °C for 40s. They reported that for heating rate of 1 °C/min the mean number of survivors was 1.4 log CFU/ml while for heating rate 10 °C /min it was 2.6 log CFU/ml. They concluded that this might be due to exposure to potentially lethal temperatures for longer during heating period. Heitzer and colleagues (1992) also studied the effect of temperature elevation from 37 to 42°C in 2 and 60 minutes on expression of heat shock gene (*htpG*) in *E. coli*. They reported that the expression pattern was strongly dependent on heating rate.

Activation energies show that VM treatments needed higher levels of energy for *E. coli* destruction, compared to the control. Since activation energy represents the minimum kinetic energy that must be possessed by a molecule in order to react, it can be concluded that destruction of *E. coli* under VM treatment occurs by a different mechanism than under the control treatment. The higher level of activation energy for microbial destruction associated

with microwave is in agreement with the study of Khalil (1987). He reported activation energy of 353.13 and 369.44 KJ/mol for *Bacillus stearothermophilus* spores and *Bacillus subtilis* spores for microwave treatment at temperature range of 95 to 100 °C. While for conventional heat treatment at the same temperature range was 316.73 and 308.36 KJ/mol for the spores of *B. stearothermophilus* and *B. subtilis.*

The dielectric constant represents the amount of microwave energy absorbed by the sample and the loss factor indicates how much of that energy will be converted to heat. Loss tangent defines the ability of the medium to convert electromagnetic energy into heat at a given frequency and temperature. Therefore, when the mixture of culture and peptone water was exposed to microwave radiation, *E. coli* with higher loss factor, produced more heat than the surrounding liquid environment (peptone water). This higher capacity of *E. coli* to generate heat may cause a slight local temperature increase inside the cell. This lends credence to the selective heating theory, one of the four predominant theories of microwave inactivation (Kozempel et al. 1998). The selective heating theory hypothesizes that microorganisms are heated more effectively by microwaves than their surrounding medium, they can be killed more rapidly (Datta & Davidson 2000). On the other hand, Sastry & Palaniappan (1991) studied the temperature difference between a microorganism and its surrounding medium using simple mathematical relationships based on heat transfer principles. Results of their analysis showed a rapid heat loss to the surrounding environment due to the high ratio of the surface area to the volume of bacteria. In the future if actual direct temperature measurements of bacteria are possible, the correlation between values predicted by Sastry & Palaniappan (1991) model and actual bacterial temperature could be used to prove or reject the selective heating theory.

4.5 Conclusion

In this study, although temperatures within experiments and between treatments were kept constant, slower inactivation at temperatures less than 53°C and higher reduction in microbial population at temperatures above 53°C for VM treated *E. coli* was observed. z value in the water bath treatment was 9 °C while for VM at 510W and 711W it was 6.0°C and 5.9 °C, suggesting that *E. coli* is more sensitive to temperature changes under microwave heating. Arrhenius calculations of the activation energies of the destruction reactions suggest that the mechanism of destruction in VM is different from that of conventional heat. Thus the presence of factor(s) other than heat involve in microwave under vacuum was established.

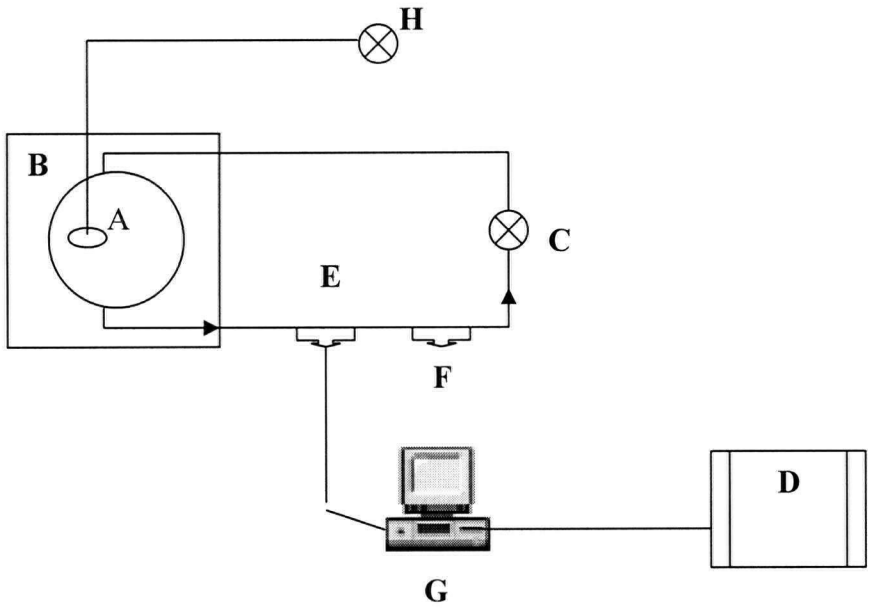

Figure 4.1. Continuous-flow vacuum system. A) glass vacuum chamber, B) heating source (microwave oven or water bath), C) micropump, D) data logger, E) thermocouple connector, F) sampling port, G) computer, H) vacuum pump.

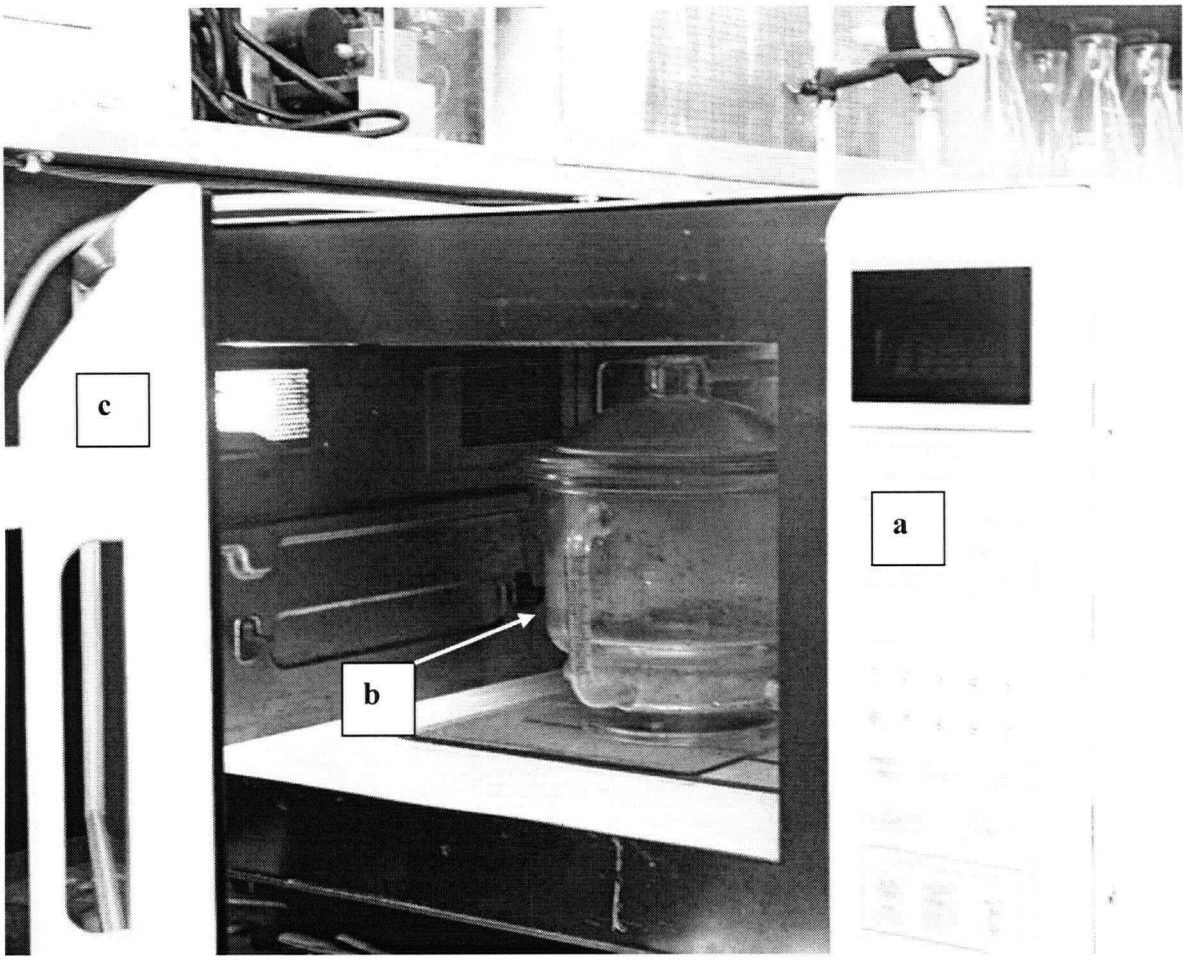

Figure 4.2. Continuous vacuum system with microwave as heating source-front view: a) microwave oven, b) glass vacuum chamber, c) microwave oven door.

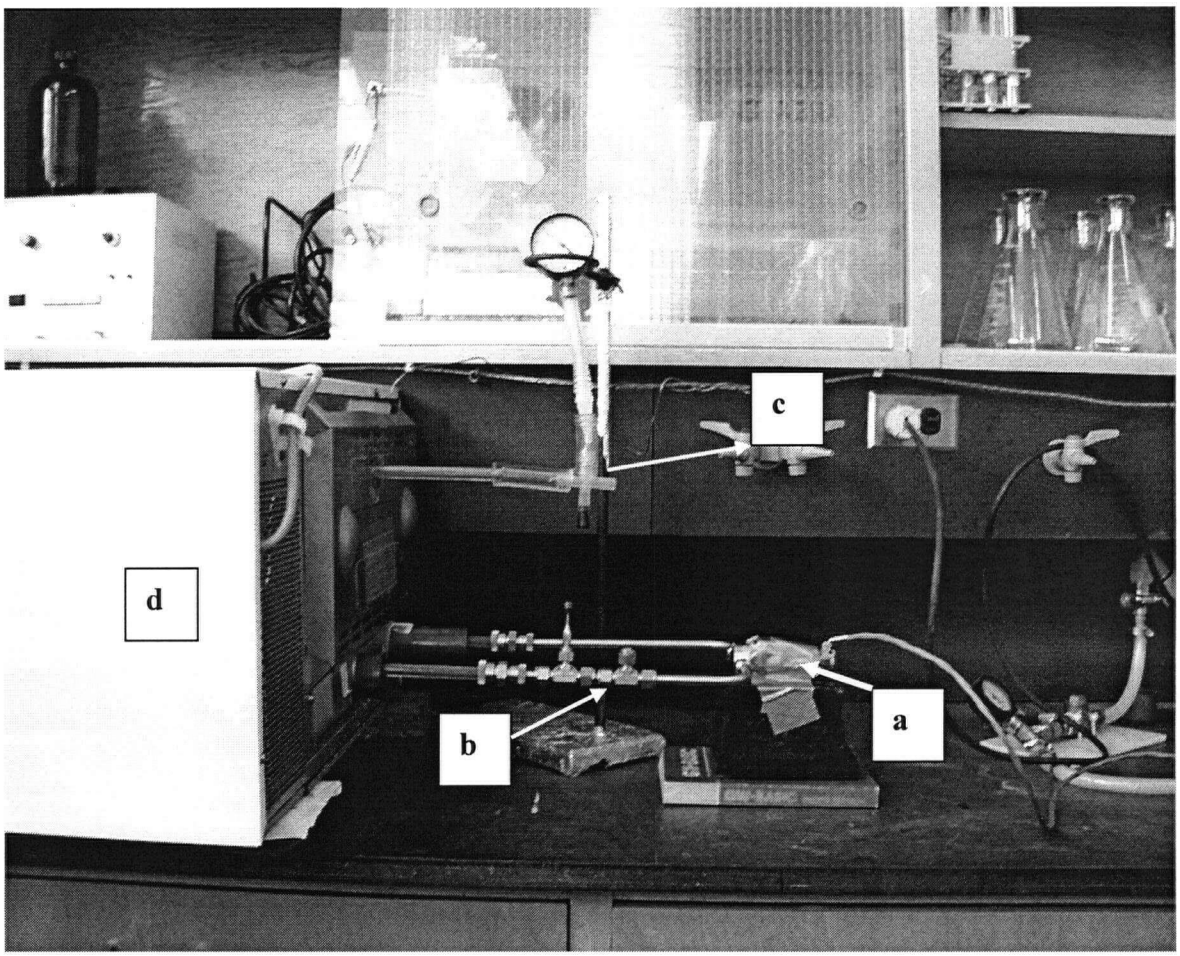

Figure 4.3. Continuous vacuum system with microwave as heating source-outside view:

a) micro-pump, b) circulation tube, c) connection to the vacuum pump, d) microwave oven.

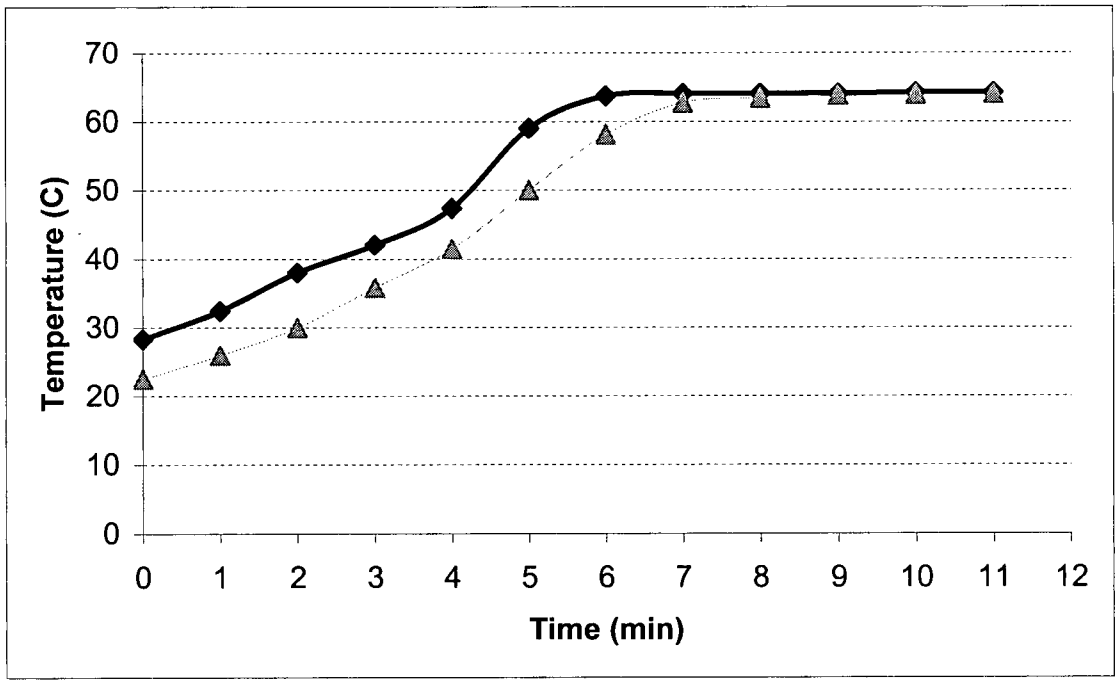

Figure 4.4. Time-temperature profile of 1000 ml distilled water in microwave (2450 MHz) under vacuum (22.5 mmHg) with fiber optic probe. Each line is the representative of one measurement.

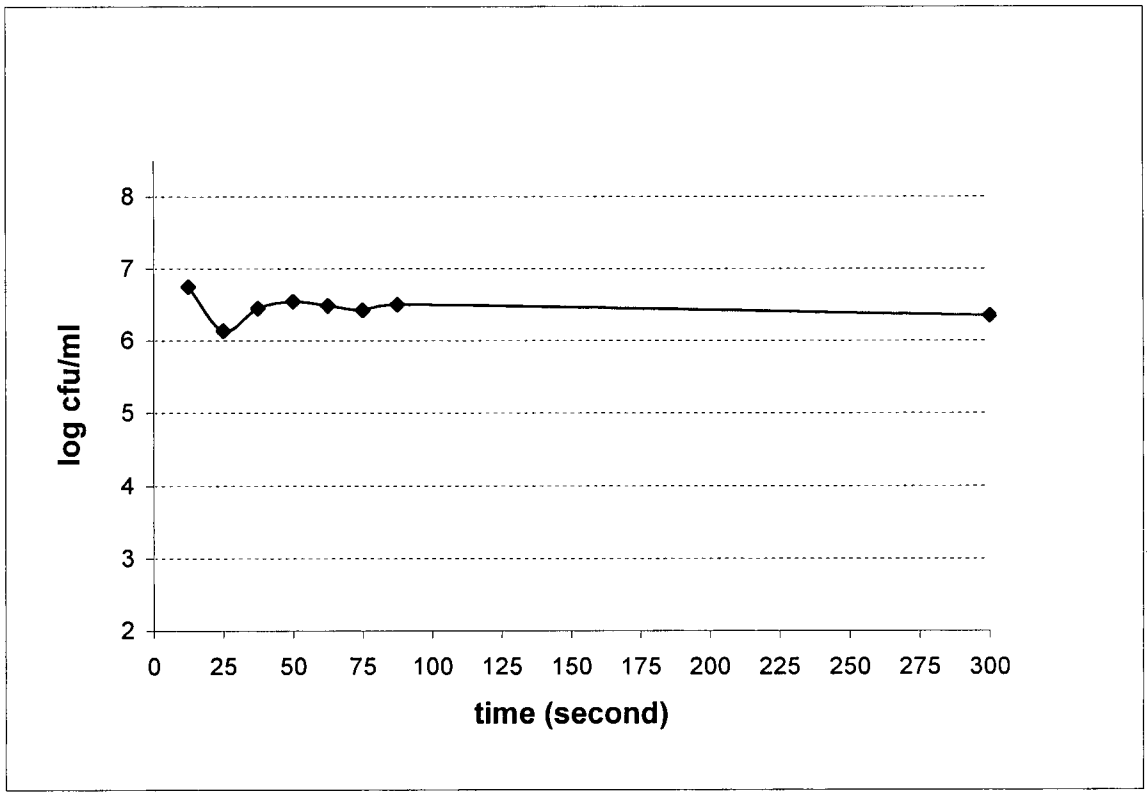

Figure 4.5. Sampled population of *E. coli* cells in the continuous-vacuum system with no heating source as a function of time indicated homogeneous mixing of injected bacteria within 30 seconds (each value is an average of two measurements).

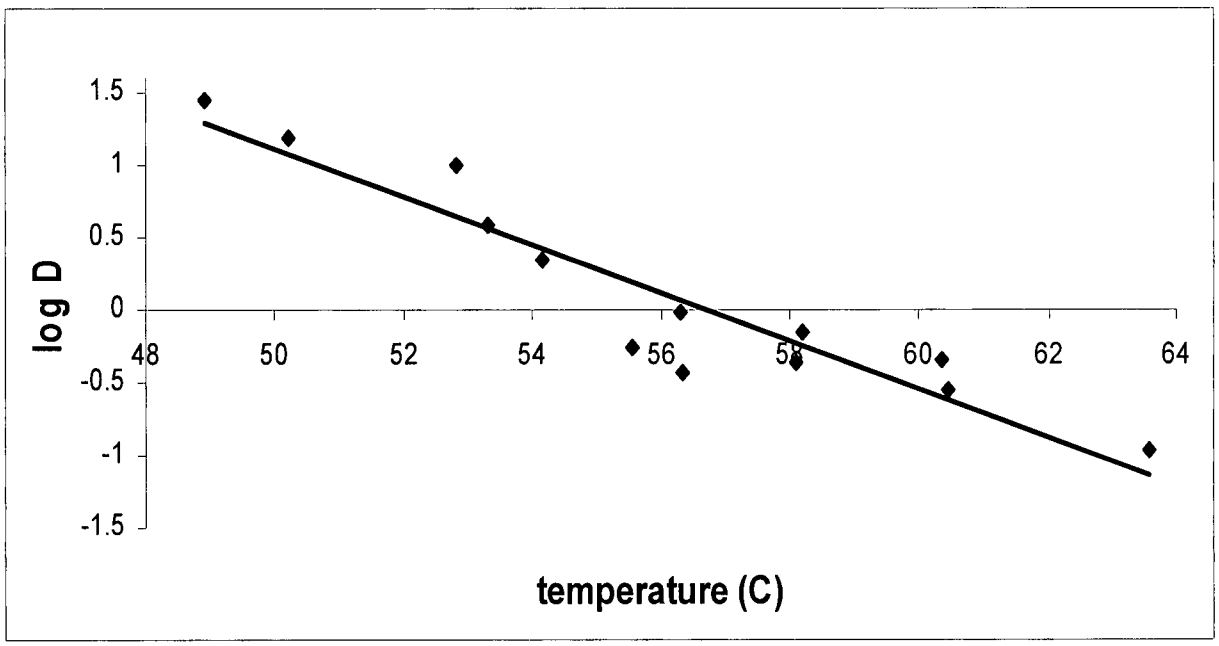

Figure 4.6.a Temperature sensitivity curves for *E. coli* treated under vacuum microwave at 510 W.

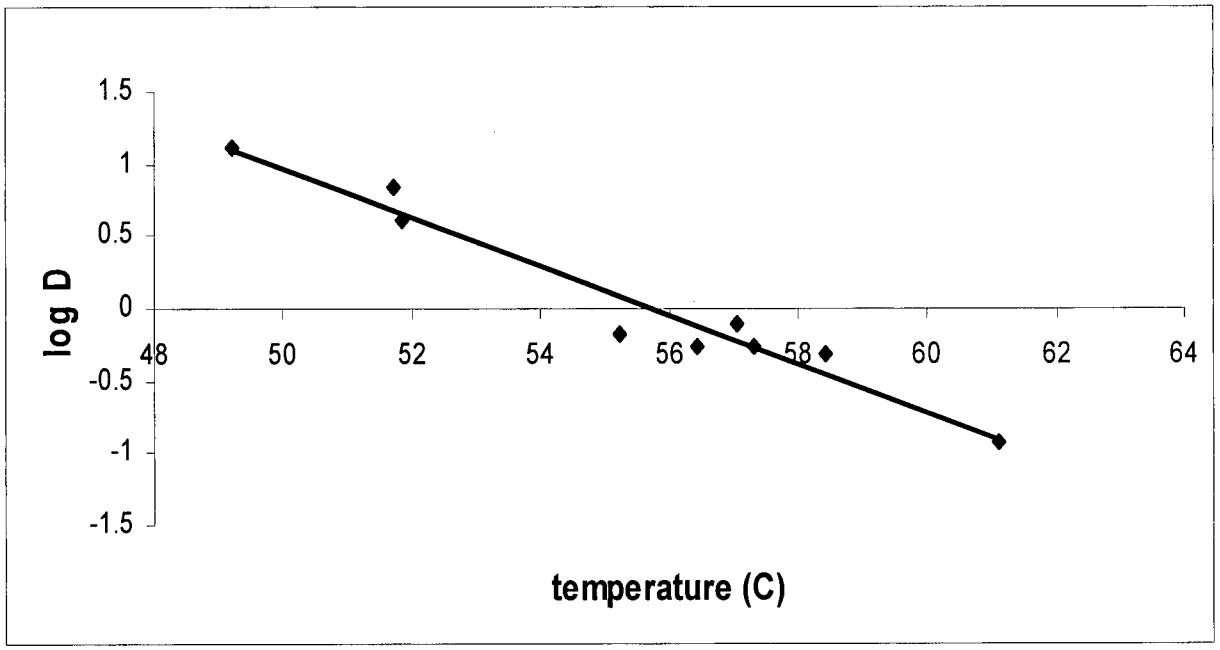

Figure 4.6.b Temperature sensitivity curves for *E. coli* treated under vacuum microwave at 711 W.

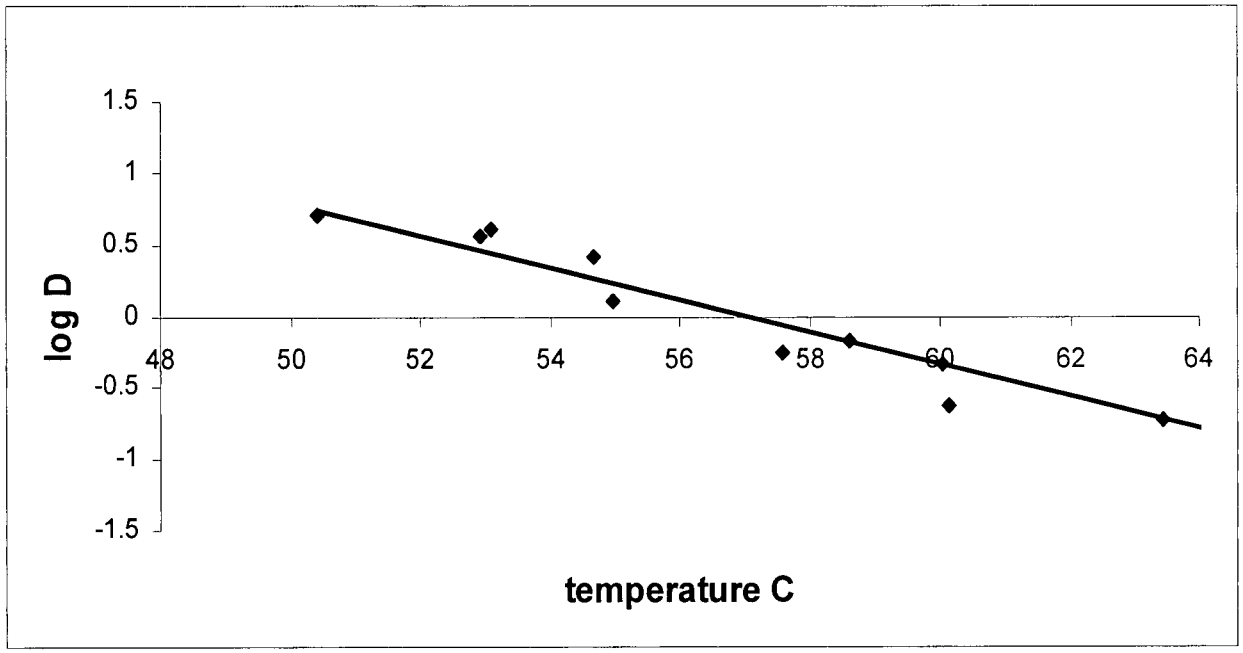

Figure 4.6.c Temperature sensitivity curves for *E. coli* treated in water-bath under vacuum.

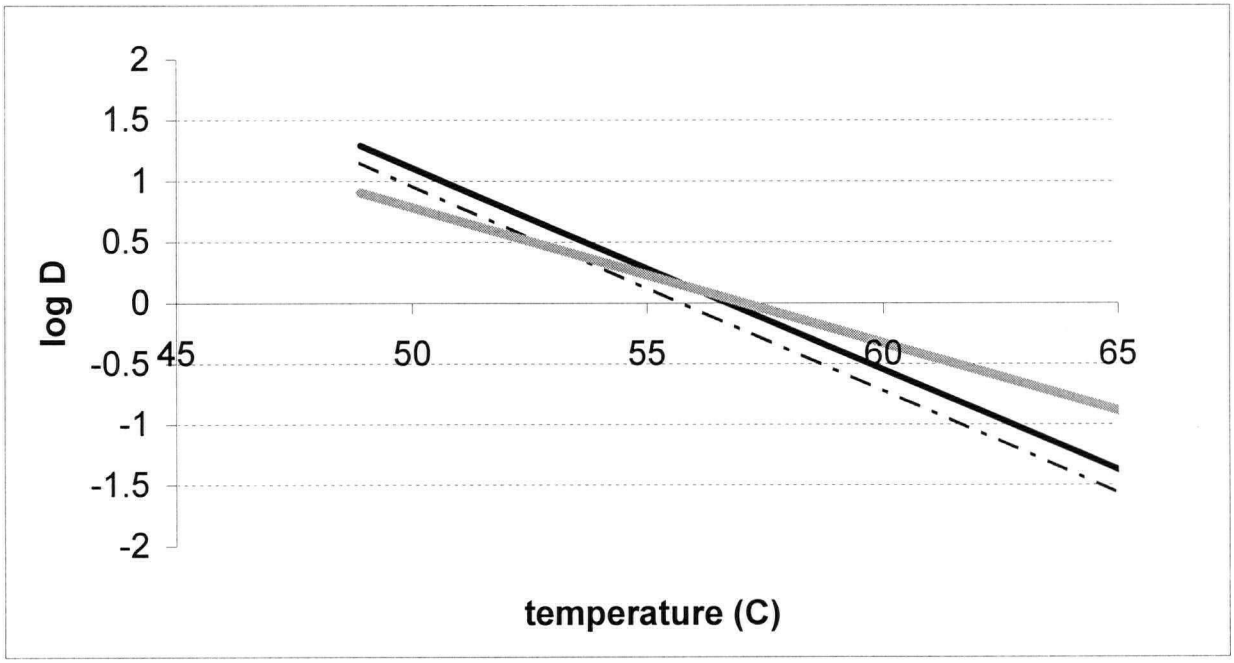

Figure 4.6.d Temperature sensitivity curves of *E. coli* treated by vacuum microwave 711 W – – – –, vacuum microwave 510 W ——— and water bath under vacuum treatment ———.

Table 4.1. Regression equations and D-values of *E. coli* exposed to vacuum microwave at 711 W treatments.

Temperature (°C)	D-value (min)	Regression Equation	r^2
49.2 +/- 0.6	13	y= -0.0013 x + 6.25	0.84
51.7 +/- 0.7	6.9	y = -0.0024 x + 6.45	0.74
51.8 +/- 0.6	4.1	y = -0.0041 x + 7.10	0.88
55.2 +/- 1.2	0.7	y = -0.025 x + 6.99	0.89
56.5 +/- 0.4	0.6	y = -0.03 x + 6.68	0.97
57.1 +/- 1.1	0.8	y = -0.021 x + 5.83	0.78
57.3 +/- 0.6	0.5	y = -0.031 x + 6.69	0.99
58.4 +/- 0.5	0.5	y = -0.034 x + 6.01	0.94
61.1 +/- 0.5	0.1	y = -0.13 x + 7.28	0.91

Table 4.2. Regression equations and D-values of *E. coli* exposed to vacuum microwave at 510 W treatments.

Temperature (°C)	D-value (min)	Regression Equation	r^2
48.9 +/- 0.7	28	y = -0.036 x + 5.41	0.94
50.2 +/- 0.5	15	y = -0.0651 x +4.83	0.54
52.8 +/- 0.5	9.8	y = -0.0017 x + 6.35	0.70
53.3 +/- 0.5	3. 8	y = -0.0044 x + 6.27	0.94
54.2 +/- 1.5	2.2	y = -0.0076 x + 6.90	0.64
55.6 +/- 0.6	0.5	y = -0.031 x + 6.82	0.98
56.3 +/- 0.4	1.0	y = -0.017 x + 6.03	0.98
56.3 +/- 0.5	0.4	y = -0.044 x + 6.18	0.91
58.1 +/- 0.8	0.4	y = -0.039 x + 6.52	0.99
58.2 +/- 0.8	0.7	y = -0.019 x + 5.72	0.87
60.3 +/- 1.0	0.5	y = -0.037 x + 6.44	0.91
60.5 +/- 0.7	0.3	y = -0.058 x + 5.64	0.76
63.6 +/- 0.7	0.1	y = -0.15 x + 6.28	0.81

Table 4.3. Regression equations and D-values of *E. coli* exposed to water-bath under vacuum treatments.

Temperature (°C)	D-value (min)	Regression Equation	r^2
50.4 +/- 0.5	5.1	y= -0.0033 x + 6.16	0.82
52.9 +/- 1.5	3.6	y = -0.0046 x + 6.09	0.38
53.1 +/- 1.5	4.1	y = -0.0041 x + 6.06	0.75
54.7 +/- 0.9	2.6	y = -0.0065 x + 5.89	0.81
55.0 +/- 1.3	1.3	y = -0.013 x + 6.45	0.97
57.6 +/- 0.5	0.6	y = -0.029 x + 6.96	0.94
58.6 +/- 0.3	0.7	y = -0.024 x + 6.41	0.94
60.0 +/- 0.5	0.5	y = -0.036 x + 5.65	0.76
60.1 +/- 0.3	0.24	y = -0.070 x + 6.52	0.87
63.5 +/- 0.1	0.19	y = -0.087 x + 5.88	0.76
65.7 +/- 0.6	0.18	y = -0.14 x + 6.52	1.00

Table 4.4 Regression equations of temperature sensitivity of *E. coli* for water-bath under vacuum treatment and vacuum microwave treatments at 510 W and 711 W.

Treatment	**z value (°C)**	**Regression Equation**	**r^2**
Water bath under vacuum (control)	9.0[a]	y = -0.11 x + 6.3241	0.92
Vacuum microwave (510 W)	6.0[b]	y = -0.17 x + 9.3883	0.88
Vacuum microwave (711 W)	5.9[b]	y = -0.17 x + 9.4155	0.96

[a,b] In each column values not sharing the common superscript are significantly different ($p < 0.05$) from each other.

Table 4.5. Regression equations of activation energy (E_a) for *E. coli* in water-bath under vacuum treatment and vacuum microwave treatments at 510 W and 711 W.

Treatment	**E_a (kJ/mole)**	**Regression equation**	**r^2**
Water bath under vacuum (control)	232^a	y = -28023 x + 85.71	0.92
Vacuum microwave (510 W)	338^b	y = -40659 x + 124.13	0.89
Vacuum microwave (711 W)	372^b	y = -44769 x + 136.93	0.95

[a,b.] In each column values not sharing the common superscript are significantly different ($p < 0.05$) from each other.

Table 4.6. Dielectric constant and loss factor of sterile peptone water, and centrifuged pellet of pure culture of *E. coli* at room temperature.

Sample	Dielectric constant	Loss factor	Loss tangent
Sterile peptone water	77.3 ± 0.60^{a}	9.86 ± 0.55^{a}	0.128 ± 0.007^{a}
E. coli pellet	55.8 ± 2.94^{b}	11.22 ± 1.14^{b}	0.179 ± 0.026^{b}

[a,b] In each column values not sharing the common superscript are significantly different ($p < 0.05$) from each other.

CHAPTER FIVE

CHANGES IN *ESCHERICHIA COLI* TRANSCRIPTOME

DUE TO

SUB-LETHAL VACUUM MICROWAVE TREATMENT

5.1 Introduction

All cells have complex signalling pathways, which help them to survive or adapt to a destructive force in their environment at some certain level. These pathways are essential to cell viability and determine resistance to individual stimuli. Therefore cellular stress responses play an important role in the sensitivity of microorganisms to any external factor (Downes et al. 1999).

Several attempts have been made to investigate the effect of microwaves on various cell function and response systems since the discovery of microwave heating properties (Mayne et al. 1999; Trošić et al. 1999). A number of studies showed evidence indicating that alternating electromagnetic fields interfere with DNA and RNA, affect molecules essential for the nervous system function, disturb the normal function of cell membrane (Mertens & Knorr 1992) and affect chromosome structure (Banik et al. 2003). In addition, changes in growth rate of *Spirulina platensis* (Pakhomov et al. 1998), *Methanoscarcina barkeri* (Banik et al. 2003), and sprouting of barley seeds (Pakhomov et al. 1998), and sensitivity of *S. aureus* to antibiotics (Bulgakova et al. 1996; Pakhomov et al. 1998) have been reported.

Galvin and colleagues (1984) exposed the whole body of pregnant mice to 2450 MHz microwave radiation at a power density of 30 mW/cm^2 for two, four hour periods per day in total for 6 and 15 days. They found no change in lymphocyte, neutrophil or monocyte numbers. Trošić and co-workers (1999), who exposed male Wistar rats (13 week old) to 2450 MHz microwave at 5-15 mW/cm^2, 2 hours per day, maximum 5 days a week for the period of 1,8,16 and 30 days, however, showed a decrease in total leukocyte count as well as lymphocyte percentage in the treated rats.

Liu and co-workers (2002) studied the effects of 2450 MHz microwave radiation at a power density of 10 mW/cm^2 on gene expression transcription of cultured human retina pigment epithelial cells. They found at temperatures that did not exceed the heat shock temperature (32°C), seven of 97 genes were up-regulated about 2.07-3.68 fold compared to cells exposed to water bath while no significant down-regulation was observed. Harada and co-workers (2001) studied the effect of magnetic field (60 Hz, 0.25-0.5 T) to Klenow enzyme-catalyzed DNA synthesis and RNA polymerase driven RNA synthesis in vitro. They indicated that neither the polymerase activity nor proof reading was affected by magnetic fields in the condition employed.

So far, published studies have focused on a specific function, cell structure or tissue of the organism under examination. To get an overview of cell response, study of the expression of the whole genome is required. DNA microarray technology has proven to be a powerful technique for investigating gene regulation by providing a system for simultaneous measurement of gene expression of the whole genome in a single hybridization assay. Genome-wide expression of *Saccharomyces cervisiae* (Wodicka et al. 1997) and *Escherichia coli* (Richmond et al. 1999) have been monitored with microarray technology. In addition, *E. coli* gene expression under a number of different conditions including minimal and rich media (Tao et al. 1999), responses to protein overproduction (Oh & Liao 2000), response to hydrogen peroxide (Zheng et al. 2001), and changes due to transition from exponential-phase to stationary phase growth in minimal medium (Wei et al. 2001) have been successfully studied.

The results of the previous section on the injury and inactivation of stationary phase *E. coli* cells during microwave heating under vacuum showed that *E. coli* cells were significantly more sensitive to temperature changes when microwaves were the medium of heat transfer. In

addition, the activation energy of *E. coli* exposed to microwaves under vacuum was significantly higher than the cells exposed to heat in water bath under vacuum while the temperature was constant. This suggests that the destruction mechanism of vacuum microwave heating could be different from water bath heating.

The aim of this section was to study the changes in gene expression of *E. coli* cells under vacuum microwave and water bath to achieve a better understanding of the mechanism of the microwave effects on microorganisms through global gene expression response. The lowest temperature that showed a difference in destruction rate was 49°C and the calculated $D_{49.5}$ from regression equations for water bath treatment and vacuum microwave at 711 W were 6.9 and 11.2 minutes respectively (Table 4.4). Thus in the present work, the sub-lethal condition of 3 minutes treatment at 49.5°C was chosen to investigate *E. coli* short-term response to vacuum microwave (VM) and water bath treatments. DNA microarray technology was used to characterize the changes in gene expressions at the transcription level as a result of short-term exposure to microwaves under vacuum compared to water bath treatment and to identify the activated and inactivated pathways involved in *E. coli* response to vacuum microwave treatment.

5.2 Materials & Methods

5.2.1 Bacterial Strain

Escherichia coli (ATCC 11775) isolated from human urine was purchased as a freeze dried sample from the American Type Culture Collection, Rockville, USA. For stock culture and inoculum preparation, refer to chapter 4, section 4.2.2.

5.2.2 Sample preparation

A stationary phase *E. coli* (10^7 to 10^8 CFU/ml) culture (75-90 ml) was aseptically transferred into six sterile, 15 ml centrifuge tubes (Fisher brand disposable sterile centrifuge tubes, seal cap, modified polystyrene, Fisher Scientific, Pittsburgh, PA, USA) and centrifuged at 2060×g at 4°C for 8 minutes (BECKMAN GS-6 centrifuge, Beckman Instrument, USA). The supernatant was discarded and cells were re-suspended in 4 ml room temperature 0.1 % (w/v) peptone water (Bacto peptone, Difco). They were mixed using a sterile syringe by passing them through a syringe several times just before the experiment.

5.2.3 Vacuum microwave (VM) and water bath under vacuum treatments

Cells were exposed to microwaves (2450 MHz, 711W) under vacuum or heated in water bath under vacuum in a continuous-flow vacuum system. For details about the system and sanitizing, refer to chapter 4, sections 4.2.6 and 4.2.8.

Peptone water (825 ± 31.62 ml; 0.1 % (w/v)) was poured into the glass chamber. The lid was sealed with vacuum grease and the vacuum pump and microwave were turned on or the chamber was immersed in the water bath. Vacuum pressure was adjusted to 24.56 ± 0.31 in Hg with an adjustable aperture connected to the vacuum trap. After the desired temperature equilibrium was achieved, 4ml of suspended *E. coli* (10^7 to 10^8 CFU/ml) was injected into the liquid system.

The length of treatment for all the experiments was 3 minutes. Next the heating source was eliminated (microwave turned off or the glass glass vacuum chamber taken out of water bath) and the sample was cooled under full vacuum (26 in Hg). The liquid medium cooled to 44°C after 40-60 s, and cooling process continued for 4-5 minutes until the sample reached

≤30°C. The final temperature of the liquid medium was measured using an infrared thermometer (Model 39650-04, Cole-Parmer Instruments, Co. Vernon Hills, USA). The detailed condition for each treatment is shown in Table 5.1. Immediately following treatment, the medium was aseptically transferred into sterile centrifuge tubes and cells were harvested at 25400×g, 4°C, 20 minutes (SORVALL RC 5B, GSA rotor, Mandel Scientific, Norwalk, USA). Each treatment was repeated three times.

5.2.4 Untreated sample

Forty eight ml stationary phase *E. coli* pure culture (10^7 CFU/ml) were aseptically transferred into six sterile 15 ml centrifuge tubes (Fisher Scientific, Pittsburgh, PA, USA) and centrifuged at 2060×g at 4°C for 8 minutes.

5.2.5 Total RNA extraction

After harvesting the cells, the supernatant was removed and 133 µl of the lysis mixture, (15 µl *Ready-Lyse Lysozyme* solution (Epicentre Technologies, Madison, WI, USA) + 785 µl Tris/EDTA (TE) buffer) were added to each tube, vortexed and incubated at room temperature (21-23°C) for 20 minutes. Lysed cell suspension was loaded onto a Qiagen RNeasy column (100 µl each column) from a Qiagen RNeasy Total RNA Isolation Mini kit (Valencia, CA, USA). Total RNA isolation was completed using the protocol supplied with the kit. Isolated RNA was diluted in TE buffer and quantified based on the absorption at 260 nm (UNICAM UV/VIS Spectrometer UV2 ATI UNICAM). The quality of RNA was checked by running the sample on an agarose/formaldehyde gel (Ausubel et al.1999) as well as calculating the ratio of absorbance at 260 / 280 nm.

The extracted total RNA was divided into 25 µg aliquots and frozen at -20°C over night then vacuum-dried using a Speed-vac (Sc110, Savant Instrument Inc. Holbrook, NY, USA), at low drying rate then stored at -20°C until further use.

5.2.6 mRNA enrichment

To enrich mRNA, Affymetrix protocols (Gene expression analysis, Affymetrix technical manual 2000 & 2001) were used with some modification as described below:

5.2.6.1 cDNA synthesis

Five tubes of 25µg total RNA were used for each sample. To each tube, 1 µl Spike control (500 pM) (Affymetrix Technical Manual 2001), 15 µl rRNA removal stock (5 µM) (16S rRNA Primers and 23S rRNA Primers, desalted, HPLC filtered, Sigma Genosys, Oakville, ON) (Table 5.2), and 24 µl of nuclease free water (Ambion, Austin, TX, USA) (total volume 40 µl) was added, mixed and incubated at: 70°C (5 min), 4°C (5min), 1 cycle, using a thermal cycler (PERKIN_ELMER, DNA Thermal cycler 480, Norwalk, CT, USA).

Then 10 µl of 10×MMLV Reverse Transcriptase buffer (Moloney Murine Leukemia Virus (MMLV) RT, Epicentre Technologies, Madison, WI, USA), 5 µl of 100 mM DTT (Epicentre Technologies, Madison, WI, USA), 2 µl of dNTP mix (25mM) (dATP, dCTP, dGTP, dTTP, Amersham Pharmacia Biotech), 2.5 µl of RNAguard (34.29 U/µl) (RNAguard RNase INHIBITOR Porcine, Amersham Biosciences corp, Piscataway, NJ, USA), 30.5 µl of nuclease free water, 10 µl of MMLV Reverse Transcriptase enzyme (50U/µl) (Epicentre Technologies, Madison, WI, USA) (total volume 60 µl) were added, mixed briefly and incubated at: 42°C (25

min), 45°C (20 min), 4°C (3 min), 1 cycle, followed by: 65°C (5 min), 4°C (5 min), 1 cycle to inactivate MMLV RT enzyme, using a thermal cycler.

5.2.6.2 rRNA digestion

rRNA digestion followed immediately after cDNA synthesis. Four µl of Rnase H (10 U/µl) (Ribonuclease H, *E. coli*, Epicentre Technologies, Madison, WI, USA), 1.7 µl of RNA guard (34.29 U/µl) and 1.3 µl of nuclease free water (total volume 7 µl) were added and each tube was incubated at: 37 °C (25 min), 4 °C (4 min), 1 cycle, using a thermal cycler.

5.2.6.3 cDNA digestion

Immediately after rRNA digestion, 1.5 µl Dnase I (10 U/µl) (Deoxyribonuclease I, Amersham Pharmacia Biotech) was mixed with 2.1 µl nuclease free water, followed by adding 1.4 µl RNA guard (34.29 U/µl) (total volume of mixture for each tube was 5µl). Tubes were incubated at 37 °C for 18 minutes. Enzyme was inactivated by adding 3 µl EDTA (500 mM) (0.5M EDTA, pH=8.0, Gibco BRL, Life Technologies, Maryland, USA). Samples were cleaned up using QIAGENE Rneasy mini column (Valencia, CA, USA) and enriched mRNA was eluted in 45µl of nuclease-free water, quantified at A_{260} nm and frozen at –20 °C before drying. Enriched mRNA samples were dried in a Speed Vac, at low drying rate, and stored at –20 °C till required for use.

5.2.7 Labeling and fragmentation

Enriched mRNA (25-38 μg) was used for fragmentation and direct labeling (Affymetrix Protocol for Prokaryotic Sample, Technical Manual, Gene Chip Expression Analysis, July 2001). First, mRNA was fragmented by heat and ion-mediated hydrolysis. Then the 5'-end RNA termini were modified by T4 polynucleotide kinase and γ-S-ATP, and a biotin group. After clean-up the quantity of enriched, fragmented, and labeled mRNA samples was checked at A_{260} nm. The efficiency of the labeling procedure was assessed using the gel-shift assay (Affymetrix technical manual 2001). Fragmented and labeled mRNA samples were stored at -20°C until use.

5.2.8 Hybridization, washing and staining

Fragmented-biotin labelled mRNA (1.5 μg) was added to a GeneChip sense *E. coli* Genome array (Affymetrix Inc., Santa Clara, CA, USA) containing 7312 probe sets, and was hybridized in an Affymetrix GeneChip Hybridization Oven 640 (16 hours, 45°C, 60 rpm). Chips were then washed and stained using a GeneChip fluidics station 400, ProkGE-WS2 protocol (Affymetrix, Santa Clara, CA USA) in three steps: (1) binding of streptavidin to biotin, (2) binding of biotin-conjugated anti-streptavidin antibody to streptavidin, and (3) binding of phycoerythrin-conjugated streptavidin to the antibody biotin.

5.2.9 Scanning

Each probe array was scanned twice in a GeneArray scanner (Agilent Technologies, Palo Alto, CA). The computer workstation automatically overlaid the two scanned-images and averaged the intensities of each probe cell.

5.2.10 Data analysis

The data for each array were collected and initially analyzed using Affymetrix Microarray Suite 5.0 software. First, the average intensity value for all probe cells was calculated. The degree of variation within the same probe cells were used to calculate the background noise. Other matrices compared the intensities of the sequence-specific Perfect Match (PM) probe cells with their control Mismatch (MM) probe cells for each probe set, and then were used in a decision matrix to determine if a transcript was Present (P), Marginal (M), or Absent (A, undetected) (Affymetrix Technical Manual 2001).

5.2.10.1 Data normalization

Log base 2 of probe set intensities and the median of the gene intensity for each array was calculated for normalization. Then the median of each set was subtracted from each probe set intensity value. This normalization method was based on the geometric midpoint (average of the logarithmic measures of the ratios) rather than the arithmetic midpoint of ratios, as the geometric midpoint accounts for down- as well as up-regulation.

5.2.10.2 Statistical analysis

The correlation among present calls of replicates was calculated using Excel (Microsoft Excel 1998). One way ANOVA was used to find genes in which expression was significantly different among treatments ($p<0.05$, $n=6$).

5.2.10.3 Calculation of fold change

The log fold change was calculated as the difference between the average log intensity of three microarrays for each treatment compared with average log intensity of three microarrays of the base (untreated samples or water bath treatment). The log fold change for the transcript is a positive value when the expression level in treatment has increased compared to base line and is a negative number when the relative expression level in the treatment has decreased. Then an antilog was performed using equations 1 and 2 for up-regulated and down-regulated genes respectively, to calculate the fold change.

For up-regulated genes:

$$\text{Fold change} = 2^{(\text{log fold change})} \qquad \text{Eq (5.1)}$$

For down-regulated genes:

$$\text{Fold change} = 1/(2^{(\text{log fold change})}) \qquad \text{Eq (5.2)}$$

5.2.10.4 Data filtering

Significant genes ($p<0.05$) had to be sorted before further analysis. Uninformative genes, genes that were expressed less than two fold and genes that were not present in any of the experiments were filtered out. Only those genes that either were present in all three replicates or evaluated twice as present and once as marginal in a data set were accepted. The probe sets related to intergenic regions were not considered. In this study the term "probe sets" refer to data on single array before data filtering which include all the 7312 probe sets and the term "gene" refers to those probe sets with a specific gene name from the *E. coli* genome.

5.2.10.5 Gene annotation

To search for activated or deactivated metabolic pathways gene, data were divided into functional groups followed by individual analysis. The following web sites were used: Affymetrix gene chip data base, Colibri (genolist.pasteur.fr/Colibri), KEGG (Kyoto Encyclopedia of Genes and Genomes, http:\\www.genome.ad.jp/kegg/), GenProtEC (Genes and Proteins of *E.coli*, http:\\www.mbl.edu/html/ecoli.html.) (Riley 1998).

5.3 Results

The A_{260}/A_{280} ratio of total RNA for all the samples was 1.97-2.1 and they showed two clear bands for 16S and 23S RNA on an agarose/formaldehyde gel. The average yield for untreated *E. coli* samples was 5.47 ± 0.27 μg total RNA/ml culture (10^7-10^8 CFU/ml) and for treated samples with VM and water bath was 2.39 ± 1.1 and 1.79 ± 0.23 μg total RNA/ml culture respectively. From each 100 μg of total RNA, 49.78 ± 7.54 μg enriched mRNA was extracted. The yield for fragmentation and labeling was 15.98 ± 7.13 %.

5.3.1 Correlation among replicates

The average correlation among present calls of replicates for each sample was 0.9 (Table 5.3). A fresh *E. coli* inoculum from the stock culture was used for each treatment. Therefore samples are biological replicates not technical replicates and the maximum calculated coefficient of variation of 5.1% and standard deviations less than 0.05 indicates that there was a good reproducibility among replicates.

5.3.2 Present, absent, and marginal probe sets in single arrays

The number of probe sets detected as present or absent or marginal after exposure to microwave and water bath and for untreated cells is shown in Table 5.4. In single arrays 57-58% of probe sets were identified as present after both treatments and 37-38% were not detected and considered as absent. In untreated cells 49% and 46% of probe sets were present and absent respectively. For all the samples 3.8-4.5% of probe sets were in the marginal area (Table 5.4). Those probe sets related to intergenic regions were not considered in this study.

5.3.3 Number of up and down-regulated genes

After water bath treatment 123 genes (1.67%) were up-regulated and 135 genes (1.85%) were down-regulated compared to untreated samples. VM caused 109 genes (1.49%) and 91 genes (1.24%) to be up and down-regulated respectively, when compared to untreated samples. In both cases ≥ 96% of genes remained unchanged. Comparison between treatments showed 55 (0.75%) up-regulated and 49 (0.67%) down-regulated genes in VM compared to water bath treatment while 98.5% of genes did not show any significant change (Table 5.5).

5.3.4 Overview of *E. coli* response

The analysis was divided into three overlapping sets of genes to obtain an overview of *E. coli* genome response. Set 1 included all previously known heat-inducible genes, set 2 included all common genes showing significant change in both treatments to identify the similar response between treatments, and set 3 includes genes that showed significant change (≥ 2 fold) in each treatment compared to untreated samples and between treatments.

5.3.4.1 Heat shock genes

A list of previously known heat shock genes along with their present/absent call and fold change for each treatment is shown in Table 5.6. Of the 76 known genes related to heat shock stress, 22 genes were not detected in any of the samples. The expression level of some heat shock related genes were up-regulated by both VM and water bath treatment. However, the degree of transcriptional response varied among the treatments as well as among heat shock genes. Only two genes significantly altered expression due to VM treatment, while the water bath treatment resulted in 6 genes that were significantly changed compared to untreated samples. Two genes showed significant change ($p<0.05$) as a result of each treatment. The *b1600* gene increased in expression by 1.45 and 1.71 fold, and the *secB* gene decreased 1.72 and 1.79 fold as a result of water bath and VM compared to untreated samples respectively.

The *htgA* (+1.56 fold), *msbB* (+1.90 fold), *uspA* (-1.70 fold) and *yfiA* (-3.57 fold) genes were significantly changed ($p<0.05$) by the water bath treatment compared to untreated samples, while these genes showed no significant change in VM treated *E. coli*. Any changes in the rest of the genes were not significant. Although a higher number of heat shock genes were altered due to water bath treatment, the change was less than two fold except in *yfiA*. None of the heat shock genes showed a significant difference between the two treatments.

5.3.4.2 Genes changed ≥ two fold in both treatments compared to untreated cells

Genes significantly altered in both treatments were studied to determine similarity in *E. coli* response to treatments (Tables 5.7 & 5.8). Of the 39 up-regulated genes, *cysW, rrlD, trpT, ybaR* were only induced by the treatments, but were not detected in the untreated sample. Of the

16 down-regulated genes, *rrfG, rrfD, rrfE, rrfF,* and *ompF* were the only genes that changed ≥ 2 fold in both treatments.

5.3.4.3 Genes changed ≥ two fold by water bath or VM treatments compared to untreated cells

Genes that altered ≥ 2 fold ($p<0.05$) due to water bath treatment in comparison to the untreated samples are shown in Tables 5.9 and 5.10. The name and description of genes changed ≥ 2 fold ($p<0.05$) due to VM treatment compared to untreated samples are shown in Tables 5.11 and 5.12. Both treatments seem to affect genes involved in amino acid metabolism, membrane transport and translation as well as genes that encode for putative proteins with unknown functions.

5.3.4.4 Genes significantly changed in VM compare to water bath treatment

All of the genes that were significantly different between the two treatments have been expressed less than two fold: 1.10-1.78 fold for down-regulated genes and 1.11-1.62 fold for up-regulated genes (Tables 5.13 & 5.14). The *fliG*, *fecA*, *b2496*, *b3694* and *ycgL* genes were induced by VM treatment, but were not expressed in the water bath treated *E. coli.* Conversely, the *rscA*, *ydgB*, *fhiA*, *b0538*, *b0878*, *b2660* and *b2999* genes were not detected in VM treated *E. coli*, but were present in the *E. coli* subjected to water bath treatment.

5.4. Discussion

5.4.1. Heat shock response

The heat shock response in *E. coli* is controlled at the transcriptional level by the sigma factor *rpoH* (sigma 32) and *rpoE* (sigma E). Although the expression level of *rpoE* and *rpoH* showed slight increases in both treatments, the change was not significant. At the same time *htgA*, a positive regulator for sigma 32, was expressed more in water bath treatment than in untreated cells, while *uspA* global regulatory gene for stress response was down-regulated in water bath treated *E. coli* and remained unchanged in VM treated cells compared to untreated cells. This suggests that the conditions employed in this study were not sufficient to stimulate the heat shock response. Thus no significant difference in expression level of *E. coli* heat shock genes was detected. The present data are in contrast with Chow & Tung (2000) who reported that heat shock proteins DnaK/J (Hsp70/40) are overproduced when *E. coli* cultures are exposed to a low frequency magnetic field (50Hz, 1 hour), while Nakasono and Saiki (2000) found no detectable change in protein synthesis of cells exposed to extremely low frequency (ELF) magnetic field (7.8-14 mT, 5-100 Hz) for (0.5-16 hours).

One possible explanation is that the temperature less than or equal to 50°C for 3 minutes is not high enough to induce a heat shock response. In addition stimulated genes may have gone back to their normal condition during the 3-4 minute cooling period. The third explanation could be related to the presence of heat shock genes in cells at stationary phase of growth. Entering stationary phase of growth is considered a mild stress condition and accompanied by production of some heat shock proteins. Stationary-phase cells of *E. coli* have the ability to survive prolonged periods of starvation and have a strong multiple-stress resistance (Hengge-Aronis

1996). Therefore, having a mild treatment along with enough time to recover from the stress may have decreased the relative expression level of stimulated genes.

5.4.2 Membrane structure and membrane transport system

The *cysW* and *ybaR* genes, related to copper and sulfate transport system respectively, were up-regulated in both treatments. *ompF*, which encodes porins and is responsible for dipeptide permease, was significantly down-regulated by both treatments. Porins, which are transmembrane proteins, associate to form small membrane holes about 1 nm in diameter for the diffusion of organic molecules through the outer membrane and into the periplasm (Madigan et al. 2003). The relative abundance of porins is regulated by the media osmotic activity and temperature (Nikaido & Vaara 1987). Chang and colleagues (2002) also reported that the outer membrane proteins encoded by *ompT* and *ompF* were down-regulated during growth arrest (Pratt & Silhavy 1996; Chang et al. 2002).

In addition *yejE*, *btuC*, *exuT*, *ycjO*, *ydiQ*, *yfcC* and b0878 involved in the membrane transport systems for peptides, vitamin B_{12}, putative S-transferase, sugar and ABC transporter system were down-regulated while *fecA*, which encodes for ferric dicitrate outer membrane receptor protein, was up-regulated in VM treated *E. coli* compared to *E. coli* exposed to water bath treatment. This suggested that transcription for genes involved in ion transfer was increased while transcription of genes involved in transfer of larger molecules including peptide, sugar and vitamin transport were decreased as a result of VM treatment. Nascimento and co-workers (2003), who reported a higher level of glucose transported into *E. coli* cells exposed to electromagnetic field (60 Hz, 8 hours, 28°C), suggested that electromagnetic field stimulated the periplasm-binding protein-dependent transport system. Liburdy and his group (1985) also found

that exposure of rabbit erythrocytes to microwaves (2450 MHz) increased sodium passive transport only at membrane phase transition.

The *fimC, fimD* and *fimG* genes, related to outer membrane protein, periplasmic chaperone and morphology of fimbriae, and *fliG*, which encodes for the flagellar motor switch, were expressed more in VM treated *E. coli* compared to water bath treated *E. coli*. The *murG* gene, that encodes for an enzyme involved in peptidoglycan biosynthesis, was expressed less in VM treated *E. coli* compared to water bath treated *E. coli*. Peptidoglycan present in the cell wall is responsible for mechanical strength and maintaining the shape of the cell (Singleton & Sainsbury 2000). This suggests that while genes related to membrane structure and transport system were affected by both treatments, the effect was greater as a result of microwave than the conventional heat treatment. This may lend credence to the dielectric cell-membrane rupture theory. This theory hypothesizes that an external electric field is induced and causes an additional trans-membrane electric potential to the normal potential of the cell, which could result in a voltage drop across the cell membrane and may be sufficient for pore formation, increased permeability, loss of cell integrity (Brunkhorst et al. 2000, Kozempel et al. 2000) or membrane rupture (Datta & Davidson 2000; Kozempel et al. 1998; Zimmermann et al. 1974).

5.4.3 Enzymatic activity

The transcription level of some genes encoding for enzymes involved in carbohydrate metabolism including *gloA* (lactoylglutathione lyase), *murD* (glutamate ligase), *glgC* (glucose-1-phosphate adenylyltransferase) and *yrfE* (putative ADP compounds hydrolase) was increased in VM treated *E. coli* compared to the water bath treated *E. coli*. The *amyA* (cytoplasmic alpha-amylase) and *murG* (N-acetylglucosamine transferase) genes from the same group were

expressed more in water bath treated cells. In addition other genes encoding other enzymes including *gpsA* (glycerol-3-phosphate dehydrogenase), *spoT* (guanosine bis-pyrophosphate pyrophosphohydrolase) and *ppiA* (peptidyl-propyl isomerase A) were expressed more in VM treatment than water bath treatment. On the other hand *menD* (2-oxoglutarate decarboxylase), *sfsA* (probable regulator for maltose metabolism) were down-regulated in VM treated *E. coli* compared to the water bath treated *E. coli*. Dreyfuss and Chipley (1980) also reported higher malate dehydrogenase, α-ketoglutarate dehydrogenase, cytochrome oxidase, and cytoplasmic ATPase activities and lower glucose-6-phosphate dehydrogenase activity in sub-lethal microwave (2450 MHz) irradiated *S. aureus* cells compared to water bath conventional heated cells at 46°C. Saffer and Profenno (1992) reported higher production of a chromophore ($A_{402.5}$) as a result of higher beta-galactosidase activity in *E. coli* cells radiated with low-level microwave radiation (10 kW/kg). Rebrova (1992) also indicated increased stimulation of fibrinolytic enzymes in *Bacillus firmus* irradiated cells.

5.4.4 Ribosomal RNA

The ribosome is a complex ribonucleoprotein responsible for translation of messenger RNAs into proteins. The *E. coli* ribosome is composed of 23S, 16S and 5S ribosomal RNA and about 53 proteins. Twenty-one of these proteins assemble with the 16S rRNA to form the 30S ribosomal subunit, while the other 31 proteins assemble with the 23S and 5S rRNA to form the 50S ribosomal subunit. Ribosomal proteins and rRNAs cooperate both in the assembly and activity of the ribosome and ribosomal functions are dependent on the presence of the major RNA species (Madigan et al. 2003).

In this study the *rrlD* gene related to 23S ribosomal RNA in rrnD operon was up-regulated about 250 and 300 fold in water bath and VM treated *E. coli* respectively. At the same time, the expression level of genes related to 5S rRNA of rrnD, rrnE and rrnG operones including *rrfD*, *rrfE*, *rrfF*, *rrfG* in *E. coli* subjected to both treatments and *rrfH* and *rrfA* in water bath treated *E. coli* was decreased. *rrsH* gene related to 16S ribosomal RNA in rrnH operon also showed down-regulation in water bath treated *E. coli*.

Hansen and colleagues (2001) investigated the level of rRNA before and after a heat shock from 30 to 43°C on exponential cells of wild-type *Lactococcous lactis* subsp. *cremoris*. They reported that the amount of 23S rRNA and 16S rRNA decreased by the same rate through the heat shock. Rosenthal and Iandolo (1970) described a heat-induced dissociation of the 30S particle and degradation of 16S rRNA in *Staphylococcus aureus* at 55°C while, the 50S ribosomal subunit and 23S rRNA appeared to be stable. Similar degradation patterns have been found in *Salmonella enterica* serovar Typhimurium due to heat treatment (Tolker-Nielsen et al. 1997). Khalil & Villota (1989) reported a selective destruction of the 16S RNA subunits after exposure of *S. aureus* cells to conventional heat whereas the destruction of the 16S RNA as well as 23S RNA subunits was reported with microwave sub-lethal heating (50°C, 30min). The present study showed down-regulation in one gene related to 16S rRNA due to conventional water bath treatment of *E. coli* while that gene remained unchanged in VM treated *E. coli* compared to untreated cells. In addition, the expression of genes related to 5S rRNA as part of 50S ribosomal subunit was down-regulated in both VM treated and water bath treated *E. coli*. But the number of down-regulated genes (6 in water bath treated *E. coli* and 4 in VM treated *E. coli*) as well as the average fold change (42.5 in water bath treated *E. coli* compared to 17.5 in

VM treated *E. coli*) was higher in water bath treated cells. The effect of sub-lethal VM treatment on ribosomal RNA may be less than conventional water bath treatment.

Simultaneously we observed a very high expression level of one gene related to 23S rRNA in VM treated and water bath treated *E. coli* while other related genes remained unchanged. This is in agreement with Lopez and colleagues (2002) who reported an increase in the occurrence of 20S RNA and 23S RNA in wild and industrial *Saccharomyces cerevisiae* after exposure to nutritional stress conditions. Those authors concluded that these RNA species could be used as indicators of yeast stress condition in industrial processing. Further studies are needed to be able to state that *rrlD* gene could be used as a stress related gene or stress indicator in *E. coli*.

More down-regulation in 5S and 16S rRNA due to water bath treatment means that ribosomal subunits in VM treated cells were affected less, and thus are more stable. This could be a reason for less destruction at 50°C of VM treated *E. coli* compared to water bath treated *E. coli*.

5.4.5 Transfer RNA (tRNA)

Transfer RNAs serve as adapter molecules matching amino acids to their codons on mRNA (Singleton & Sainsbury 2000). The tRNA and its related amino acids are brought together by amino-acyl-tRNA synthetases, which ensure a particular tRNA receives its correct amino acid (Madigan et al. 2003).

In this study, genes related to tRNAs specific to glutamine, tryptophan and leucine including *glnV*, *trpT* in VM and *glnX*, *trpT* and *leuX* in water bath treated *E. coli* were up-regulated compared to untreated samples. The *glnA*, which encode for glutamine synthetase,

was not detected in water bath treated cells while remaining unchanged in VM treated *E. coli*. In addition the comparison between treatments showed that the expression of *glnS* coding for glutaminyl-tRNA synthetase was significantly higher (1.34 fold) in VM treated *E. coli* compared to water bath treated *E. coli*. In VM treated cells induction of *glnA* gene could activate L-glutamine synthesis by assimilation of ammonia and converting L-glutamate to L-glutamine. In addition, in the amino acyl-tRNA biosynthesis pathway, higher expression of *glnS* could increase the connection of the L-glutamine to tRNA (glutamine) and as a result increase production of L-glutaminyl-tRNA (glutamine). Thus higher production of glutamine in VM treated *E. coli* compared to water bath treated *E. coli* would be expected (Figure 5.1).

Almost all the nitrogenous compounds in an enteric bacterium derive their nitrogen atoms from either glutamate or glutamine. About 88% of the cellular nitrogen in *E. coli* is derived from glutamate and the remaining 12% is derived from glutamine (Reitzer 1996). Thus, glutamine is one of the key intermediates in cellular nitrogen metabolism.

5.4.6 Cell respiration

The *E. coli* respiratory chain contains a number of dehydrogenase and oxidase complexes (Poole & Ingledew 1987). Quinones are non-protein electron carriers which can diffuse freely through the membrane and mediate electron transfer between protein components of the respiratory chain, generally by transferring electrons from iron-sulfate proteins to cytochromes (Madigan et al. 2003; Gennis & Stewart 1996). *E. coli* can synthesize three types of quinones including ubiquinone and menaquinone and demethylmenaquinone (Gennis & Stewart 1996). The amount of quinone and menaquinone in the cell depends on growth conditions (Singleton & Sainsbury 2000) especially the presence of oxygen in the growth environment (Poole &

Ingledew 1987). Studies have shown that quinone is used for oxygen respiration, while both quinone and menaquinone are used for nitrate respiration but menaquinone and demethylmenaquinone are used for anaerobic respiration with acceptors other than nitrate (Gennis & Stewart 1996). In general, under aerobic conditions, ubiquinone is predominant while menaquinone is dominant at reduced oxygen levels (Wallace & Young 1977; Hollander 1976).

The *ubiB* gene is one of the genes responsible for ubiquinone biosynthesis and was expressed 1.28 fold more in VM treated *E. coli* compared to water bath treated *E. coli*. On the other hand, *menD* is one among five genes (*menA,B,C,D* and *E*) necessary for menaquinone synthesis that were expressed 1.32 fold less in VM treated *E. coli* compared to water bath treated *E. coli*. *fliG* expression level showed 1.2 fold decrease in water bath treated *E. coli* as well, meaning flagellar motility was reduced. This is in agreement with Poole & Ingledew (1987) who reported mutation in quinone biosynthesis gives rise to immobility and lack of flagella. At the same time, in water bath treated *E. coli*, genes involved in energy metabolism through oxidative phosphorylation (*ppa)* and nitrogen metabolism (*aspA)* were not detected or were expressed less compared to untreated *E. coli* while these genes remained unchanged after VM treatment. Simultaneously, transcription levels for genes related to copper, sulfate and ferric, ions functioning in electron acceptors in anaerobic respiration were shown to be up-regulated for copper and sulfate in both VM treated and water bath treated *E. coli*, and ferric just in VM treated *E. coli*.

These data suggest that although in both treatments *E. coli* responded to vacuum by higher expression in transcription level of genes related to anaerobic respiration, the evidence for the start of anaerobic respiration is higher for water bath treated *E. coli* than for VM treated *E. coli*.

5.5 Conclusion

This study was designed to investigate the effect of microwaves on cell stress response in a sub-lethal condition. The very first step was to check for heat shock genes as an indicator of heat stress and general stress response. Although some of the heat shock genes were altered significantly in treated *E. coli*, in general the result of this experiment did not show any major change in heat shock gene expression levels.

VM treatment had larger effects on genes related to membrane structure and membrane transport systems as well as the activity of enzymes related to metabolism of carbohydrates, lipids and amino acids. Meanwhile, the effect of conventional water bath treatment on ribosomal subunits was higher. Interestingly, although both treatments employed vacuum and signs of anaerobic respiration would be expected, water bath treated *E. coli* showed more evidence at transcriptional level for the start of anaerobic respiration.

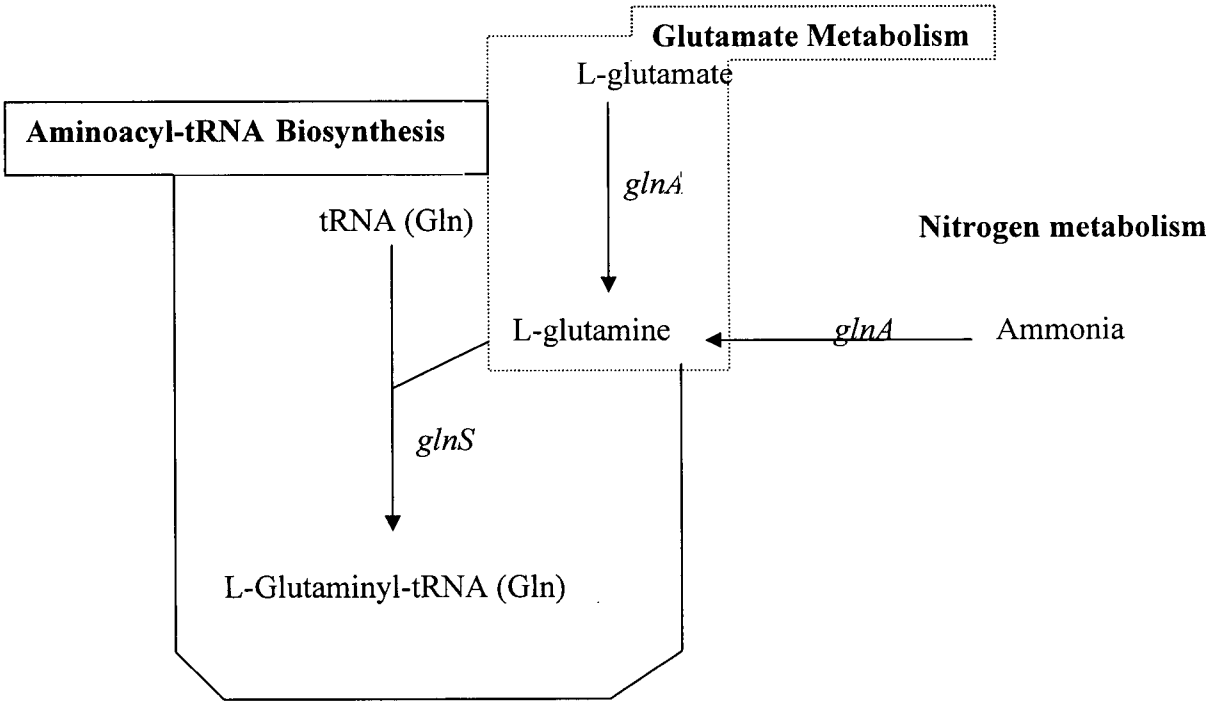

Figure 5.1. Simplified flow diagram of role of *glnS* and *glnA* in glutamine synthesis

Table 5.1. Treatment conditions for vacuum microwave (VM) and water bath under vacuum.

Treatment		Treatment temperature (°C)	Final temperature after cooling (°C)
VM 711W	1	49.6±0.22	30.0
	2	49.9±0.32	28.3
	3	49.9±0.99	30.0
Water bath under vacuum	1	49.7±2.89	27.6
	2	49.9±1.58	30.1
	3	50.4±0.25	30.0

Table 5.2. Sequence of primers for 16S and 23S rRNA used in this study (Affymetrix manual 2000).

Name	Sequence
16S rRNA Primers	
16S1514	5' –CCTAC GGTTA CCTTG TT-3'
16S889	5' –TTAAC CTTGC GGCCG TACTC-3'
16S541	5' –TCCGA TTAAC GCTTG CACCC-3'
23S rRNA Primers	
23S2878	5' –CCTCA CGGTT CATTA GT-3'
23SEco2064	5' –CTATA GTAAA GGTTC ACGGG-3'
23SEco1595	5' –CCTGT GTCGG TTTGG GGT-3'
23S539	5' –CCATT ATACA AAAGG TAC-3'

Table 5.3. Correlation among replicates for treated and untreated *E. coli*.

Treatment	r^2	Average
Untreated *E. coli*	0.92 0.86 0.95	0.91+/-0.04
Vacuum microwave treated *E. coli*	0.90 0.97 0.88	0.92+/-0.04
Water bath treated *E. coli*	0.93 0.95 0.88	0.92+/-0.03

Table 5.4. *E. coli* array probe set signals from *E. coli* exposed to water bath under vacuum, vacuum microwave treatments, untreated stationary phase *E. coli* cells.

Samples	**Present call (%)**	**Absent call (%)**	**Marginal call (%)**
Untreated *E. coli*	49.20+/-15.02	46.30+/-14.72	4.50+/-0.32
Water bath under vacuum	57.26+/-12.43	38.47+/-11.73	4.27+/-0.72
Vacuum microwave 711W	58.61+/-16.00	37.5+/-14.77	3.89+/-1.23

Table 5.5. Number of significantly up-regulated, down-regulated or unchanged genes ($p < 0.05$) between treatments.

	Up-regulated genes		Down-regulated genes	
	Total	**≥ 2 fold**	**Total**	**≥ 2 fold**
Water bath compare to untreated	123	10	135	6
VM compared to untreated	109	12	91	15
VM compared to water bath	55	0	49	0

Table 5.6. List of previously known heat shock genes and their calls in untreated, water bath and vacuum microwave treated *E. coli*.

Gene name	b No.	Fold change			Call			Description
		W-U*	VM-U**	VM-W***	untreated	water bath	VM	
apaH	b0049	1.04	-1.29	-1.34	P[6]	P	A[7]	Diadenosine tetraphosphatase[1,2]; stress response; complex operon[1]
	b1599	1.34	1.28	-1.05	P	P	P	Possible chaperone[2]
	b1600	1.45†	1.71†	1.18	P	P	P	Possible chaperone[2]
cbpA	b1000	1.11	1.02	-1.09	A	P	P	Recognizes a curved DNA sequence similarity to DnaJ[1]; curved DNA-binding protein; functions closely related to DnaJ[2]
clpB	b2592	1.10	1.05	-1.04	P	P	P	ClpB protease, ATP dependent[1]; heat shock protein[2,3] clpB protein (heat shock protein f84.1)[5]
clpP	b0437	-1.22	-1.04	1.18	P	P	P	ClpP ATP-dependent protease proteolytic subunit[1]; heat shock protein F21.5[2]; ATP-dependent proteolytic subunit of clpA-clpP serine protease[3]
clpX	b0438	1.00	1.10	1.09	P	P	P	ClpX protease, which activates ClpP[1]; ATP-dependent specificity component of clpP serine protease[2,3], chaperone[2]
cstA	b0598	-1.26	-1.52	-1.21	P	P	P	Starvation induced stress response protein[1,2]
ddg	b2378	1.41	1.67	1.18	P	P	P	Acetyltransferase[1]; putative heat shock protein[2,5]
dksA	b0145	-1.32	-1.38	-1.05	P	P	P	High copy suppresses muK and TS growth and filamentation of dnaK mutant[1]; dnaK suppressor protein[2]
dnaJ	b0015	-1.04	-1.14	-1.09	P	P	P	Chaperone with DnaK[1,2,3]; DNA chain elongation[1]; stress-related DNA biosynthesis[1], responsive to heat shock[1]; heat shock protein[2,3]
dnaK	b0014	1.10	-1.02	-1.12	P	P	P	HSP-70-type molecular chaperone[1,2,3], with DnaJ[1]; DNA biosynthesis[3]; stress-related heat-shock DNA biosynthesis[1,2]; ATP-regulated binding and release of polypeptide substrates[1]; auto-regulated heat shock protein[2]; dnaK protein (heat shock protein 70) (HSP70)[5]
dps	b0812	-1.02	-1.29	-1.27	P	P	P	Stress response DNA-binding protein; starvation induced resistance to H_2O_2 phase[1]; global regulator, starvation condition[2]

Table 5.6. Continued.

Gene name	b No.	Fold change			Call			Description
		W-U*	VM-U**	VM-W***	untreated	water bath	VM	
ecpD	b0140	------	------	------	A	A	A	Possible pilin chaperone[1]; probable pilin chaperone similar to PapD[2]
fimC	b4316	-1.08	1.45	1.12	P	P	P	Biosynthesis of fimbriae; periplasmic chaperone for type 1 fimbriae[1,2]
flgM	b1071	------	------	------	A	A	A	Anti-sigma F factor (FliA) [1,2]; regulator of FlhD; also known as RflB protein[2]
fliA	b1922	------	------	------	A	A	A	Transcription sigma factor for class 3a and 3b operons; regulation of late gene expression[1]; flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons[2]
ftsJ	b3179	1.47	1.46	-1.01	P	P	P	Cell division and growth; heat inducible[1], cell division protein[2,3]
grpE	b2614	-1.00	1.02	1.02	P	P	P	Heat shock protein[1,2]; mutant survives induction of prophage lambda; stimulates DnaK ATPase; nucleotide exchange function[1]; phage lambda replication; host DNA synthesis; heat shock protein[2,3]; protein repair[2] heat shock protein grpE (heat shock protein b25.3) (HSP24)[5]
hflX	b4173	-1.32	1.02	1.35	P	A	P	Subunit of protease specific for phage λ CII repressor [3]; HflX GTpase, putative[1]; GTP - binding subunit of protease specific for phage lambda cII repressor[2]
hfq	b4172	-0.97	1.28	1.24	P	P	P	Host factor I for bacteriophase Q β replication[1,3]
hscA	b2526	1.21	1.28	1.02	A	P	P	Stress response gene[1]; Hsp70 family; heat shock protein[1,2], chaperone[2]; heat shock protein hscA (HSC66)[5]
hslJ	b1379	1.01	1.10	1.09	P	P	P	Heat-inducible; regulatory gene[1], heat shock protein hsIJ[2,4]
hslU	b3931	-1.48	-1.26	1.29	P	P	P	Heat-inducible ATP-dependent protease HslVU; heat shock protein D48.5[1]:heat shock protein hslVU[2,3,5], ATPase subunit[2,3], homologous to chaperones[2]
hslV	b3932	1.80	1.22	1.04	P	P	P	Heat shock regulon[1]; heat shock protein hslVU[2,3,5], proteasome-related peptidase subunit[2,3]

Table 5.6. Continued.

Gene name	b No.	Fold change			Call			Description
		W-U*	VM-U**	VM-W***	untreated	water bath	VM	
htgA	b0012	1.56†	1.21	-1.29	P	P	P	Positive regulator for sigma 32 heat shock promoters[2]; heat shock protein htgA (heat shock protein htpy)[5]
htpG	b0473	-1.41	-1.06	1.40	P	A	P	Heat shock protein C62.5[1,2,5] ; chaperone[1]; chaperone Hsp90[2,3] ;heat shock protein htpG (high temperature protein G) (heat shock protein c62.5)[5]
htpX	b1829	1.04	1.08	1.04	P	P	P	Protein expressed as heat shock regulon member[1]; heat shock protein, integral membrane protein[2,3] ; probable protease htpX (heat shock protein htpX) [5]
htrA	b0161	------	------	------	A	A	A	Periplasmic serine protease Do; heat shock protein HtrA[2]
htrB	b1054	1.27	1.22	-1.04	P	P	P	Not under heat shock regulation; membrane protein affecting cell division, growth, and high-temperature survival[1]; heat shock protein[2]
htrC	b3989	------	------	------	A	A	A	Essential for growth at high temperature, under sigma 32 (heat shock) regulation[1]; heat shock protein htrC[2] ; heat shock protein C[5]
htrE	b139	------	------	------	A	A	A	Outer membrane usher protein htrE precursor (heat shock protein E)[5]; probable outer membrane porin protein involved in fimbrial assembly[2]; Sequence homology with pilin protein PapC[1]
hyfR	b2491	------	------	------	A	A	A	Formate-sensing regulator for hyf operon[1]; putative 2-component regulator, interaction with sigma 54[2]
ibpA	b3687	-1.31	1.03	1.34	P	P	P	Chaperone, heat-inducible protein of HSP20 family[1]; heat shock protein[2] Inclusion body protein A[3]; 16 kD heat shock protein A[5]
ibpB	b3686	-1.46	-1.12	1.30	P	P	P	Chaperone, heat-inducible protein of HSP20 family[1]; heat shock protein[2] Inclusion body protein B[3]; 16 kD heat shock protein B[5]
inaA	b2237	-1.16	1.18	1.36	P	P	P	Protein induced by acid, independent of SoxRS regulation[1]; pH-inducible protein involved in stress response[2]

Table 5.6. Continued.

Gene name	b No.	Fold change			Call			Description
		W-U*	VM-U**	VM-W***	untreated	water bath	VM	
lon	b0439	1.21	1.38	1.14	P	P	P	DNA-binding, ATP-dependent protease LA[1,2,3]; lon mutants form long cells[1]; heat shock K-protein[2,3]; nucleic acid – binding heat shock protein[4]
lysU	b4129	1.04	1.13	1.08	A	P	A	Lysyl tRNA synthetase, inducible[1,2]; heat shock protein[2];nucleic acid-binding heat shock protein[4]
miaA	b4171	-1.02	1.07	1.09	P	P	P	Δ(2)- Isopentenyl pyrophosphate tRNA-adenosine transferase [2,3]; 2-methylthio-N6-isopentyladenosine tRNA hypermodification[1]
mopA	b4143	-1.31	-1.36	-1.04	P	P	P	GroEL, chaperone Hsp60[2,3]; peptide-dependent ATPase; heat shock protein[2]
mopB	b4142	1.04	-1.19	-1.24	P	P	P	GroES, 10 KDalton chaperone binds to Hsp60[2,3] in pres. Mg-ATP, suppressing its ATPase activity[2]
msbB	b1855	1.90†	1.65	-1.15	A	P	P	Role in outer membrane structure or function[1]; suppressor of htrB, heat shock protein[2]
narJ	b1226	------	------	------	A	A	A	Nitrate reductase delta-subunit[1,2]; chaperone[1]; nitrate reductase 1, delta subunit, assembly function[2]
nhaA	b0019	1.62	1.22	-1.33	A	A	P	Na+/H+ antiporter[1,2]; stress response to high salinity and pH[1]; pH dependent[2]
pphA	b1838	------	------	------	A	A	A	Phosphoprotein phosphatase involved in signalling protein misfolding[1,2]; heat shock regulon[1]; protein phosphatase 1 [2]
pphB	b2734	1.06	1.25	1.19	P	P	P	Phosphoprotein phosphatase involved in signalling protein misfolding; heat shock regulon[1]; protein phosphatase 2[2]
rpoD	b3067	-1.13	1.06	1.20	P	P	P	RNA polymerase, sigma 70[3,1]; sigma suc-unit, initiates most exponential phase transcription[1]
rpoE	b2573	1.90	1.41	-1.34	P	P	P	RNA polymerase, sigma E-subunit[1,2,3], high-temperature transcription[1]; heat shock and oxidative stress[2,3]
rpoH	b3461	2.12	1.68	-1.27	P	P	P	RNA polymerase,sigma 32-subunit[1,2], heat-shock transcription[1]; regulation of proteins induced at high temperatures[2]

Table 5.6. Continued.

Gene name	b No.	Fold change			Call			Description
		W-U*	VM-U**	VM-W***	untreated	water bath	VM	
rpoS	b2741	1.71	1.48	-1.16	P	P	P	RNA polymerase[1,2] sigma S-subunit[1], sigma S (sigma38) factor[2] stationary phase[1]; synthesis of many growth phase related proteins[2]
rseA	b2572	1.29	-1.00	-1.29	P	P	P	Membrane protein[1,2], negative regulator of sigma E[1,2,3];
rseB	b2571	-1.00	1.03	1.04	P	P	P	Binds rseA, negative regulation of sigma E[1]; regulates activity of sigma-E factor[2]
rseC	b2570	------	------	------	A	A	A	Deletion does not affect sigma E activity[1]; sigma-E factor, negative regulatory protein[2]
secB	b3609	-1.72†	-1.79†	-1.04	P	P	P	Protein export[1,2]; chaperone SecB[1];molecular chaperone; may bind to signal sequence[2]
sfmC	b0531	------	------	------	A	A	A	Salmonella fimbriae gene homolog[1]; putative chaperone[2]
sspA	b3229	1.26	1.08	-1.17	P	P	A	Stress response protein[1]; regulator of transcription; stringent starvation protein A[2]
sspB	b3228	1.25	1.37	1.10	P	P	P	Stress response protein[1]; stringent starvation protein B[2]
stpA	b2669	------	------	------	A	A	A	Hns-like protein[1,2], suppresses T4 tf mutant[1] ; DNA-binding protein; chaperone activity; RNA splicing? [2]
sugE	b4148	1.18	1.02	-1.15	A	P	A	Suppresses groL mutation and mimics effects of gro overexpression[1]; suppresses groEL, may be chaperone[2]
suhB	b2533	-1.32	-1.57	-1.19	P	A	A	Inositol monophosphate[1]; enhances synthesis of sigma32 in mutant; extragenic suppressor, may modulate RNAse III lethal action[2]
tig	b0436	-1.49	-1.37	1.08	P	P	P	Trigger factor; chaperone[1,2]; a molecular chaperone involved in cell division[2]
topA	b1274	-1.10	1.06	1.17	P	P	P	DNA topoisomerase type I, Ω protein[3]; Topoisomerase I, Omega protein I [1]
uspA	b3495	-1.70†	-1.36	1.25	P	P	P	Global regulatory gene for stress response[1]; broad regulatory function? [2]
ybeW	b0650	------	------	------	A	A	A	Function unknown[1];putative dnaK protein[2]
ybgP	b0717	------	------	------	A	A	A	Function unknown[1]; putative chaperone[2]

Table 5.6. Continued.

Gene name	b No.	Fold change			Call			Description
		W-U*	VM-U**	VM-W***	untreated	water bath	VM	
ycaL	b0909	------	------	------	A	A	A	Function unknown[1]; putative heat shock protein[2]
ycbF	b0944	------	------	------	A	A	A	Function unknown[1]; putative chaperone[2]
ycbR	b0939	------	------	------	A	A	A	Function unknown[1]; putative chaperone[2]
yciM	b1280	1.13	-1.31	-1.47	P	P	A	Function unknown[1]; putative heat shock protein[2]
yegD	b2069	------	------	------	A	A	A	Function unknown[1]; putative heat shock protein[2]
yehC	b2110	------	------	------	A	A	A	Function unknown[1]; putative chaperone[2]
yfcS	b2336	------	------	------	A	A	A	Function unknown[1]; putative chaperone[2]
yfiA	b2597	-3.57†	-2.91	1.23	P	P	P	Function unknown[1];putative yhbH sigma 54 modulator[2]
yhcA	b3215	------	------	------	A	A	A	Function unknown[1]; putative chaperone[2]
yraI	b3143	------	------	------	A	A	A	Function unknown[1]; putative chaperone[2]
yrfH	b3400	-1.13	-1.06	1.07	P	P	P	Binding nucleic acid-heat shock protein[4]; orf, hypothetical protein[2]; Function unknown[1]

[1]http://genolist.pasteur.fr/Colibri/
[2]Affymetrix gene chip data base
[3]Richmond et al. 1999
[4]Korber et al. 1999
[5]http://www.genome.ad.jp/kegg/
†significant genes ($p<0.05$)
* W-U= water bath treatment compare to untreated *E. coli*
** VM-U= VM711W compare to untreated *E. coli*
*** VM-W= VM711W compare to water bath treatment
[6]P= present, detected
[7]A= absent, not detected

Table 5.7. Genes displaying up-regulation in vacuum microwave and water bath under vacuum treated cells compared to untreated stationary phase *E. coli* cells.

Gene name	b no	Fold change		Call			Description
		VM-U**	W-U*	untreated	VM	water bath	
aceE	b0114	1.71	1.83	P[3]	P	P	pyruvate dehydrogenase (decarboxylase component)[1]
argC	b3958	1.36	1.69	A[4]	P	P	N-acetyl-gamma-glutamylphosphate reductase[1]
	b0257	1.67	1.83	P	P	P	putative transposase[1]
	b0845	1.7	1.92	A	P	P	putative DEOR-type transcriptional regulator[1]
	b1342	1.39	1.31	A	P	P	orf, hypothetical protein[1]
	b1600	1.71	1.45	A	P	P	possible chaperone[1]
	b1680	1.54	1.44	P	P	P	orf, hypothetical protein[1]
	b1754	1.55	1.59	A	P	P	orf, hypothetical protein[1]
	b2899	1.52	1.35	P	P	P	putative oxidoreductase[1]
chaA	b1216	1.36	1.51	A	P	P	sodium-calcium/proton antiporter[1]
cysW	b2423	2.48	2.33	A	P	P	sulfate transport system permease W protein[1]
fdx	b2525	1.24	1.22	A	P	P	[2FE-2S] ferredoxin, electron carrier protein[1]
fhuB	b0153	1.1	1.22	A	P	P	hydroxamate-dependent iron uptake, cytoplasmic membrane component[1]
gloB	b0212	1.77	1.82	P	P	P	probable hydroxyacylglutathione hydrolase[1]
hdhA	b1619	1.68	1.75	A	P	P	NAD-dependent 7alpha-hydroxysteroid dehydrogenase, dehydroxylation of bile acids[1]
marR	b1530	1.46	1.91	A	P	P	multiple antibiotic resistance protein; repressor of mar operon[1]
msbB	b1855	1.65	1.9	A	P	P	suppressor of htrB, heat shock protein[1]

Table 5.7. Continued.

Gene name	b no	Fold change		Call			Description
		VM-U**	W-U*	untreated	VM	water bath	
murG	b0090	1.31	1.44	A	P	P	UDP-N-acetylglucosamine:N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase[1]
pabC	b1096	1.76	1.87	A	P	P	4-amino-4-deoxychorismate lyase[1]
pgpA	b0418	1.34	1.48	P	P	P	phosphatidylglycerophosphatase[1]
ptrB	b1845	1.51	1.44	A	P	P	protease II[1]
rrlD	b3275	299.90	250.41	A	P	P	23S rRNA of rrnD operon[1]
trpT	b3761	3.95	4.11	A	P	P	Tryptophan tRNA[1]
yaaJ	b0007	1.64	1.71	P	P	P	inner membrane transport protein[1]
yafN	b0232	1.39	1.31	P	P	P	orf, hypothetical protein[1]
ybaR	b0484	3.19	4.14	A	P	P	putative ATPase[1]
ybdF	b0579	1.41	1.46	P	P	P	orf, hypothetical protein[1]
ybgA	b0707	1.47	1.29	P	P	P	orf, hypothetical protein[1]
ybhR	b0792	1.41	1.30	P	P	P	orf, hypothetical protein[1]
ybiM	b0806	1.28	1.70	A	P	P	orf, hypothetical protein[1]
ybiO	b0808	1.40	1.30	P	P	P	putative transport protein[1]
ycfR	b1112	2.27	1.84	A	P	P	orf, hypothetical protein[1]
ycgJ	b1177	1.61	1.54	P	P	P	orf, hypothetical protein[1]
yeiO	b2170	1.33	1.60	A	P	P	putative transport[1]
yhiI	b3487	1.35	1.49	P	P	P	putative membrane protein[1]
yi21_6	b4272	1.52	1.55	A	P	P	IS2 hypothetical protein[1]
yjcB	b4060	1.86	1.95	P	P	P	orf, hypothetical protein[1]
yqjA	b3095	1.19	1.30	P	P	P	orf, hypothetical protein[1]

[1] Affymetrix web site

* W-U= water bath treatment compare to untreated *E. coli*

** VM-U= VM711W compare to untreated *E. coli*

[3]P= present, detected

[4]A= absent, not detected

Table 5.8. Genes displaying down-regulation in vacuum microwave and water bath under vacuum treated cells compared to untreated *E. coli* cells.

Gene name	b no	Fold change		Call			Description
		VM-U**	W-U*	untreated	VM	water bath	
fbp	b4232	1.65	1.85	P	P	P	fructose-bisphosphatase[1]
folE	b2153	1.51	1.56	P	P	P	GTP cyclohydrolase I[1]
glpT	b2240	1.64	1.65	P	A	P	sn-glycerol-3-phosphate permease[1]
hisP	b2306	1.54	1.31	P	P	P	ATP-binding component of histidine transport[1]
ompF	b0929	2.37	2.04	P	P	P	outer membrane protein 1a (Ia;b;F) [1]
purC	b2476	1.75	1.93	P	P	P	phosphoribosylaminoimidazole-succinocarboxamide synthetase[1]
rrfD	b3274	29.90	75.75	P	P	P	5S rRNA of rrnD operon[1]
rrfE	b4010	9.85	24.12	P	P	P	5S rRNA of rrnE operon[1]
rrfF	b3272	19.42	71.88	P	P	P	5S rRNA of rrnD operon[1]
rrfG	b2588	11.22	74.23	P	P	P	5S rRNA of rrnG operon[1]
secB	b3609	1.788	1.72	P	P	P	protein export; molecular chaperone; may bind to signel sequence[1]
slp	b3506	1.627	1.41	P	P	P	outer membrane protein induced after carbon starvation[1]
smf_2	b3285	1.43	1.40	P	P	P	orf, fragment 2[1]
tsr	b4355	1.38	1.35	P	P	P	methyl-accepting chemotaxis protein I, serine sensor receptor[1]
ynaF	b1376	1.84	2.10	P	P	P	putative filament protein[1]
ytfJ	b4216	1.53	1.64	P	P	P	orf, hypothetical protein[1]

[1] Affymetrix web site

* W-U= water bath treatment compare to untreated *E. coli*

** VM-U= VM711W compare to untreated *E. coli*

[3]P= present, detected

[4]A= absent, not detected

Table 5.9. Genes down-regulated (≥2 fold) in water bath under vacuum treated *E. coli* compared to untreated stationary phase *E. coli* cells ($p<0.05$).

Gene name	b number	Fold change	Call		Description
			untreated	water bath	
aspA	b4139	2.38	P[3]	P	aspartate ammonia-lyase (aspartase)[1,2]
glnA	b3870	3.52	P	A[4]	glutamine synthetase[1,2] (glutamate--ammonia ligase)[1]
glpF	b3927	2.16	P	P	glycerol uptake facilitator protein[1], facilitated diffusion of glycerol[2]
glpQ	b2239	2.58	P	P	glycerophosphoryl diester phosphodiesterase periplasmic precursor[1](glycerophosphodiester phosphodiesterase)[1,2]
ompF	b0929	2.04	P	P	outer membrane protein F precursor (outer membrane protein 1a, ia, or B)[1], outer membrane protein 1a (Ia;b;F)[2]
ppa	b4226	1.96	P	A	inorganic pyrophosphatase[1,2]
rrfA	b3855	3.70	P	P	5S ribosomal RNA[1], 5S rRNA of rrnA operon[2]
rrfD	b3274	75.75	P	P	5S ribosomal RNA[1], 5S rRNA of rrnD operon[2]
rrfE	b4010	24.12	P	P	5S ribosomal RNA[1], 5S rRNA of rrnE operon[2]
rrfF	b3272	71.88	P	P	5S ribosomal RNA[1], 5S rRNA of rrnD operon[2]
rrfG	b2588	74.23	P	P	5S ribosomal RNA[1], 5S rRNA of rrnG operon[2]
rrfH	b0205	5.79	P	P	5S ribosomal RNA[1], 5S rRNA of rrnH operon[2]
rrsH	b0201	2.64	P	P	16S ribosomal RNA[1], 16S RNA of rrnH operon[2]
yfiA	b2597	3.57	P	P	12.7 kD protein in sfhB-pheL intergenic region (URF1) (ORFS54)[1], putative yhbH sigma 54 modulator[2]
ynaF	b1376	2.09	P	P	putative filament protein[1,2]

[1]http://www.genome.ad.jp/kegg/
[2] Affymetrix gene chip data base
[3]P= present, detected
[4]A= absent, not detected

Table 5.10. Genes up-regulated (≥2 fold) in water bath under vacuum treated *E. coli* compared to untreated stationary phase *E. coli* cells ($p<0.05$).

Gene name	b no.	Fold change	Call		Description
			untreated	water bath	
	b1657	1.99	A[4]	P[3]	putative transport protein[1,2]
cysW	b2423	2.33	A	P	sulfate transport system permease protein[1], sulfate transport system permease W protein[2]
glnX	b0664	5.67	A	P	glutamine tRNA 2[1,2]
leuX	b4270	2.29	A	P	leucine tRNA 5[1], Leucine tRNA5 (amber [UAG] suppressor)[2]
pdhR	b0113	2.59	A	P	pyruvate dehydrogenase complex repressor[1], transcriptional regulator for pyruvate dehydrogenase complex[2]
rcsA	b1951	2.05	A	P	colanic acid capsular biosynthesis activation protein A[1], positive regulator for ctr capsule biosynthesis, positive transcription factor[2]
rrlD	b3275	250.41	A	P	23S ribosomal RNA[1], 23S rRNA of rrnD operon[2]
trpT	b3761	4.11	A	P	tryptophan tRNA[1,2]
ybaR	b0484	4.14	A	P	probable copper-transporting ATPase[1], putative ATPase[2]
ycfC	b1132	1.98	A	P	hypothetical 22.9 kD protein in purB-icdA intergenic region (ORF- 23)[1], orf, hypothetical protein[2]
yjcB	b4060	1.95	P	P	hypothetical 13.0 kD protein in ssb-soxS intergenic region (F116)[1], orf, hypothetical protein[2]
yjgN	b4257	2.10	A	P	hypothetical 44.4 kD protein in argI-valS intergenic region[1], orf, hypothetical protein[2]

[1]http://www.genome.ad.jp/kegg/
[2] Affymetrix gene chip data base
[3]P= present, detected
[4]A= absent, not detected

Table 5.11. Genes down-regulated (≥2 fold) in VM treated cells compared to untreated stationary phase *E. coli* cells ($p<0.05$).

Gene name	b no.	Fold change	Call		Description
			untreated	VM	
	b1746	2.27	P[3]	A[4]	putative aldehyde dehydrogenase[1,2]
ompF	b0929	2.37	P	P	outer membrane protein F precursor (outer membrane protein 1a, ia, or B) [1], outer membrane protein 1a (Ia;b;F) [2]
rrfD	b3274	29.90	P	P	5S ribosomal RNA[1], 5S rRNA of rrnD operon[2]
rrfE	b4010	9.85	P	P	5S ribosomal RNA[1], 5S rRNA of rrnE operon[2]
rrfF	b3272	19.42	P	P	5S ribosomal RNA[1], 5S rRNA of rrnD operon[2]
rrfG	b2588	11.22	P	P	5S ribosomal RNA[1], 5S rRNA of rrnG operon[2]

[1]http://www.genome.ad.jp/kegg/
[2] Affymetrix gene chip data base
[3]P= present, detected
[4]A= absent, not detected

Table 5.12. Genes up-regulated (≥2 fold) in VM treated cells compared to untreated stationary phase *E. coli* cells ($p<0.05$).

Gene name	b no.	Fold change	Call		Description
			untreated	VM	
cysW	b2423	2.48	A[4]	P[3]	sulfate transport system permease protein[1], sulfate transport system permease W protein[2]
glnV	b0665	3.16	A	P	glutamine tRNA 2[1,2]
hype	b2730	2.53	A	P	hydrogenase isoenzymes formation protein hypE[1], plays structural role in maturation of all 3 hydrogenases[2]
rrlD	b3275	299.90	A	P	23S ribosomal RNA[1], 23S rRNA of rrnD operon[2]
soxR	b4063	1.98	A	P	soxR protein[1], redox-sensing activator of soxS[2]
trpT	b3761	3.95	A	P	tryptophan tRNA[1,2]
ybaR	b0484	3.19	A	P	probable copper-transporting ATPase[1], putative ATPase[2]
ycfR	b1112	2.27	A	P	hypothetical 8.8 kD protein in ndh-mfd intergenic region[1], orf, hypothetical protein[2]
ycgB	b1188	2.04	A	P	putative sporulation protein[1,2]
yhbU	b3158	3.68	A	P	putative protease in sohA-mtr intergenic region precursor[1], putative collagenase[2]

[1]http://www.genome.ad.jp/kegg/
[2]Affymetrix gene chip data base
[3]P= present, detected
[4]A= absent, not detected

Table 5.13. *E. coli* genes down-regulated in VM treatment compared to water bath under vacuum treatment ($p<0.05$).

Gene name	b no.	Fold change	Call		Description
			water bath	VM	
	b0878	1.78	P[3]	A[4]	putative membrane protein[1,2]
	b1844	1.40	P	P	exodeoxyribonuclease X[1], orf, hypothetical protein[2]
	b0538	1.40	P	A	putative sensory transduction regulator[1,2]
	b1501	1.34	P	P	putative oxidoreductase, major subunit, putative oxidoreductase, major subunit[1,2]
	b1837	1.37	P	P	orf[2], hypothetical protein[1,2]
	b2999	1.26	P	A	orf[2], hypothetical protein[1,2]
amyA	b1927	1.22	P	P	cytoplasmic alpha-amylase[1,2], (1,4-alpha-D-glucan glucanohydrolase)[1]
btuC	b1711	1.24	P	P	vitamin B_{12} transport permease protein[1,2]
deoR	b0840	1.46	P	P	deoxyribose operon repressor[1], transcriptional repressor for deo operon, tsx, nupG[2]
exuT	b3093	1.21	P	P	hexuronate transporter [1,2]
fhiA	b0229	1.15	P	A	flagellar biosynthesis[2], fhiA protein[1]
marR	b1530	1.31	P	P	multiple antibiotic resistance protein [1,2]; repressor of mar operon[2], marR [1]
menD	b2264	1.32	P	P	2-oxoglutarate decarboxylase; SHCHC synthase [2], 2-oxoglutarate decarboxylase / 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase[1]
murG	b0090	1.10	P	P	UDP-N-acetylglucosamine:N-acetylmuramyl- (pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase[1,2]
rcsA	b1951	1.41	P	A	colanic acid capsular biosynthesis activation protein A[1], positive regulator for ctr capsule biosynthesis, positive transcription factor[2]
sfsA	b0146	1.40	P	P	probable regulator for maltose metabolism[2], sugar fermentation stimulation protein[1],
yacC	b0122	1.24	P	P	hypothetical 12.8 kD protein in speE-gcd intergenic region precursor[1], orf, hypothetical protein[2]
yadD	b0132	1.37	P	P	hypothetical 34.6 kD protein in panD-panC intergenic region[1], orf, hypothetical protein[2]

Table 5.13. Continued.

Gene name	b no.	Fold change	Call		Description
			water bath	VM	
ybjD	b0876	1.48	P	P	orf, hypothetical protein[2] , hypothetical 63.6 kD protein in aqpZ-cspD intergenic region[1]
ycdT	b1025	1.63	P	P	orf, hypothetical protein[2], hypothetical 51.8 kD protein in phoH-csgG intergenic region[1]
ycjO	b1311	1.57	P	P	multiple sugar transport system permease protein[1], putative binding-protein dependent transport protein[2]
ydgB	b1606	1.60	P	A	hypothetical oxidoreductase in pntA-rstA intergenic region[1], putative oxidoreductase[2]
ydiQ	b1697	1.26	P	P	putative transport protein[2], putative electron transfer flavoprotein subunit ydiq[1]
yeaJ	b1786	1.21	P	P	orf, hypothetical protein[2], hypothetical 63.2 kD protein in gapA-rnd intergenic region[1]
yeaQ	b1795	1.30	P	P	hypothetical 8.7 kD protein in gapA-rnd intergenic region[1], orf, hypothetical protein[2]
yeiG	b2154	1.55	P	P	putative esterase[1,2] (EC 3.1.1.-) [1]
yejE	b2179	1.19	P	P	peptide transport system permease protein[1], putative transport system permease protein[2]
yfcC	b2298	1.16	P	P	putative S-transferase[1,2]
ygaF	b2660	1.21	P	A	hypothetical 48.6 kD protein in alpA-gabP intergenic region[1], orf, hypothetical protein[2]
yihG	b3862	1.32	P	P	hypothetical 36.3 kD protein in dsbA-polA intergenic region[1], putative endonuclease[2]
ylbA	b0515	1.47	P	P	hypothetical 28.7 kD protein in GIP-fdrA intergenic region[1], orf, hypothetical protein[2]
ymfA	b1122	1.39	P	P	orf, hypothetical protein[2], hypothetical 17.8 kD protein in cobb-potB intergenic region[1]

Table 5.13. Continued.

Gene name	b no.	Fold change	Call		Description
			water bath	VM	
ymfN	b1149	1.21	P	P	orf, hypothetical protein[2], hypothetical 50.9 kD protein in inte-pin intergenic region [1]
yrhA	b3443	1.60	P	A	hypothetical 16.0 kD protein in gntR-ggt intergenic region (O138) [1], orf, hypothetical protein[2]

[1]http://www.genome.ad.jp/kegg/
[2]Affymetrix gene chip data base
[3]P= present, detected
[4]A= absent, not detected

Table 5.14. *E. coli* genes up-regulated in VM treatment compared to water bath under vacuum treatment ($p<0.05$).

Gene name	b no.	Fold change	Call		Description
			water bath	*VM*	
	b2496	1.29	A[4]	P[3]	putative DNA replication factor[1,2]
	b2174	1.16	P	P	orf[2], hypothetical protein[1,2]
	b2511	1.29	P	P	putative GTP-binding factor[1,2]
	b2595	1.20	P	A	orf[2], hypothetical protein[1,2]
	b2689	1.32	P	P	orf[2], hypothetical protein[1,2]
	b3051	1.22	P	P	putative membrane protein[1,2]
	b3694	1.48	A	P	putative FADA-type transcriptional regulator[1,2]
argR	b3237	1.16	P	P	repressor of arg regulon; cer-mediated site specific recombination[2], arginine repressor[1]
fecA	b4291	1.33	A	P	ferric citrate outer membrane receptor protein[1], outer membrane receptor; citrate-dependent iron transport, outer membrane receptor[2]
fimC	b4316	1.58	P	P	periplasmic chaperone, required for type 1 fimbriae[2], chaperone protein fimC precursor[1]
fimD	b4317	1.54	P	P	outer membrane protein; export and assembly of type 1 fimbriae, interrupted[2], outer membrane usher protein fimD precursor[1]
fimG	b4319	1.62	P	P	fimbrial morphology[2], fimG protein precursor[1]
fliG	b1939	1.27	A	P	flagellar motor switch protein fliG[1], flagellar biosynthesis, component of motor switching and energizing, enabling rotation and determining its direction[2]
glgC	b3430	1.33	P	P	glucose-1-phosphate adenylyltransferase[1,2]
glnS	b0680	1.34	P	P	glutamine tRNA synthetase[2], glutaminyl-tRNA synthetase[1]
gloA	b1651	1.22	P	P	lactoylglutathione lyase [1,2]
gpsA	b3608	1.47	P	P	glycerol-3-phosphate dehydrogenase (NAD+)[1,2]
mreB	b3251	1.33	P	P	regulator of ftsI, penicillin binding protein 3, septation function[2], rod shape-determining protein mreB[1]

Table 5.14. Continued.

Gene name	b no.	Fold change	Call		Description
			water bath	*VM*	
murD	b0088	1.45	P	P	UDP-N-acetylmuramoylalanine-D-glutamate ligase[1,2] (UDP-N-acetylmuranoyl-L-alanyl-D-glutamate synthetase)[1]
phoP	b1130	1.39	P	P	transcriptional regulatory protein[1,2] phoP[1]
ppiA	b3363	1.24	P	P	peptidyl-prolyl cis-trans isomerase A[1,2] precursor (ppiase A)[1] (rotamase A)[1,2] (cyclophilin A)[1]
spoT	b3650	1.41	P	P	guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase[1], (p)ppGpp synthetase II; also guanosine-3 ,5 -bis pyrophosphate 3 –pyrophosphohydrolase[2]
ubiB	b3844	1.28	P	P	NAD(P)H-flavin reductase (ferrisiderophore reductase C)[1,2],(NADPH:flavin oxidoreductase)[2]
ycgL	b1179	1.35	A	P	orf, hypothetical protein[2], hypothetical 12.4 kD protein in minC-shea intergenic region[1]
yeaA	b1778	1.15	P	P	orf, hypothetical protein[2], peptide methionine sulfoxide reductase[1]
yghB	b3009	1.11	P	P	orf, hypothetical protein[2], hypothetical 24.1 kD protein in metC-sufI intergenic region[1]
yhcS	b3243	1.41	P	P	putative transcriptional regulator LYSR-type[2], hypothetical transcriptional regulator in argR-cafA intergenic region[1]
yhfA	b3356	1.27	P	P	orf, hypothetical protein[2], hypothetical 14.5 kD protein in prkB-CRP intergenic region (F134)[1]
yhhF	b3465	1.31	P	P	putative methylase[1], orf, hypothetical protein[2]
yhhL	b3466	1.22	P	P	orf, hypothetical protein[2], hypothetical 10.3 kD protein in ftsY-nikA intergenic region[1]
yicH	b3655	1.17	P	P	orf, hypothetical protein[2], hypothetical 62.3 kD protein in gltS-selC intergenic region[1]
yjjA	b4360	1.25	P	P	putative glycoprotein/receptor[2], hypothetical 17.5 kD protein in mdoB-dnaC intergenic region precursor (protein P-18) (F165)[1]

Table 5.14. Continued.

Gene name	b no.	Fold change	Call		Description
			water bath	*VM*	
ytfB	b4206	1.35	P	P	orf, hypothetical protein[2], hypothetical 24.9 kD protein in rplI-cpdB intergenic region (F224) [1]
yrfE	b3397	1.53	P	P	orf, hypothetical protein[2], ADP compounds hydrolase[1]

[1]http://www.genome.ad.jp/kegg/
[2]Affymetrix gene chip data base
[3]P= present, detected
[4]A= absent, not detected

CHAPTER SIX

ESCHERICHIA COLI TRANSCRIPTOME IN LATE-LOG AND MID-STATIONARY PHASE OF GROWTH

6.1 Introduction

Bacteria in natural environments are constantly challenged by the need to adapt to changes in nutrient availability and stress conditions. Bacterial cells growing in an optimal condition are not an exception. They are also exposed to a continuous change of environment due to constant consumption of nutrients and accumulation of waste products (Singleton & Sainsbury 2000). Thus referring to a bacterial cell without mentioning their growth condition is as meaningless as talking about them without specifying their strains or their stage of growth (Neidhardt & Umbarger 1996).

Most researchers use the mid-logarithmic phase as their experimental control for studying cell physiology or response either at the transcription or translation level, because cells in this stage are in a steady state and well-defined physiological phase, thus the experimental variability can be minimized and identified easily (Conway & Schoolnik 2003). On the other hand, in nature, bacterial cells spend most of their life under conditions in which the amount of available nutrients is limited and they rarely encounter an environment that allows exponential growth. In addition, in the food industry the focus is on stationary phase bacteria that are able to survive stress conditions better than cells in the logarithmic phase. The ability of stationary phase cells to survive prolonged periods of starvation as well as their higher resistance to variable stress conditions compared to cells in exponential phase of growth has been demonstrated (Hengge-Aronis 1996).

The stationary-phase influences the entire cell physiology (Hengge-Aronis 1996). In the log phase cells the primary metabolisms such as energy metabolism and synthesis of cell components are the most active metabolism while in stationary phase cells the secondary metabolisms are dominant (Singleton & Sainsbury 2000). Thompson and colleagues (2003)

have studied growth-phase-dependent gene expression in *Helicobacter pylori* using a high-density DNA microarray. They conducted their experiment on cells during the late log-to-stationary phase and reported changes between two stages of growth in genes involved in iron homeostasis and iron-storage protein, neutrophil activating protein and the major flagellin subunit (*flaA*). de Saizieu and co-workers (1998) compared RNA samples from exponentially growing *Streptococcus pneumoniae* cells to early stationary phase cells and reported that genes related to the polysaccharide capsule biosynthesis, long-chain fatty acid biosynthesis and cell division were transcribed three to eight times less in stationary phase cells.

The primary response of *E. coli* cells to the limitation of a specific nutrient is activation of certain groups of genes for higher uptake of other nutrients that are present in low concentration, or for the utilization of other substances (Hengge-Aronis 1996). In contrast to the specific response, the stationary phase response is not dependent on the type of the limiting nutrient (Hengge-Aronis 1996).

A number of morphological and physiological changes have been identified in stationary phase *E. coli* cells, including thickened cell wall, condensed cytoplasm (Makinoshima et al. 2002), accumulation of polyphosphates (Kornberg 1995), variation in the compositions and proportions of RNA polymerase σ subunit , modulation of nucleoid (Ishihama 1999), decrease in DNA superhelicity (Jaworski et al. 1991; Kusano et al. 1996), smaller cells with a spherical rather than a rod-shaped morphology, increased tendency to form aggregates (Hengge-Aronis 1996), differential protein degradation (Lange & Hengge-Aronis 1994), and alterations in ribosome assembly (Wada et al. 1990).

In this section, DNA microarray technology was used to investigate the differences in gene expression of *E. coli* at mid-stationary phase compared to late-log phase. The goal was to

investigate the effect of growth phase on *E. coli* genome at the transcription level and to identify *E. coli* metabolism and functions involved in late-log and mid-stationary stage of growth through activation or inactivation of biochemical pathways.

6.2 Materials & Methods

6.2.1 Bacterial Strain

Escherichia coli (ATCC 11775) isolated from urine was purchased as freeze dried sample from American Type Culture Collection, Rockville, USA. For stock culture and inoculum preparation refer to chapter 4, section 4.2.2.

6.2.2 Growth determination

To determine the pattern of *E. coli* growth under conditions employed for cell growth and maintenance in chapters 4 and 5, one ml stationary phase culture was transferred into 50 ml Nutrient Broth (Difco) and incubated at 37°C. A 1 ml sample was taken hourly for 21 hours and serial dilutions were prepared with peptone water 0.1% (w/v). The duplicate dilutions were spread plated on Plate Count Agar and incubated at 37°C for 21 hours before enumeration. The average number of colonies for each dilution was calculated and the results were plotted as time versus log of colony forming unit per ml. The experiment was repeated twice.

6.2.3 Batch cultures

To prepare samples, one ml stationary phase culture was transferred into 50 ml of Nutrient Broth and incubated for 5 or 16 hours for late-log and mid-stationary phase samples, respectively. Then 48 ml *E. coli* pure culture containing 10^7 or 10^8 CFU/ml for late-log and mid-

stationary phase samples respectively was aseptically transferred into six sterile 15 ml centrifuge tubes (Fisher brand disposable sterile centrifuge tubes, seal cap, modified polystyrene, Fisher Scientific, Pittsburgh, PA, USA) and centrifuged at 2060×g at 4°C for 8 minutes (BECKMAN GS-6 centrifuge, Beckman Instrument, USA). Total RNA samples were extracted immediately from the pellet using a Qiagen RNeasy total RNA Isolation Mini kit (Valencia, CA, USA) (Chapter 5, section 5.2.5).

6.2.4 DNA microarray analysis

Reverse transcriptase and primers specific to 16S and 23S rRNA were used to synthesize complementary cDNAs. Then rRNA was removed enzymatically by Rnase H, which exclusively digested RNA within an RNA:DNA hybrid. The cDNA molecules are removed with Dnase I digestion and the enriched mRNA was purified on Qiagen RNEasy columns. The procedure for total RNA extraction, mRNA enrichment, fragmentation, labelling and hybridization were described previously (Chapter 5, sections 5.2.6, 5.2.7, 5.2.8, 5.2.9).

6.2.5 Statistical analysis

Linear regression (SYSTAT 1998) was used to determine the linear section of growth. One way ANOVA was used to find genes which expression was significantly different among treatments ($p<0.05$, $n=6$). (Chapter 5, section 5.2.10)

6.3 Results

To verify the stage of growth, the number of cells was plotted on a logarithmic scale against time on an arithmetic scale (Figure 6.1). The result of linear regression test of the data

points for the first 6 hours of growth showed a straight line with r^2 of 0.966 and $p<0.01$. The straight line in a semilogarithmic graph of bacteria growth is an immediate indicator of cells that are growing exponentially (Madigan et al. 2003). In addition, calculation of generation time showed that by 6 hours *E. coli* population have gone through four generation times, which means that despite the high initial population, cells were in log phase of growth and should be homogenous. Therefore the first 6 hours of growth were considered as exponential phase and a sample taken at 5 hours was considered as late-exponential phase.

The correlation among present calls of replicates for each sample showed an average of 0.90 ± 0.08. In single arrays, 49% and 46% of probe sets were identified as present and absent respectively in mid-stationary phase cells, while the values for late-exponential phase samples were 46% and 49% respectively. For all the samples, 4.3-4.5% of probe sets were in the marginal category (Table 6.1).

A comparison of the mid-stationary phase expression data with that obtained from the late-exponential growth identified 494 down-regulated (11.22%) and 84 up-regulated (1.91%) genes. Of these, 304 genes were down-regulated and 12 genes were up-regulated more than two fold in mid-stationary phase *E. coli* cells. Most of genes (86.88%) remained unchanged between the two growth phases. A list of genes altered less than two fold among samples is presented in appendix VII (Tables 9.4, 9.5). The probe sets related to intergenic regions were not considered in this study.

6.3.1 Genes up-regulated (≥2 fold) in mid-stationary phase cells

Seven of the 12 genes that displayed ≥2 fold up-regulation in stationary phase cells are related to hypothetical proteins, which are not assigned to any known pathways. The *csgA*, *csgB*

and *csgD* genes involved in curli synthesis were induced in stationary phase cells by 14 and 12 fold for *csgA* and *csgB* respectively, and 2.25 fold for *csgD*. In addition *guaB* which encodes the enzyme inosine-monophosphate dehydrogenase and is related to purine metabolism, was expressed 3.54 fold more in stationary phase cells. (Table 6.3).

6.3.2 Genes down-regulated (≥2 fold) in mid-stationary phase cells

The name and description of genes down-regulated more than 2 fold in mid-stationary phase along with their fold change is shown in Table 6.4. The majority of down-regulated genes were assigned to six functional groups, namely translation, amino acid metabolism, carbohydrate metabolism, energy metabolism, cell motility and membrane transport (Table 6.2). About 30% of the expressed genes do not have functional annotation and are not assigned to any specific pathway.

6.3.2.1 Translation and transcription

The expression of genes that encode for ribosomal proteins was affected most in mid-stationary versus late-exponential growth phase. These genes showed the highest reduction in mid-stationary phase cells. Of the 85 genes related to translation, 55 genes were significantly down-regulated in mid-stationary samples (2.01-19.95 fold change). Those genes were mostly related to 30S and 50S ribosomal proteins. In addition, 6 other genes, from 13 genes that encode for translation factors, were also down-regulated in mid-stationary phase cells.

Genes that encode for RNA polymerase enzymes including *rpoA*, *rpoB* and *rpoC*, which are involved in transcriptional functions, were expressed more in cells at late-exponential phase of growth.

6.3.2.2 Energy metabolism

From 9 genes involved in ATP synthesis, 8 genes showed lower expression in mid-stationary cells. Twenty of the 41 genes related to oxidative phosphorylation were down-regulated more than two fold and 5 of the others were expressed 1.6-1.92 fold less in mid-stationary phase.

6.3.2.3 Cell motility

Bacterial cell genes involved in chemotaxis and flagellar assembly are responsible for cell motility (Madigan et al. 2003). Of the 41 genes known to be involved in flagellar assembly, 32 genes were down-regulated more than two fold in mid-stationary phase *E. coli*. Six other motility genes were not detected in any of the samples while 3 remained unchanged. Eleven of 20 genes involved in bacterial chemotaxis were down-regulated more than two fold in mid-stationary *E. coli* cells, while 3 were not detected in any of the samples, and the change in the rest was not significant.

6.3.2.4 Carbohydrate metabolism

The *aceE*, *aceF*, *lpdA*, *eno*, *fba* and *tdcD* genes which are involved in glycolysis, *caiB*, *sucC* and *sucD* which are involved in propanoate metabolism were expressed less in mid-stationary phase of growth. In addition, the *lpdA*, *acnB*, *gltA*, *sdhA*, *sucA*, *sucB*, *sucC* and *sucD* which are involved in citrate cycle were down-regulated in mid-stationary *E. coli* cells.

6.3.2.5 Fatty acid biosynthesis

Six genes from the fatty acid biosynthesis pathway were expressed more in late-exponential phase of *E. coli* growth, while 5 genes were not detected in any samples and one gene (*fabH*) remained unchanged. de Saizieu and co-workers (1998) showed that the *accC* gene, which is involved in long-chain fatty acid biosynthesis, was transcribed three to eight fold lower in stationary phase *Streptococcus pneumoniae* cells than in exponential phase cells.

6.3.2.6 Membrane and transport systems

Genes involved in fimbriae proteins were transcribed less in mid-stationary phase *E. coli* cells. Also *opmA*, *ompC*, *ompF*, *tolC* and *acrB* genes involved in membrane transport through pore ion channels were down-regulated in mid-stationary phase *E. coli* cells.

6.4 Discussion

6.4.1 Curli synthesis

Curli, thin fibres tending to coil up into a fuzzy mass on the surface of bacteria, are one of the adhesive organelles in *E. coli* (Hultgren et al. 1996). Curli also promote clumping of bacterial cells in culture and binding to abiotic surfaces such as glass and polystyrene, making them important for biofilm formation (Vidal et al. 1998; Austin et al. 1998; Prigent-Combaret et al. 2000).

The *csg* genes are required for curli synthesis (Chirwa & Herrington 2003). Studies have shown that the expression of *csg* genes is related to temperature, osmolarity, and the availability of the nutrients, oxygen and iron (Olsén et al. 1998; Gerstel & Römling 2001). Polymerization

of the curlin subunit to insoluble curli is dependent on the presence of a specific protein encoded by the *csgB* gene (CsgB) (Hammar et al. 1996). The *csgA* and *csgB* genes are co-transcribed (Arnqvist et al. 1994) and *csgD* encodes for a lipoprotein involved in secretion of curlin and CsgB (Hammar et al. 1995, 1996; Loferer et al. 1997). Others have reported that some regulatory proteins including RpoS, OmpR, and Cpx are responsible for CsgD expression (Arnqvist et al. 1994; Prigent-Combaret et al., 2001; Chirwa & Herrington 2003). In this study the *csgA*, *csgB* and *csgD* genes were induced in mid-stationary phase *E. coli* cells. The expression of *rpoS*, *ompR* genes displayed no significant change between mid-stationary and exponential *E. coli* samples and *cpx* was not detected in either phase of *E. coli* growth. This shows that curli synthesis was started in stationary phase of growth and may suggest that other regulatory systems could be involved in the activation of these genes. Hultgren and colleagues (1996) also reported cells develop curli in their stationary phase of growth.

Since curlin contains high amounts of glycine, Chirwa & Herrington (2003) proposed that up-regulation of *glyA* is an essential response for curli formation. In this study, although *glyA* was detected in all samples, its transcription level did not show any significant change between the two stages of growth. It is possible that *glyA* was expressed in the late-log phase to produce amino acid necessary for rapid cell growth and its expression continued in the stationary phase to provide necessary glycine for curli synthesis, whereas other genes involved in amino acid biosynthesis were down-regulated.

6.4.2 Cell motility

About 61 genes in *E. coli* are required for flagellar synthesis, chemotaxis and subsequent motility. These genes have several functions, including encoding structural proteins of the

flagellar apparatus, export of flagellar components through the membrane to the outside of the cell, and regulation of the many biochemical events surrounding the synthesis of new flagella (Madigan et al. 2003). In *E. coli* cells in the mid-stationary phase of growth, 70.5% of these genes were down-regulated or were not expressed at all. This indicates that *E. coli* cells in mid-stationary phase of growth may have less tendency to be mobile.

6.4.3 Transcription and translation

The expression profiles of genes involved in transcription and translation, including the major subunits of RNA polymerase, ribosomal proteins, and translation factors showed down-regulation in mid-stationary phase *E. coli*. The decrease in the overall translation activity or protein synthesis, has been reported to occur along with the transition from the exponential growth to the stationary phase in *E. coli* cells (Wada et al. 1990). Selinger and colleagues (2000) reported a decreased of expression for genes involved in protein synthesis (rRNA, tRNA and ribosomal protein) in stationary *E. coli* MG1655 cells compared to exponential cells. These genes are also reported to be down-regulated due to growth arrest (Chang et al. 2002).

6.4.4 Regulatory systems

Fis and Rpos are two regulatory proteins which co-ordinately control the expression of some of the genes during late-log and stationary phase of growth. While Fis expression is at its maximum in early-to-mid log, the expression of Rpos is turned on in late exponential and stationary phase. Fis reduces the expression of specific genes required for growth under sub-optimal nutrient conditions (Xu & Johnson 1995) and the expression of *fis* has also been reported to decrease during growth arrest (Chang et al. 2002). Rpos is required for expression of genes

important under starvation or stationary phase conditions (Xu & Johnson 1995). In this experiment the expression of *fis* gene was 2.25 fold higher in late-exponential phase, but interestingly, although the *rpoS* gene was detected as present in late-log and mid-stationary of *E. coli*, its expression did not show any significant change between two stages of growth. Selinger and colleagues (2000) also did not find any increase in *rpoS* in *E. coli* K-12 stationary cell compared to exponenetial cells.

In addition, Xu & Johnson (1995) reported that the products of *xylF* and *mglA* are required for *E. coli* growth under nutrient-poor conditions in which *fis* levels are low. The result of this experiment supports Xu & Johnson as expression of the *xylF* gene was 1.57 fold higher in mid-stationary phase. However the *mglA* gene had a higher expression in late-exponential phase of growth where *fis* level was not reduced. Xu & Johnson (1995) also reported the presence of *rpoS* reduced the expression of *xylF*, *mglA* and *sdhA*. The present study showed that while the expression of *rpoS* did not change, the expression of other three genes were altered between the two stages of growth. This could suggest that other regulatory genes are involved in the induction or repression of these genes in different stages of growth. Schellhorn and co-workers (1998) also found that the expression of some of the growth dependent genes can be changed without the presence of *rpoS* and they concluded that probably many growth-phase-regulated functions in *E. coli* do not require RpoS for expression. Another explanation is that most changes reported in literature were detected at the protein level and the correlation between gene transcript and protein activity is not expected to be perfect (Selinger et al. 2000).

6.4.5 Early stationary phase genes

Previous studies have shown that the expression of genes which encode basic proteins that non-specifically bind DNA (*hupA*, *hupB* and *hlpA*) (Dersch et al. 1993; Weglenska et al. 1996), and genes that encode for the integration host factor (*himA* and *himD*) (Claret & Rouviere-Yaniv 1997) increases upon entry into stationary phase. The study of Chang and colleagues (2002) also showed up-regulation for the above genes as a result of growth arrest. In the present study, transcription of *hupA* (2.42 fold), *hupB* (3.33 fold), and *hlpA* (3.26 fold) showed down-regulation in mid-stationary phase *E. coli* cells. In addition the transcription of *himA* and *himD* decreased 2.68 and 1.81 fold in mid-stationary phase *E. coli*. The transition of *E. coli* cells to stationary phase of growth has been described as a general stress response (Hengge-Aronis 1996, 1999). Changes in gene expression at early stationary phase is a short-term response to external stress factors while mid and late stationary phase are long-term stress responses. Differences in short-term and long term stress response, in this case early and mid stationary phase, would be expected. Azam and co-workers (1999) also reported some changes in gene expression of *E. coli* cells in early and late stationary phase.

6.5 Conclusion

In the mid-stationary phase of growth, genes encoding for energy metabolism as well as amino acid and carbohydrate metabolism were down-regulated. In addition, transcription of genes involved in fimbriae synthesis was reduced while genes encoding for curli synthesis were induced in stationary phase cells. Thus, *E. coli* cells may be less mobile and may have more tendency to clump or stick to surfaces. Interestingly some genes reported to up-regulate upon entry into stationary phase were shown to be down-regulated in mid-stationary phase cells,

indicating that the active metabolic pathways involved in early, mid and late stationary phase varied.

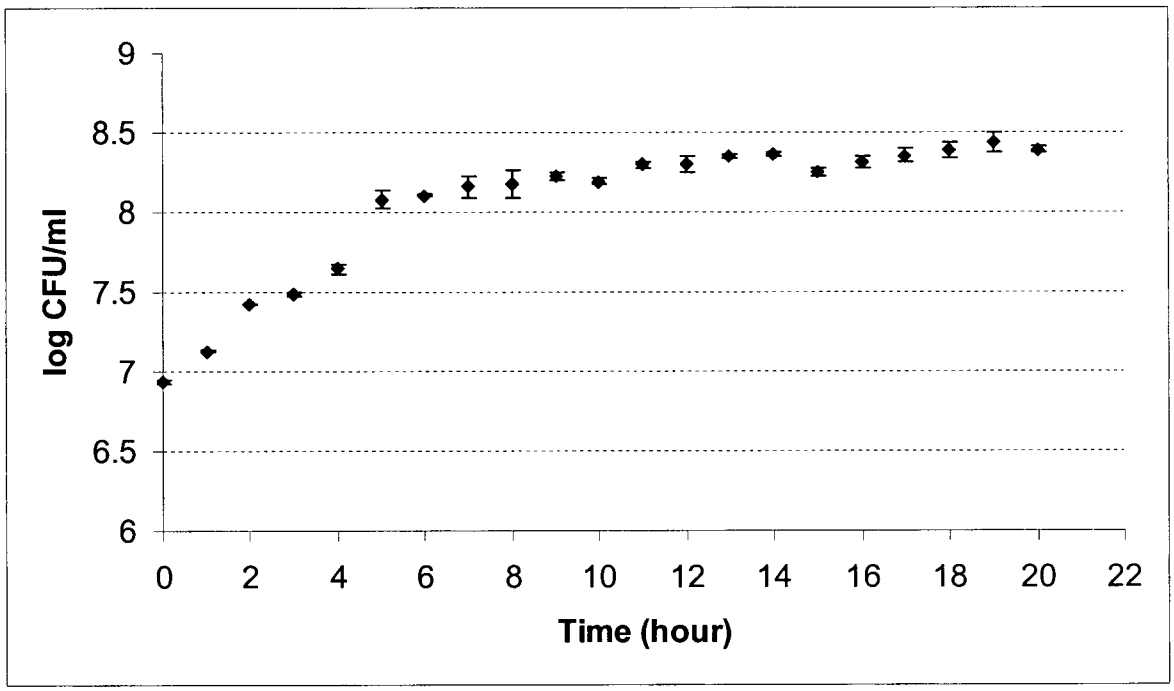

Figure 6.1. Growth of 10^7 CFU/ml stationary phase *E. coli* (ATCC 11775) transferred to 50ml Nutrient Broth at 37°C over 22 hours.

Table 6.1. *E. coli* probe sets signals in mid-stationary phase and late-log phase cells.

Samples	**Present call (%)**	**Absent call (%)**	**Marginal call (%)**
Mid-stationary phase *E. coli*	49.20+/-15.02	46.30+/-14.72	4.50+/-0.32
Late-log phase *E. coli*	46.25+/-3.27	49.38+/-3.39	4.36+/-0.13

Table 6.2. Distribution of gene transcription in mid-stationary phase *E. coli*, compared to late-log phase *E. coli*.

Gene function	Down-regulated ≥ 2 fold		Up-regulated ≥ 2 fold	
	number of genes		**number of genes**	
Translation	71	(23.36 %)	0	0
Amino acid metabolism	48	(15.79 %)	0	0
Carbohydrate metabolism	46	(15.13 %)	0	0
Energy metabolism	45	(14.80 %)	0	0
Cell Motility	42	(13.82 %)	0	0
Membrane transport	16	(5.26 %)	0	0
Nucleotide metabolism	12	(3.95 %)	1	(7.69 %)
Metabolism of cofactors and vitamins	11	(3.62 %)	0	0
Lipid metabolism	9	(2.96 %)	0	0
Sorting and degradation	9	(2.96 %)	0	0
Signal transduction	5	(1.64 %)	0	0
Transcription	4	(1.32 %)	0	0
Biodegradation of xenobiotics	2	(0.66 %)	0	0
Biosysnthesis of secondary metabolite	1	(0.33 %)	0	0
Unassigned	93	(30.59 %)	12	(92.31 %)

Table 6.3. Genes up-regulated (≥2 fold) in mid-stationary phase cells compared to late-log phase *E. coli* cells ($p<0.05$).

Gene name	b no.	Fold change	Call		Description
			Stationary phase	Log phase	
	b2670	1.96	P[3]	P	orf[2], hypothetical protein[1,2]
bfr	b3336	2.19	P	A[4]	bacterioferritin[1,2] (BFR) [1] (cytochrome B-1) (cytochrome B-557)[1], an iron storage homoprotein[1]
csgA	b1042	14.06	P	A	major curlin subunit precursor[1,2],coiled surface structures; cryptic[2]
csgB	b1041	11.79	P	A	minor curlin subunit precursor[1,2], similar ro CsgA[2]
csgD	b1040	2.25	P	A	probable csgab operon transcriptional regulatory protein[1], putative 2-component transcriptional regulator for 2nd curli operon[2]
guaB	b2508	3.54	P	P	inosine-5'-monophosphate dehydrogenase [1](IMP dehydrogenase)[1,2],(IMPDH) (IMPD)[1]
ybhH	b0769	2.15	P	A	hypothetical 37.1 kD protein in modC-bioA intergenic region[1], orf, hypothetical protein[2]
yceO	b1058	2.25	P	A	hypothetical 5.9 kD protein in waam-solA intergenic region[1], orf, hypothetical protein[2]
ygiP	b3060	2.30	P	A	hypothetical transcriptional regulator in bacA-ttdA intergenic region[1], putative transcriptional regulator LYSR-type[2]
yhcN	b3238	2.46	P	P	hypothetical 11.2 kD protein in argR-cafA intergenic region[1], orf, hypothetical protein[2]
yhcR	b3242	2.32	P	P	hypothetical 10.3 kD protein in argR-cafA intergenic region (F90) [1], orf, hypothetical protein[2]
yjjB	b4363	1.99	P	P	P14 protein[1], orf, hypothetical protein[2]

[1]http://www.genome.ad.jp/kegg/
[2]Affymetrix gene chip data base
[3]P= Present (gene was detected)
[4]A= Absent (gene was not detected)

Table 6.4. Genes down-regulated (≥2 fold) in mid-stationary phase cells compared to late-log phase *E. coli* cells ($p<0.05$).

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
	b1199	2.33	A[4]	P[3]	putative dihydroxyacetone kinase[1]
	b1200	2.77	P	P	putative dihydroxyacetone kinase[1]
	b1604	2.18	P	P	hypothetical protein[1]
	b1722	2.43	A	P	hypothetical protein[1]
	b1839	2.33	P	P	hypothetical protein[1]
	b2255	3.24	P	P	putative transformylase[1,2]
	b2512	4.75	A	P	putative dehydrogenase[1,2]
	b2529	2.37	P	P	orf[2], hypothetical protein[1,2]
	b2595	2.45	A	P	orf[2], hypothetical protein[1,2]
	b2736	2.84	A	P	putative dehydrogenase[1,2]
	b2737	2.25	P	P	orf[2], hypothetical protein[1,2]
	b2878	2.37	A	P	putative oxidoreductase, Fe-S subunit[1,2]
	b2881	3.11	A	P	putative dehydrogenase[1,2]
accC	b3256	2.37	P	P	biotin carboxylase[1], acetyl CoA carboxylase, biotin carboxylase subunit[2]
aceE	b0114	4.27	P	P	pyruvate dehydrogenase[1,2] E1 component[1], (decarboxylase component)[2]
aceF	b0115	2.76	A	P	dihydrolipoamide acetyltransferase component (E2) of pyruvate dehydrogenase complex[1,2]
acnB	b0118	2.83	P	P	aconitate hydratase 2[1], aconitate hydrase B[2]
acpP	b1094	2.72	P	P	acyl carrier protein[1,2]
acrA	b0463	2.05	P	P	acriflavin resistance protein A precursor[1]
acrB	b0462	2.50			acriflavin resistance protein B[1]
aer	b3072	2.31	A	P	aerotaxis receptor[1]
ahpC	b0605	2.88	P	P	alkyl hydroperoxide reductase c22 protein (scrp-23) (sulfate starvation-induced protein 8) (SSI8)[1]
ahpF	b0606	2.31	A	P	alkyl hydroperoxide reductase f52a protein[1]
alaS	b2697	2.79	P	P	alanyl-tRNA synthetase[1]
aroK	b3390	2.73	P	P	shikimate kinase I (SKI)[1]
artI	b0863	2.86	P	P	L-arginine transport system substrate-binding protein[1]
asd	b3433	2.49	P	P	aspartate-semialdehyde dehydrogenase (AsA dehydrogenase)[1]
asnA	b3744	2.76	P	P	aspartate--ammonia ligase[1] (asparagine synthetase A)[1,2]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
asnB	b0674	3.33	P	P	asparagine synthetase B[1,2] (glutamine-hydrolyzing) [1]
aspC	b0928	3.45	P	P	aspartate aminotransferase[1]
atpA	b3734	3.44	P	P	ATP synthase alpha chain[1], membrane-bound ATP synthase, F1 sector, alpha-subunit[2]
atpB	b3738	2.40	P	P	ATP synthase A chain (protein 6) [1], membrane-bound ATP synthase, F0 sector, subunit a[2]
atpC	b3731	5.73	P	P	ATP synthase epsilon chain[1], membrane-bound ATP synthase, F1 sector, epsilon-subunit [2]
atpD	b3732	5.82	P	P	ATP synthase beta chain[1], membrane-bound ATP synthase, F1 sector, beta-subunit[2]
atpE	b3737	2.59	P	P	ATP synthase C chain (lipid-binding protein) (dicyclohexylcarbodiimide-binding protein) [1], membrane-bound ATP synthase, F0 sector, subunit c[2]
atpF	b3736	2.48	P	P	ATP synthase B chain[1], membrane-bound ATP synthase, F0 sector, subunit b[2]
atpG	b3733	3.63	P	P	ATP synthase gamma chain[1], membrane-bound ATP synthase, F1 sector, gamma-subunit[2]
atpH	b3735	3.57	P	P	ATP synthase delta chain[1], membrane-bound ATP synthase, F1 sector, delta-subunit[2]
caiA	b0039	2.77	A	P	probable carnitine operon oxidoreductase [1,2]; caiA[1]
caiB	b0038	2.72	P	P	crotonobetainyl-CoA:carnitine CoA-transferase[1]; l-carnitine dehydratase[2]
caiE	b0035	3.39	P	P	carnitine operon protein caiE[1]
cheA	b1888	2.80	A	P	chemotaxis protein cheA[1]
cheR	b1884	1.97	P	P	chemotaxis protein methyltransferase[1]
cheW	b1887	2.73	A	P	purine-binding chemotaxis protein[1]
cheY	b1882	2.92	A	P	chemotaxis protein cheY[1]
cheZ	b1881	4.59	A	P	chemotaxis protein cheZ[1]
cspA	b3556	3.97	P	P	cold shock protein cspA (7.4 kD cold shock protein) (CS7.4) [1,2], transcriptional activator of hns[2]
cspG	b0990	4.43	P	P	cold shock-like protein cspg[1]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
cydA	b0733	2.28	P	P	cytochrome d ubiquinol oxidase[1,2] subunit I,(cytochrome bd-I oxidase subunit I) [1], polypeptide subunit I [2]
cydB	b0734	2.69	P	P	cytochrome d ubiquinol oxidase[1,2] subunit II (cytochrome bd-I oxidase subunit II) [1], polypeptide subunit II [2]
cyoC	b0430	2.80	P	P	cytochrome o ubiquinol oxidase subunit III[1,2]
cyoD	b0429	3.32	A	P	cytochrome o ubiquinol oxidase operon protein cyoD[1], subunit IV [2]
cyoE	b0428	2.87	A	P	protoheme IX farnesyltransferase[1,2], (haeme O biosynthesis) [2]
cysK	b2414	6.21	P	P	cysteine synthase A (O-acetylserine sulfhydrylase A)[1,2] (O- acetylserine (THIOL)-lyase A) (csase A) (sulfate starvation-induced protein 5) (SSI5) [1]
cysP	b2425	3.79	A	P	sulfate transport system thiosulfate-binding protein [1]
eno	b2779	2.72	P	P	enolase[1,2]
fabD	b1092	3.58	A	P	malonyl CoA-acyl carrier protein transacylase (MCT) [1,2]
fabF	b1095	3.15	P	P	3-oxoacyl-[acyl-carrier-protein synthase II][1,2]
fba	b2925	3.25	P	P	fructose-bisphosphate aldolase, class II[1,2]
fdhF	b4079	2.49	A	P	formate dehydrogenase, major subunit (formate dehydrogenase alpha subunit) [1], selenopolypeptide subunit of formate dehydrogenase H[2]
fdoG	b3894	2.19	P	P	formate dehydrogenase, major subunit[1,2] (formate dehydrogenase alpha subunit)[1],formate dehydrogenase-O [2]
fdoH	b3893	2.36	P	P	formate dehydrogenase, iron-sulfur subunit (formate dehydrogenase beta subunit) [1,2], formate dehydrogenase-O[2]
fimC	b4316	3.40	P	P	chaperone protein fimC precursor[1], periplasmic chaperone, required for type 1 fimbriae[2]
fimD	b4317	3.16	P	P	outer membrane usher protein fimD precursor[1], outer membrane protein; export and assembly of type 1 fimbriae, interrupted[2]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
fimG	b4319	2.11	P	P	fimG protein precursor[1], fimbrial morphology[2]
fimI	b4315	3.21	P	P	fimbrin-like protein fimI[1], fimbrial protein[2]
fis	b3261	2.25	P	P	factor-for-inversion stimulation protein[1], site-specific DNA inversion stimulation factor; DNA-binding protein; a trans activator for transcription[2]
fixA	b0041	3.63	A	P	fixA protein[1]
fixB	b0042	3.66	A	P	fixB protein[1]
fixX	b0044	3.02	A	P	ferredoxin like protein[1]
flgA	b1072	2.99	A	P	flagella basal body P-ring formation protein flgA precursor[1], flagellar biosynthesis; assembly of basal-body periplasmic P ring[2]
flgB	b1073	5.04	A	P	flagellar basal-body rod protein flgB[1], flagellar biosynthesis, cell-proximal portion of basal-body rod[2]
flgC	b1074	6.06	A	P	flagellar basal-body rod protein flgC[1], flagellar biosynthesis, cell-proximal portion of basal-body rod[2]
flgD	b1075	6.96	A	P	basal-body rod modification protein flgD[1], flagellar biosynthesis, initiation of hook assembly[2]
flgE	b1076	7.43	A	P	flagellar hook protein flgE[1], flagellar biosynthesis, hook protein[2]
flgF	b1077	4.96	A	P	flagellar basal-body rod protein flgF[1], flagellar biosynthesis, cell-proximal portion of basal-body rod[2]
flgG	b1078	4.92	A	P	flagellar basal-body rod protein flgG[1], flagellar biosynthesis, cell-distal portion of basal-body rod[2]
flgH	b1079	2.09	A	P	flagellar L-ring protein precursor[1], flagellar biosynthesis, basal-body outer-membrane L (lipopolysaccharide layer) ring protein[2]
flgJ	b1081	2.00	P	P	flagellar protein flgJ[1], flagellar biosynthesis[2]
flgK	b1082	5.13	P	P	flagellar hook-associated protein 1 (HAP1)[1,2], flagellar biosynthesis[2]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
flgL	b1083	3.21	A	P	flagellar hook-associated protein 3 (HAP3)[1] (hook- filament junction protein) [1,2], flagellar biosynthesis[2]
flgM	b1071	3.08	A	P	negative regulator of flagellin synthesis (anti-sigma factor) [1], also known as RflB protein[2]
flgN	b1070	3.71	P	P	flagella synthesis protein flgN[1,2]
flhC	b1891	2.14	P	P	flagellar transcriptional activator flhC[1], regulator of flagellar biosynthesis acting on class 2 operons; transcription initiation factor[2]
flhD	b1892	2.24	P	P	flagellar transcriptional activator flhD[1], regulator of flagellar biosynthesis, acting on class 2 operons; transcriptional initiation factor[2]
fliA	b1922	4.86	A	P	RNA polymerase sigma factor for flagellar operon[1], flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons[2]
fliC	b1923	5.02	A	P	flagellin[1,2], flagellar biosynthesis; filament structural protein[2]
fliD	b1924	3.53	A	P	flagellar hook-associated protein 2 (HAP2) (filament CAP protein) [1], flagellar biosynthesis; filament capping protein; enables filament assembly[2]
fliE	b1937	2.58	P	P	flagellar hook-basal body complex protein fliE[1], flagellar biosynthesis; basal-body component, possibly at (MS-ring)-rod junction[2]
fliG	b1939	4.10	A	P	flagellar motor switch protein fliG[1]
fliH	b1940	3.17	A	P	flagellar assembly protein fliH[1], flagellar biosynthesis; export of flagellar proteins?[2]
fliK	b1943	2.24	A	P	flagellar hook-length control protein[1,2]
fliL	b1944	3.27	A	P	flagellar fliL protein[1], flagellar biosynthesis[2]
fliM	b1945	3.46	P	P	flagellar motor switch protein fliM[1], flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction[2]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
fliN	b1946	8.45	A	P	flagellar motor switch protein fliN[1], flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction[2]
fliO	b1947	3.05	A	P	flagellar protein fliO[1], flagellar biosynthesis[2]
fliP	b1948	2.35	A	P	flagellar biosynthetic protein fliP[1], flagellar biosynthesis[2]
fliS	b1925	4.39	A	P	flagellar protein fliS[1], flagellar biosynthesis; repressor of class 3a and 3b operons (RflA activity)[2]
fliY	b1920	3.01	P	P	putative polar amino acid transport system substrate-binding protein[1], putative periplasmic binding transport protein[2]
fliZ	b1921	2.15	A	P	fliZ protein[1], orf, hypothetical protein[2]
fusA	b3340	4.31	P	P	elongation factor EF-G[1]
galK	b0757	1.97	A	P	galactokinase[1,2]
gcvP	b2903	2.25	P	P	glycine dehydrogenase[1]
glnA	b3870	2.73	P	P	glutamine synthetase[1,2] (glutamate--ammonia ligase) [1]
gloA	b1651	1.96	P	P	lactoylglutathione lyase[1,2]
glpK	b3926	2.03	P	P	glycerol kinase[1]
glpQ	b2239	1.96	P	P	glycerophosphoryl diester phosphodiesterase periplasmic precursor (glycerophosphodiester phosphodiesterase) [1]
glpT	b2240	2.04	P	P	glycerol-3-phosphate transporter (G-3-P transporter) (G-3-P permease) [1]
gltA	b0720	1.96	P	P	citrate synthase[1,2]
gltL	b0652	2.01	P	P	glutamate/aspartate transport system ATP-binding protein[1]
gnd	b2029	2.725	P	P	6-phosphogluconate dehydrogenase, decarboxylating[1,2]
gpsA	b3608	2.29	P	P	glycerol-3-phosphate dehydrogenase (NAD+)[1]
gyrA	b2231	2.33	P	P	DNA gyrase subunit A[1]
hflK	b4174	2.32	P	P	hflK protein[1], protease specific for phage lambda cII repressor[2]
himA	b1712	2.68	P	P	integration host factor alpha-subunit[1]
hisS	b2514	2.04	P	P	histidyl-tRNA synthetase[1]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
hlpA	b0178	3.26	P	P	histone-like protein hlp-1 precursor (DNA-binding 17 kD protein) [1]
hscA	b2526	1.97	A	P	heat shock protein hscA (HSC66) [1], heat shock protein, chaperone, member of Hsp70 protein family[2]
hupA	b4000	2.42	P	P	DNA-binding protein hu-alpha (HU-2)[1,2](NS2)[1]
hupB	b0440	3.33	P	P	DNA-binding protein hu-beta (NS1) (HU-1) [1]
hybE	b2992	1.97	P	P	hydrogenase-2 operon protein hybE[1], member of hyb operon[2]
ileS	b0026	3.65	A	P	isoleucyl-tRNA synthetase[1]
inaA	b2237	2.37	P	P	inaA protein[1]
infB	b3168	4.06	P	P	translation initiation factor IF-2[1]
leuL	b0075	2.19	P	P	Leu operon leader peptide[1]
lipA	b0628	1.96	P	P	lipoic acid synthetase[1]
lon	b0439	2.22	P	P	ATP-dependent protease La[1]
lpdA	b0116	3.02	P	P	dihydrolipoamide dehydrogenase (e3 component of pyruvate and 2-oxoglutarate dehydrogenases complexes) (glycine cleavage system L protein) [1]
lpxD	b0179	2.88	P	P	UDP-3-O-[3-hydroxymyristoyl glucosamine N- acyltransferase (firA protein) (rifampicin resistance protein) [1]
lrp	b0889	3.14	A	P	leucine-responsive regulatory protein[1]
lysS	b2890	2.66	P	P	lysyl-tRNA synthetase[1]
metG	b2114	2.03	A	P	methionyl-tRNA synthetase[1]
metT	b0673	2.43	P	P	methionine tRNA-m[1,2]; duplicate gene [2]
mglA	b2149	2.00	P	P	D-galactose transport system ATP-binding protein[1]
modA	b0763	2.00	A	P	molybdate transport system substrate-binding protein [1]
motA	b1890	2.27	P	P	chemotaxis motA protein (motility protein A) [1]
mukE	b0923	2.63	A	P	mukE protein (kica protein) [1]
murD	b0088	2.95	A	P	UDP-N-acetylmuramoylalanine--D-glutamate ligase (UDP-N-acetylmuranoyl-L-alanyl-D-glutamate synthetase) [1]
murE	b0085	2.18	A	P	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase [1]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
murG	b0090	2.22	A	P	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase [1]
nlpB	b2477	2.45	P	P	lipoprotein-34[1,2] precursor[1]
ntpA	b1865	2.36	P	P	dATP pyrophosphohydrolase[1]
nuoC	b2286	2.48	P	P	NADH dehydrogenase I chain C, chainD[1,2]
nuoF	b2284	2.64	P	P	NADH dehydrogenase I chain F[1]
nuoI	b2281	2.62	A	P	NADH dehydrogenase I chain I[1]
nuoJ	b2280	2.62	P	P	NADH dehydrogenase I chain J[1,2]
nuoL	b2278	2.81	A	P	NADH dehydrogenase I chain L (NADH-ubiquinone oxidoreductase chain 12) (NUO12) [1]
nuoM	b2277	2.33	A	P	NADH dehydrogenase I chain M[1,2] (NADH-ubiquinone oxidoreductase chain 13) (NUO13) [1]
nusA	b3169	3.30	P	P	N utilization substance protein A[1]
nusB	b0416	2.59	A	P	N utilization substance protein B (nusB protein) [1]
ompA	b0957	2.66	P	P	outer membrane protein A precursor (outer membrane protein II*)[1]
ompC	b2215	4.70	P	P	outer membrane protein C precursor (outer membrane protein 1b) [1]
ompF	b0929	4.57	P	P	outer membrane protein F precursor (outer membrane protein 1a, ia, or B) [1]
oppA	b1243	2.32	P	P	oligopeptide transport system substrate-binding protein[1]
pal	b0741	4.82	P	P	peptidoglycan-associated lipoprotein precursor[1]
pdxA	b0052	2.04	A	P	4-hydroxythreonine-4-phosphate dehydrogenase[1]
pflB	b0903	3.30	P	P	formate acetyltransferase 1[1,2] (pyruvate formate-lyase 1)[1]
pheT	b1713	2.56	P	P	phenylalanyl-tRNA synthetase beta chain[1]
plsX	b1090	2.05	P	P	fatty acid/phospholipid synthesis protein[1]
pnp	b3164	3.17	P	P	polyribonucleotide nucleotidyltransferase (polynucleotide phosphorylase[1,2]) (PNPase) [1], cytidylate kinase activity[2]
pntA	b1603	2.23	P	P	NAD(P) transhydrogenase subunit alpha[1]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
potD	b1123	2.26	A	P	spermidine/putrescine transport system substrate-binding protein[1]
priB	b4201	6.90	P	P	primosomal replication protein N[1]
prsA	b1207	3.74	P	P	ribose-phosphate pyrophosphokinase[1], phosphoribosylpyrophosphate synthetase[2]
pyrG	b2780	2.26	P	P	CTP synthase (UTP--ammonia ligase) (CTP synthetase) [1]
pyrH	b0171	2.14	P	P	uridylate kinase[1]
rbfA	b3167	2.66	A	P	ribosome-binding factor A[1]
relF	b1562	3.46	P	P	relF protein[1]
ribH	b0415	2.33	P	P	riboflavin synthase beta chain[1]
rimM	b2608	6.19	P	P	16s rRNA processing protein rimm[1]
rplA	b3984	6.02	P	P	50S ribosomal protein L1[1]
rplB	b3317	5.96	P	P	50S ribosomal protein L2[1]
rplC	b3320	5.57	A	P	50S ribosomal protein L3[1]
rplD	b3319	6.27	P	P	50S ribosomal protein L4[1]
rplE	b3308	4.37	P	P	50S ribosomal protein L5[1]
rplF	b3305	6.63	P	P	50S ribosomal protein L6[1]
rplI	b4203	6.63	P	P	50S ribosomal protein L9[1]
rplJ	b3985	7.23	P	P	50S ribosomal protein L10[1]
rplK	b3983	5.48	P	P	50S ribosomal protein L11[1]
rplL	b3986	4.83	P	P	50S ribosomal protein L7/L12[1]
rplM	b3231	4.45	P	P	50S ribosomal protein L13[1]
rplN	b3310	3.92	P	P	50S ribosomal protein L14[1]
rplO	b3301	6.21	P	P	50S ribosomal protein L15[1]
rplP	b3313	7.76	P	P	50S ribosomal protein L16[1]
rplQ	b3294	6.16	P	P	50S ribosomal protein L17[1]
rplR	b3304	5.71	P	P	50S ribosomal protein L18[1]
rplS	b2606	3.84	P	P	50S ribosomal protein L19[1]
rplT	b1716	5.38	P	P	50S ribosomal protein L20[1]
rplV	b3315	7.17	P	P	50S ribosomal protein L22[1]
rplW	b3318	6.38	P	P	50S ribosomal protein L23[1]
rplX	b3309	3.93	P	P	50S ribosomal protein L24[1]
rplY	b2185	3.87	P	P	50S ribosomal protein L25[1]
rpmA	b3185	3.31	P	P	50S ribosomal protein L27[1]
rpmB	b3637	2.75	P	P	50S ribosomal protein L28[1]
rpmC	b3312	19.95	A	P	50S ribosomal protein L29[1]
rpmD	b3302	5.96	P	P	50S ribosomal protein L30[1]
rpmE	b3936	2.98	P	P	50S ribosomal protein L31[1]
rpmF	b1089	4.04	P	P	50S ribosomal protein L32[1]
rpmG	b3636	2.42	P	P	50S ribosomal protein L33[1]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
rpmH	b3703	2.01	P	P	50S ribosomal protein L34[1]
rpmI	b1717	7.144	P	P	50S ribosomal protein L35[1]
rpmJ	b3299	4.02	P	P	50S ribosomal protein L36[1]
rpoA	b3295	6.64	P	P	DNA-directed RNA polymerase alpha chain[1], RNA polymerase, alpha subunit[2]
rpoB	b3987	5.51	P	P	DNA-directed RNA polymerase beta chain[1], RNA polymerase, beta subunit[2]
rpoC	b3988	5.51	A	P	DNA-directed RNA polymerase beta' chain[1], RNA polymerase, beta subunit[2]
rpsA	b0911	5.21	P	P	30S ribosomal protein S1[1]
rpsB	b0169	6.24	P	P	30S ribosomal protein S2[1]
rpsC	b3314	9.77	P	P	30S ribosomal protein S3[1]
rpsD	b3296	5.20	P	P	30S ribosomal protein S4[1]
rpsE	b3303	5.88	P	P	30S ribosomal protein S5[1]
rpsF	b4200	4.35	P	P	30S ribosomal protein S6[1]
rpsG	b3341	4.61	P	P	30S ribosomal protein S7[1]
rpsH	b3306	6.19	P	P	30S ribosomal protein S8[1]
rpsI	b3230	5.42	P	P	30S ribosomal protein S9[1]
rpsJ	b3321	5.50	P	P	30S ribosomal protein S10[1]
rpsK	b3297	5.18	P	P	30S ribosomal protein S11[1]
rpsL	b3342	3.66	P	P	30S ribosomal protein S12[1]
rpsM	b3298	4.82	P	P	30S ribosomal protein S13[1]
rpsN	b3307	4.62	P	P	30S ribosomal protein S14[1]
rpsO	b3165	3.64	P	P	30S ribosomal protein S15[1]
rpsP	b2609	5.22	P	P	30S ribosomal protein S16[1]
rpsQ	b3311	8.97	P	P	30S ribosomal protein S17[1]
rpsR	b4202	3.58	P	P	30S ribosomal protein S18[1]
rpsS	b3316	7.84	P	P	30S ribosomal protein S19[1]
rpsT	b0023	5.30	P	P	30S ribosomal protein S20[1]
sbp	b3917	2.01	P	P	sulfate transport system sulfate-binding protein[1]
sdhA	b0723	2.61	P	P	succinate dehydrogenase flavoprotein subunit[1,2]
secA	b3609	2.00	P	P	preprotein translocase secA subunit[1],
secD	b0408	2.33	P	P	protein-export membrane protein secD[1]
secF	b0409	2.20	A	P	protein-export membrane protein secF[1]
secG	b3175	2.98	P	P	protein-export membrane protein secG (preprotein translocase band 1 subunit) (P12)[1]
secY	b3300	4.36	P	P	preprotein translocase SecY subunit[1]
serC	b0907	3.14	A	P	phosphoserine aminotransferase[1]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
serS	b0893	2.78	P	P	seryl-tRNA synthetase[1]
sodB	b1656	3.13	P	P	superoxide dismutase (FE) [1]
speA	b2938	2.29	A	P	biosynthetic arginine decarboxylase (ADC) [1]
spr	b2175	2.86	P	P	lipoprotein spr precursor[1]
sspA	b3229	2.04	P	P	stringent starvation protein A[1,2], regulator of transcription[2]
sspB	b3228	2.68	P	P	stringent starvation protein B[1,2]
sucA	b0726	3.25	P	P	2-oxoglutarate dehydrogenase[1,2] E1 component[1], (decarboxylase component) [2]
sucB	b0727	2.64	P	P	2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase) [1]
sucC	b0728	2.99	P	P	succinyl-CoA synthetase beta chain[1,2], beta subunit[2]
sucD	b0729	3.51	A	P	succinyl-CoA synthetase alpha chain[1,2], alpha subunit[2]
surA	b0053	2.68	P	P	survival protein surA precursor (peptidyl-prolyl cis-trans isomerase surA) (PPIase) (rotamase C)[1]
talB	b0008	3.17	P	P	transaldolase B[1,2]
tdcD	b3115	2.11	P	P	propionate kinase[1], putative kinase[2]
tdcE	b3114	2.79	A	P	keto-acid formate acetyltransferase (keto-acid formate-lyase) [1], probable formate acetyltransferase 3[2]
tgt	b0406	2.28	P	P	queuine tRNA-ribosyltransferase[1]
thrA	b0002	2.49	P	P	aspartokinase I / homoserine dehydrogenase I[1]
thrC	b0004	2.46	P	P	threonine synthase[1]
thrS	b1719	3.85	P	P	threonyl-tRNA synthetase[1]
tig	b0436	3.93	P	P	trigger factor[1]
tolA	b0739	2.70	P	P	tolA protein[1]
tolB	b0740	3.14	P	P	tolB protein precursor[1]
tolC	b3035	2.42	P	P	outer membrane protein tolC precursor [1]
tpx	b1324	2.24	A	P	thiol peroxidase (scavengase p20) [1]
trmD	b2607	5.93	P	P	tRNA (guanine-n1)-methyltransferase (m1g-methyltransferase) [1]
trpA	b1260	9.26	A	P	tryptophan synthase alpha chain[1]
trpB	b1261	8.90	A	P	tryptophan synthase beta chain[1]
trpD	b1263	5.08	A	P	anthranilate phosphoribosyltransferase / anthranilate synthase component II[1]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
trpL	b1265	2.41	P	P	Trp operon leader peptide[1]
trpT	b3761	4.71	A	P	tryptophan tRNA[1,2]
truB	b3166	2.24	A	P	tRNA pseudouridine 55 synthase[1,2]
tsf	b0170	7.31	P	P	elongation factor EF-Ts[1]
tsr	b4355	3.67	P	P	methyl-accepting chemotaxis protein I (MCP-I) (serine chemoreceptor protein) [1]
tufA	b3339	2.55	P	P	elongation factor EF-Tu[1]
tufB	b3980	3.25	P	P	elongation factor EF-Tu[1]
vacB	b4179	2.22	P	P	ribonuclease R[1]
valS	b4258	2.29	P	P	valyl-tRNA synthetase[1]
yaeL	b0176	2.45	P	P	protease ecfE[1]
yaeS	b0174	2.25	P	P	undecaprenyl pyrophosphate synthetase[1]
yaeT	b0177	2.51	A	P	unknown protein from 2d-PAGE precursor (spots m62/m63/o3/o9/t35) [1]
yaiE	b0391	2.87	P	P	hypothetical 10.2 kD protein in aroM-araJ intergenic region[1]
yajC	b0407	2.23	A	P	hypothetical 11.9 kD protein in tgt-secD intergenic region (ORF12) [1]
ybgE	b0735	2.58	P	P	10.9 kD protein in cydB-tolQ intergenic region(ORFD) [1]
ybgF	b0742	3.70	P	P	hypothetical 28.2 kD protein in pal-lysT intergenic region precursor[1]
yceD	b1088	3.92	P	P	hypothetical 19.3 kD protein in rne-rpmF intergenic region (G30K) [1]
ydgQ	b1632	2.33	A	P	hypothetical 24.5 kD protein in add-nth intergenic region[1]
ydgR	b1634	2.33	P	P	putative proton-dependent oligopeptide transporter[1]
yebC	b1864	2.06	P	P	hypothetical 26.4 kD protein in ruvC-aspS intergenic region[1]
yebJ	b1831	2.83	P	P	hypothetical 4.2 kD protein in prc-prpA intergenic region[1]
yeeD	b2012	4.58	A	P	hypothetical 8.1 kD protein in sbcB-hisL intergenic region[1]
yeeF	b2014	2.36	P	P	hypothetical 49.8 kD transport protein in sbcB-hisL intergenic region[1]
yfgA	b2516	2.15	P	P	hypothetical 36.2 kD protein in ndk-gcpE intergenic region[1], putative membrane protein[2]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
yfgB	b2517	1.99	P	P	hypothetical 43.1 kD protein in ndk-gcpE intergenic region[1], orf, hypothetical protein[2]
yfiF	b2581	2.13	A	P	hypothetical tRNA/rRNA methyltransferase yfiF[1]
ygbL	b2738	3.86	A	P	hypothetical 23.2 kD protein in prpB-rpoS intergenic region[1], putative epimerase/aldolase[2]
ygbM	b2739	2.26	P	P	hypothetical 29.2 kD protein in mutS-rpoS intergenic region (O258) [1], orf, hypothetical protein[2]
ygeW	b2870	5.88	A	P	hypothetical 40.2 kD protein in kduI-lysS intergenic region[1], putative carbamoyl transferase[2]
ygeX	b2871	4.18	A	P	putative diaminopropionate ammonia-lyase (diaminopropionatase) [1]
ygeY	b2872	6.33	A	P	hypothetical 44.8 kD protein in kduI-lysS intergenic region[1], putative deacetylase[2]
ygiB	b3037	2.00	P	P	hypothetical 24.9 kD protein in tolC-ribB intergenic region (ORFD) (O234) [1], orf, hypothetical protein[2]
yhaR	b3113	3.47	A	P	hypothetical 16.3 kD protein in exuR-tdcC intergenic region[1], orf, hypothetical protein[2]
yhbY	b3180	2.50	P	P	hypothetical 10.8 kD protein in ftsJ-greA intergenic region (O97) [1], orf, hypothetical protein[2]
yhdG	b3260	2.58	P	P	hypothetical 35.9 kD protein in pmra-fis intergenic region (ORF1) [1], putative dehydrogenase[2]
yhfS	b3376	2.02	A	P	hypothetical 38.6 kD protein in cysG-trpS intergenic region (F361) [1], orf, hypothetical protein[2]
yicC	b3644	1.99	P	P	33.2 kD protein in dinD-rph intergenic region (ORF X) [1], putative alpha helix protein[2]
yihK	b3871	2.86	P	P	GTP-binding protein TypA/BipA (tyrosine phosphorylated protein A) [1], putative GTP-binding factor[2]

Table 6.4. Continued.

Gene name	b no.	Fold change	Call		Description
			stationary phase	log phase	
yjdA	b4109	2.43	A	P	hypothetical 84.2 kD protein in phnA-proP intergenic region (ORF742) [1], putative vimentin[2]
ykgG	b0308	2.02	P	P	hypothetical 31.1 kD protein in eaeH-betA intergenic region[1],
yqeA	b2874	4.43	A	P	carbamate kinase[1], putative kinase[2]
yqgB	b2939	2.23	P	P	hypothetical 5.4 kD protein in speA-metK intergenic region (F48) [1], orf, hypothetical protein[2]
yqgC	b2940	2.04	P	P	hypothetical 8.1 kD protein in speA-metK intergenic region (O71) [1], orf, hypothetical protein[2]
znuA	b1857	2.35	P	P	high-affinity zinc transport system substrate-binding protein[1]

[1]http://www.genome.ad.jp/kegg/
[2]Affymetrix gene chip data base
[3]P= Present (gene was detected)
[4]A= Absent (gene was not detected)

CHAPTER SEVEN

GENERAL DISCUSSION, GENERAL CONCLUSION

AND

RECOMMENDATIONS FOR FUTURE STUDIES

7.1 General discussion

7.1.1 Effect of VM on *E. coli* cells

In the present work an attempt was made to verify whether the lethal effect of vacuum microwave on microorgnisms was entirely due to heat or whether other effects were associated with microwave radiation. For this purpose *E. coli* was chosen as a model microorganism because of its relatively simple structure, and well known physiology and genome sequence. *E. coli* kinetic parameters under lethal and sub-lethal conditions were determined, followed by a study of the *E. coli* transcriptional response to sub-lethal treatment with vacuum microwave and convective heating.

The larger D-values for VM, at lower temperatures and small D-values at higher temperatures compared to conventional heat treatments, provided evidence of a factor(s) involved in VM other than heat in *E. coli* inactivation. One possible source of the difference in lethality was the difference in heating rates of bacteria in the microwave treatment compared to the water bath under vacuum treatment. Kaur and colleagues (1998) studied the effect of heating rate on the survival of *E. coli* at 60°C for 40s. They reported that for heating rate of 1 °C/min the mean number of survivors was 1.4 log CFU/ml while for heating rate 10 °C /min it was 2.6 log CFU/ml. They concluded that this might be due to exposure to potentially lethal temperatures for longer during heating period. Therefore higher inactivation with slower heating rate would be expected.

Another hypothesis for lower destruction rates at lower temperatures is that direct heating of microorganisms with microwaves enhances the production of heat shock proteins, thereby increasing their resistance compared to the control. Other researchers also reported that heat resistance of some bacteria increases upon exposure to temperatures slightly higher than their

optimum (Foster & Spector 1995; Kaur et al. 1998). Kusukawa & Yura (1988) proposed that this higher resistance is due to expression of heat shock proteins. The result of the present work on *E. coli* transcriptional response at 50°C did not support this hypothesis and showed no significant difference on the expression of heat shock genes between vacuum microwave and water bath under vacuum treatment. During the kinetic study at 50°C, *E. coli* cells were exposed to VM for 20-25 minutes, whereas the transcriptional response was determined after three minutes of exposure to vacuum microwave. It is possible the expression of heat shock genes and production of heat shock proteins might happen after a longer exposure time. Thus, here we can only say that heat shock genes were not significantly expressed as a result of short-time exposure to sub-lethal vacuum microwave treatment. At the same time, more down-regulation in 5S and 16S rRNA due to water bath treatment showed that ribosomal subunits in VM treated cells were affected less and thus were more stable. This could be a reason for less destruction at 50°C in VM treatment compared to water bath treatment.

Activation energies showed that VM treated cells needed higher levels of energy for destruction, compared to the water bath under vacuum treatment. Since activation energy represents the minimum kinetic energy that must be possessed by a molecule in order to react, it can be concluded that destruction of *E. coli* under VM treatment occurs by a different mechanism than under the convection heat treatment.

Dielectric loss tangent and loss factor were higher for the centrifuged *E. coli* pellet compared to peptone water. Therefore, when the mixture of culture and peptone water was exposed to microwave radiation, *E. coli* produced more heat than the surrounding liquid environment (peptone water). This may cause a slight local temperature increase inside the cells. This lends credence to the selective heating theory, one of the four predominant theories of

nonthermal inactivation of microwave (Kozempel et al. 1998). The selective heating theory hypothesizes that microorganisms are heated more effectively by microwaves than their surrounding medium and therefore can be killed more rapidly (Datta & Davidson 2000).

The presence of evidence for the existence of a factor or factors other than heat associated to VM led to further investigation. To search for these factors at the molecular level, *E. coli* cell transcriptional responses to sub-lethal VM and water bath treatment at 50°C for 3 minutes were studied. The number of genes altered through water bath treatment was higher than VM, indicating that water bath treatment had a greater impact. Since the D value for water bath treatment at 50°C was shorter than for VM, more severe changes in cells exposed to water bath treatment would indeed be expected.

Some differences in *E. coli* responses at transcriptional level to VM compared to water bath treatment were observed, such as the effects on genes involved in cell membrane and cell transport systems. The *cysW* and *ybaR* genes related to copper and sulfate transport respectively and the *ompF* gene encoding porins and responsible for dipeptide permease, were significantly altered during both treatments. Simultaneously the *yejE, btuC, exuT, ycjO, ydiQ, yfcC* and b0878 genes involved in membrane transport of peptide, vitamin B_{12}, galacturonate and glucuronate, putative S-transferase, multiple sugar and ABC transporter were all down-regulated while *fecA* encoding for ferric citrate outer membrane receptor protein was up-regulated in VM treated *E. coli* compared to the water bath treated *E. coli*. This suggests that due to VM, transcription for genes involved in ion transfer were increased while transport for larger molecules including peptides, multiple sugars and vitamins was decreased. Effect of electromagnetic field on periplasm-binding protein-dependent transport system were previously reported. Nascimento and colleagues (2003) showed higher amounts of glucose transported into

the *E. coli* cells exposed to electromagnetic field (60 Hz, 8 hours, 28°C), and Liburdy and co-workers (1985) reported an increase in sodium passive transport at the membrane of rabbit erythrocytes exposed to microwaves (2450 MHz, 400 mW/g) within a narrow range (17.7 to 19.5°C) of temperature.

In addition *fimC, fimD* and *fimG* genes related to outer membrane protein, periplasmic chaperone and morphology of fimbriae and *fliG* encoding for the flagellar motor switch were expressed more in VM treated *E. coli* compared to water bath treated *E. coli*. The *murG* gene encoding for an enzyme involved in peptidoglycan biosynthesis was expressed less in VM compare to water bath treated *E. coli*. Peptidoglycan in the cell wall is responsible for mechanical strength and maintaining the shape of the cell (Singleton & Sainsbury 2000). Thus it could suggest that while genes related to membrane structure and transport systems were affected in both treatments, the effect of the VM treatment was greater than conventional heat treatment. This may lend credence to dielectric cell-membrane rupture theory. This theory hypothesizes that an external electric field induces an additional trans-membrane electric potential in addition to the normal potential of the cell which in turn results in a voltage drop across the cell membrane sufficient for membrane rupture (Datta & Davidson 2000; Kozempel et al. 1998; Zimmermann et al. 1974) or pore formation, increased permeability, and loss of cell integrity (Brunkhorst et al. 2000, Kozempel et al. 2000).

The other difference noted in this study was in tRNA synthesis. Although genes related to tRNAs specific to glutamine, tryptophan and leucine were up-regulated by both treatments compared to untreated *E. coli* cells, genes encoding for glutamine synthetase and glutaminyl-tRNA synthetase were significantly higher in VM compared to water bath treated *E. coli*. This indicates that glutamine synthesis was more active in *E. coli* after VM treatment.

Higher expression of the gene responsible for ubiquinone biosynthesis and lower expression of the gene involved in menaquinone synthesis, along with increased expression of genes involved in flagellar motility in VM compare to water bath treated *E. coli* are signs of aerobic respiration in VM treated cells. It has been reported that mutation in the quinone biosynthesis pathway gives rise to immobility and lack of flagellum (Poole & Ingledew 1987). At the same time, in water bath treated cells, genes involved in energy metabolism through oxidative phosphorylation and nitrogen metabolism were not detected or were expressed less compared to untreated samples while these genes remained unchanged after VM treatment. Simultaneously, transcription levels for genes related to copper and sulfate ions functioning as electron acceptors in anaerobic respiration were shown to be up-regulated for both treatments while gene for ferric ion were up-regulated in VM treated *E. coli*. This may suggest that although *E. coli* showed signs of anaerobic respiration in both treatments, the transition to anaerobic respiration was more advanced in water bath treated *E. coli* than in VM treated *E. coli*.

In this study *rrlD* gene related to 23S ribosomal RNA in the rrnD operon was up-regulated about 250 and 300 fold in water bath and VM treated *E. coli* respectively. This could be explained as an exposure to stress conditions as reported by other researchers for *Saccharomyces cerevisiae* (Lopez et al. 2002) and *S. typhimurium* (Tolker-Nielsen et al. 1997). At the same time the expression of 5S rRNA genes were down-regulated in both treatments. But the number of down-regulated genes (6 in water bath treated *E. coli* and 4 in VM treated *E. coli*) as well as the average fold change (42.5 in water bath compare to 17.5 in VM) was higher for the water bath treated *E. coli*. In addition, one gene related to 16S rRNA showed down-regulation during water bath treatment while the level of gene expression remained unchanged in VM treated *E. coli* compared to untreated *E. coli*. Therefore, the effect of sub-lethal VM treatment

on ribosomal RNA was less pronounced than water bath under vacuum treatment. Since ribosomes are responsible for translation of messenger RNAs into proteins (Madigan et al. 2003), this could suggest that the process of translation for protein production was affected less by VM treatment than by water bath heat treatment.

7.1.2 Effect of growth phase on *E. coli* transcriptome

In the present work, kinetic studies and gene transcription studies were of stationary cells while most gene expression studies use cells from exponential stages of growth. Thermal death kinetics of bacteria are typically studied on stationary phase cells, for bacteria cells in this stage are in their most resistant form. Thus to close the loop, the effect of growth phase on *E. coli* transcription was examined. The gene expression of *E. coli* cells from mid-stationary phase were compared with that of late-log phase cells.

The expression profiles of genes involved in transcription and translation, including the major subunits of RNA polymerase, ribosomal proteins, and translation factors were down-regulated in mid-stationary phase. A decrease in the overall translation activity or protein synthesis was previously reported to occur during transition from the exponential growth to the stationary phase in *E. coli* cells (Wada et al. 1990).

In addition, the *csg* genes required for curli synthesis (Chirwa & Herrington 2003) were induced in mid-stationary phase cells. Curli, one of the adhesive organelles in *E. coli,* promote clumping of bacterial cells in culture and are important for biofilm formation (Vidal et al. 1998; Austin et al. 1998; Prigent-Combaret et al. 2000). Since curli synthesis was started in stationary phase cells, the expression of *rpoS*, *ompR* and *cpx* genes reported to be responsible for *csg* genes expression (Arnqvist et al. 1994; Prigent-Combaret et al., 2001; Chirwa & Herrington 2003)

expected to be up-regulated. At the present work the expression of *csg* genes either displayed no significant change between late-log and mid-stationary *E. coli* or were not detected in neither phase of the growth, which indicated that other regulatory systems could be involved in the activation of *csg* genes.

As curlin contains high amounts of glycine, Chirwa & Herrington (2003) proposed that up-regulation of *glyA* is an essential response for curli formation. But in this study, although *glyA* was detected in all samples, its transcription level did not show any significant change between the two stages of growth. It is possible that *glyA* was already sufficiently expressed in the late-log phase to produce amino acid necessary for rapid cell growth, and its expression continued in the stationary phase to provide necessary glycine for curli synthesis, where other genes involved in amino acid biosynthesis were down-regulated.

In cells at mid-stationary phase of growth, 70.5% of the genes required for flagellar synthesis, chemotaxis and subsequent motility were down-regulated or were not expressed at all. These *E. coli* cells in the mid-stationary phase of growth would have less capacity for motility.

Some unexpected changes in regulatory events were observed. Fis and Rpos are two regulatory proteins which co-ordinately control the expression of some of the genes during late log and stationary phase. While Fis expression is said to be at its maximum in the early-to-mid log condition, the expression of Rpos is believed to be turned on in late exponential and stationary phase and is required for expression of genes important under starvation conditions or stationary phase (Xu & Johnson 1995). In this experiment the expression of *fis* gene was 2.25 fold higher in late-exponential phase, but interestingly, although the *rpoS* gene was detected as present in both phases, its expression did not show any significant change between the two stages of growth. Xu & Johnson (1995) reported that the products of *xylF* and *mglA* are required

for growth under nutrient-poor conditions, in which *fis* levels are low. The result of this experiment was in agreement with Xu & Johnson (1995) for expression of *xylF* gene, which was 1.57 fold higher in mid-stationary phase but was in disagreement for the *mglA* gene. The *mglA* gene showed a higher expression in late-exponential growth where the *fis* level of expression was not reduced. Xu & Johnson (1995) also reported that the presence of *rpoS* reduced the expression of *xylF*, *mglA* and *sdhA*. The present study showed that while the expression of *rpoS* did not change, the expression of these three genes were altered between the two stages of growth. Perhaps other regulatory genes are involved in the induction or repression of *xylF*, *mglA* and *sdhA* in different stages of growth. On the other hand, most of reported changes in literature were detected at the protein level, and gene transcript and protein activity do not have a linear correlation, thus some discrepancies would be expected.

A comparison between the expression of previously known genes involved in early stationary phase and their expression in this study, mid-stationary phase, showed some interesting results. In the present work, the transcription of *hupA*, *hupB*, *hlpA*, *himA* and *himD* was 2.42, 3.33, 3.26, 2.68 and 1.81 fold down-regulated respectively in mid-stationary phase cells while other researchers reported a higher transcription for these genes upon entry into stationary phase (Dersch et al. 1993; Weglenska et al. 1996; Claret & Rouviere-Yaniv 1997). The transition to stationary phase has been described as a general stress response in *E. coli* cells (Hengge-Aronis 1996, 1999). Thus early stationary phase could be viewed as a short-term response to the external stress factor while the gene expression at mid and late stationary phase could be referred to as a long-term stress response and difference between short-term and long-term stress response would be expected.

7.2 General conclusion

The present work revealed that although there is much similarity between conventional heat treatment and VM, there is evidence for the presence of an inactivation mechanism other than heat associated with VM.

While temperature within experiments and between treatments, was kept constant, slower inactivation at temperatures less than 53°C and higher reduction in microbial population at temperatures above 53°C for VM treated *E. coli* was observed, along with significant differences in activation energy and temperature sensitivity between VM and water bath treated *E. coli*. The impact of temperature on lethal rate of *E. coli* was different when microwaves were the medium of heat transfer and the destruction mechanism of VM was therefore different from that of water bath heating. Thus the presence of factor(s) other than heat involve in microwave under vacuum was established.

At 50°C, VM had a larger effect on transcription of genes related to membrane structure and membrane transport system, as well as genes related to metabolism of carbohydrates, lipids and amino acids than the water bath treatment. On the other hand, the effect of conventional water bath treatment on ribosomal subunits was greater. Interestingly, although both treatments included equal vacuum and signs of anaerobic respiration would be expected, water bath treated *E. coli* shown more evidence for the start of anaerobic respiration at transcriptional level than VM treated *E. coli*.

In addition, this work identified some differences in transcriptional response of *E. coli* in late-log and mid-stationary phase of growth. In mid-stationary phase, genes encoding for energy metabolism as well as amino acids and carbohydrate metabolism were down-regulated. In addition, the *E. coli* response in transcriptional level showed lower expression for genes involved

in cell motility and higher espression for genes involved in curli synthesis in mid-stationary *E. coli* compared to late-log cells. Interestingly some genes reported by other researchers to up-regulate upon entry into stationary phase showed down-regulation in mid-stationary phase cells suggesting that the mechanisms involved in cell behaviour are not only different between lag, log and stationary phase of growth but may differ in early, mid and late stationary phase.

7.3 Proposed theories

The data presented in this study is not sufficient to elucidate the mechanism of *E. coli* destruction upon exposure to microwave radiation under vacuum. Nonetheless, based on the present findings the following theories can be proposed:

This study showed that VM treatment affected the transcription of genes related to membrane structure. This could indicate that VM damages the *E. coli* cell membrane. Findings on the changes in transcription of genes related to the cell membrane transport system could suggest that cell transportation systems are disturbed. *E. coli* could then be faced with a lack of essential substrate or excess of unnecessary substrate. This imbalance of material could affect the cell function and result in cell destruction. Changes in transcription of genes related to aerobic respiration due to VM treatment could act to favour cell growth or not depending on the environmental conditions. If the environmental conditions require oxidative respiration, *E. coli* with up-regulated aerobic respiration would have more chance to survive, but if environmental conditions require anaerobic respiration, *E. coli* cells with more tendency for aerobic respiration will have less chance to survive.

7.4 Recommendations for future studies

There is no doubt that more studies are needed to reach a comprehensive explanation about the effect of microwaves on living bacterial cells. The following are some recommendation for future directions:

- Investigate the expression of *rrlD* gene under different stress conditions such as acid stress, starvation and or cold stress to find that whether this gene can be recognized as an stress indicator in *E. coli*.
- Investigate the *E. coli* response to longer exposure time for example 6 and 9 minutes, to microwave radiation and conventional heat treatment to monitor the changes in transcription of genes related to membrane structure and transport system.
- Investigate the effect of microwave on *E. coli* cells on membrane genes at translation level using a proteomic approach.
- Investigate the effects of microwaves on *E. coli* cells from the exponential growth phase. Cells in this stage are more active and less resistant to any stress, therefore the cell response to microwave radiation as a stress factor would be expected to be greater. In addition, since more pathways are active in this stage, more changes could be expected.
- Investigate the effect of microwave radiation (2450 MHz and/or 915 MHz) on the gene expression of human epithelial cells, as the primary tissue of humans exposed to microwaves.

- Any of the above studies could be conducted with prolonged, repeated exposure to check for a cumulative effect of microwave radiation.
- A comparison of genes involved in different stages of stationary phase (early, mid and late) using DNA microarray would be informative
- A study of the genes involved in early, mid and late exponential phases of *E. coli* growth would be informative.

The two latter studies could help researchers better understand the growth phases and the physiological functions involved in each stage of growth and allow us to design appropriate conditions to promote or prevent the growth of a target microorganism.

CHAPTER EIGHT

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CHAPTER NINE

APPENDICES

9.1 Appendix I: Checking the purity of culture

Table 9.1. Tests and outcomes for checking the purity of the *E. coli* culture.

Test	Results
Gram stain	Small rod shape cell, Pink colour
Lactose fermentaion[1] (Lauryl Sulfate Tryptose Broth)	Gas formation
Lactose fermentation[1] (Brilliant Green Bile Broth)	Gas formation
Glucuronidase activity[2] (Violet Red Bile MUG Agar)	Red colonies surrounded by turbid zone + scatter blue light under UV
Beta-galactosidase and Beta-glucoronidase activity (Chromocult Coliform Agar)[3,4]	Dark blue to violet colonies

[1] Kornacki & Johnson 2001.
[2] Linton et al. 1997.
[3] Alonso et al. 1998.
[4] Finney et al. 2003.

9.2 Appendix II: Continuous Vacuum System: Schematics and suppliers

1. Microwave oven (General Electric- JE435, Mississauga, Canada) or water bath
2. Dessiccator (Pyrex brand-Fisher Scientific), I.D. 160mm modified by Sandfire Scientific Ltd. Mission, BC) (Figures 9.1-9.6)
3. Micropump (Pressure-loaded compact low-flow pump head without canister, Micropump, Inc. WA, USA)
4. Vacuum pump (SIHI pumps Ltd. Guelph, Canada)
5. Stainless steel Tubing & Connectors (Columbia Valve & Fitting Ltd., North Vancouver, Canada)

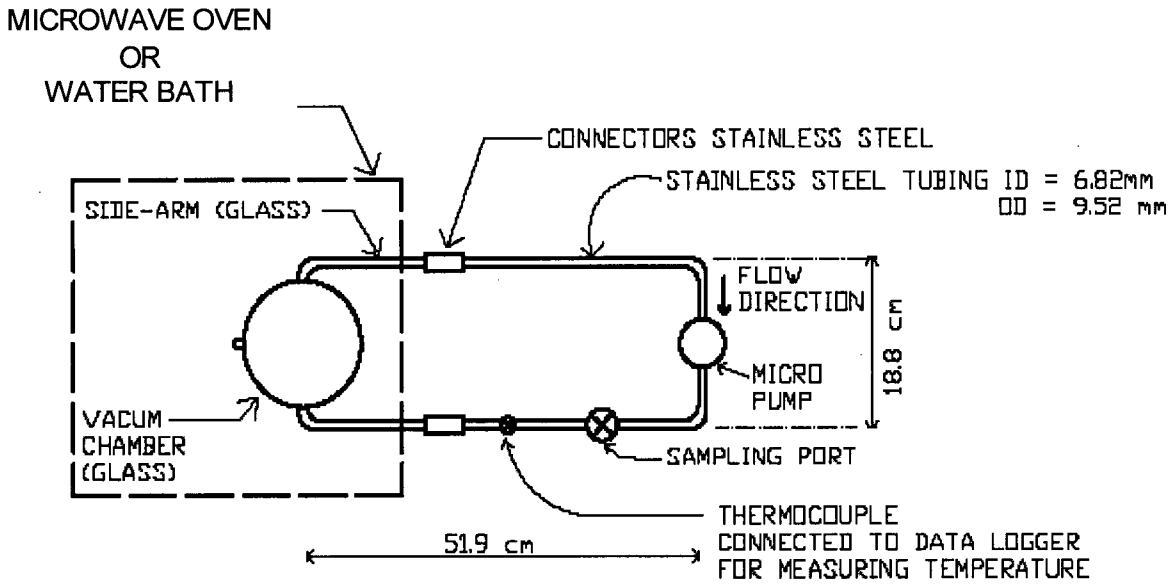

Figure 9.1. Overview of Continuous Vacuum System

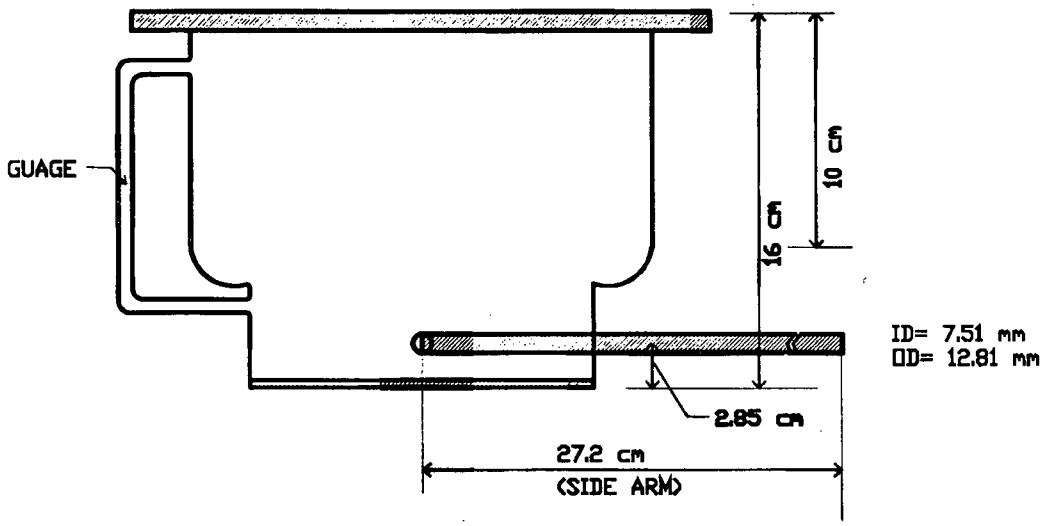

Figure 9.2. Glass vacuum chamber body; Side-view.

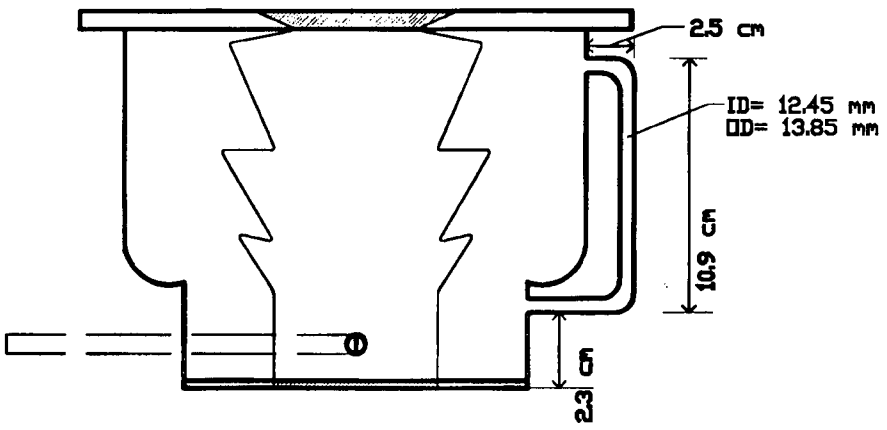

Figure 9.3. Glass vacuum chamber body; Inside-view.

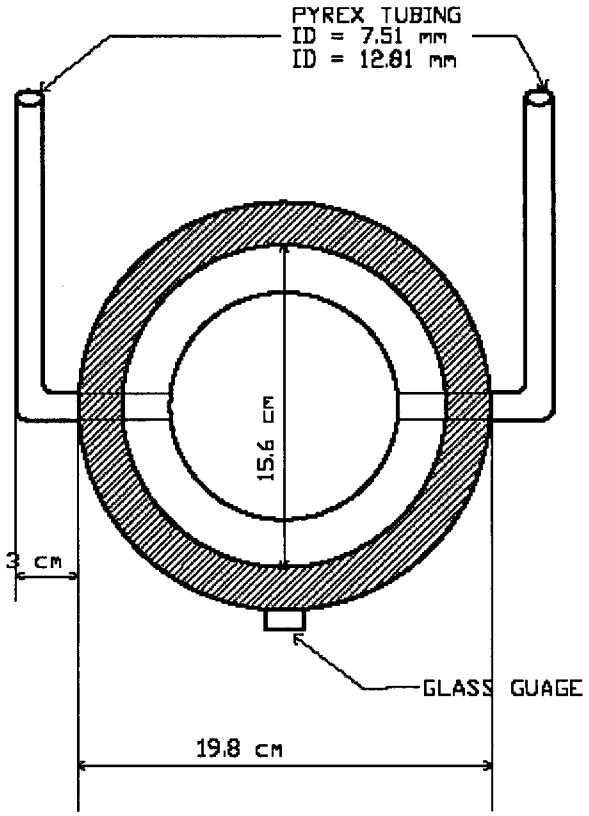

Figure 9.4. Glass vacuum chamber body; Top-view

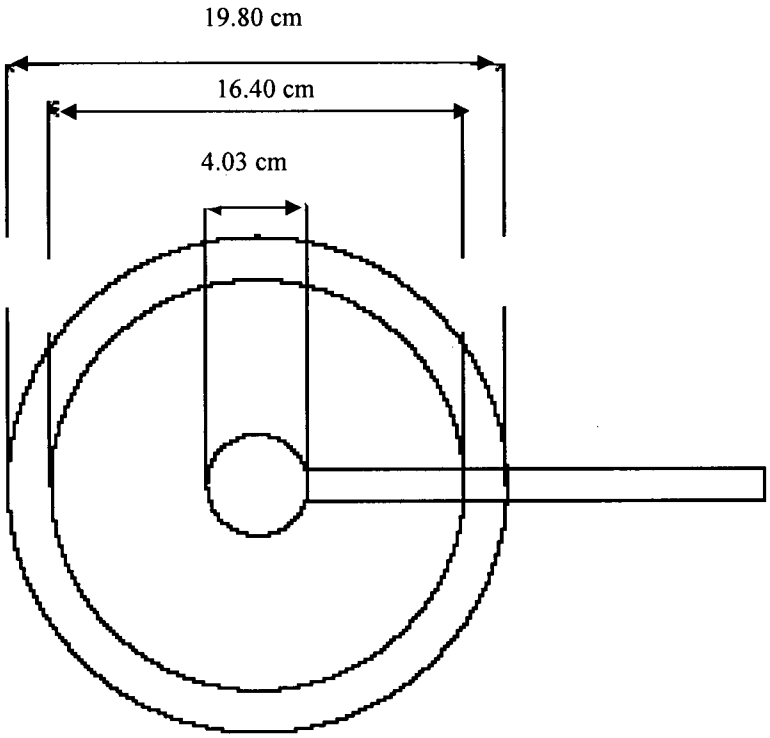

Figure 9.5. Glass vacuum Chamber lid; Top-view

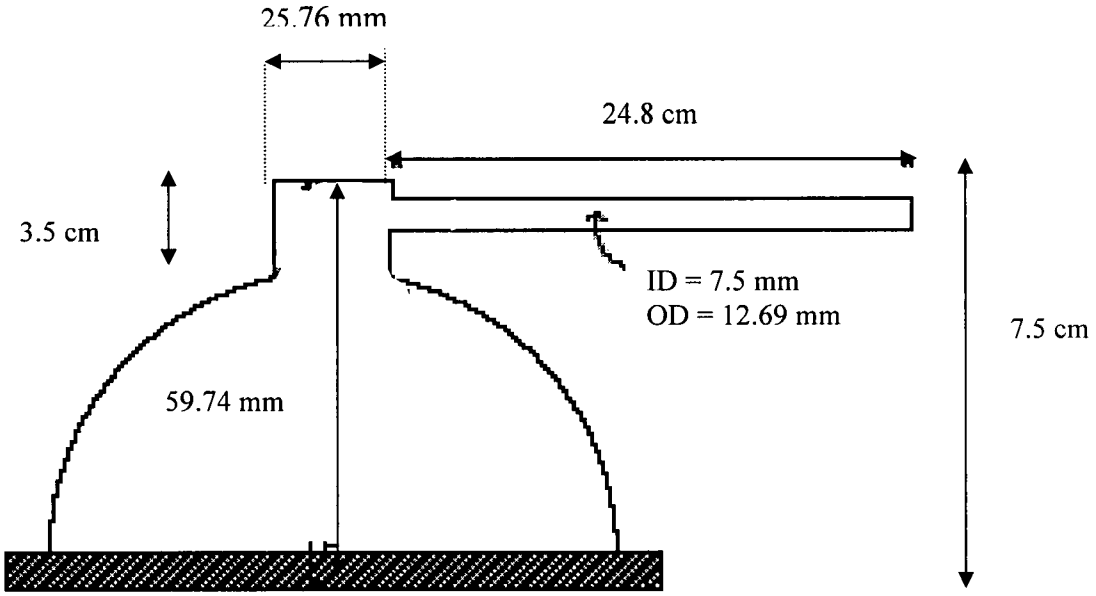

Figure 9.6. Glass vacuum Chamber lid; Side-view.

9.3 Appendix III: Microwave power determinations

The power is calculated from the following formula:

$$P = 70 \times (\Delta T_1 + \Delta T_2)/ 2 \qquad \text{Eq (9.1)}$$

Where P = power (W)
ΔT_1 and ΔT_2 = Temperature rise of the water in the two beakers (°C).

Table 9.2. Microwave power determined using IMPI2-Liter test (Buffler 1993).

Microwave Oven setting	**Microwave Power (W)**			**mean ± stdv**
10	700.00	700.00	735.00	711.67 ± 20.21
9	682.50	656.25	638.75	659.17 ± 22.02
8	507.50	507.50	516.25	510.42 ± 5.05
7	393.75	376.25	376.25	382.08 ± 10.10
6	253.75	253.75	245.00	250.83 ± 5.05
5	201.25	210.00	218.75	210.00 ± 8.75
4	175.10	192.50	192.50	186.70 ± 10.05
3	87.50	87.50	87.50	87.50 ± 0.00
2	70.00	70.00	70.00	70.00 ± 0.00
1	35.00	35.00	35.00	35.00 ± 0.00

9.4 Appendix IV: Micro pump flow rate determinations

Table 9.3. The flow rate of micro pump was determined under normal atmosphere and vacuum (22, 24 and 26 inHg). Each value is the mean of three measurements.

Pump setting	**Flow rate ml/min**			
	no vacuum	**26 inHg**	**24 inHg**	**22 inHg**
10	626.3	582.5	602.5	642.5
9	620.0	580.0	593.3	633.3
8	495.0	435.0	485	583.3
7	410.0	422.2	396.7	453.3
6	286.3	257.5	316.7	340.0
5	220.0	233.3	220.0	203.3

9.5 Appendix V: Thermocouple calibration

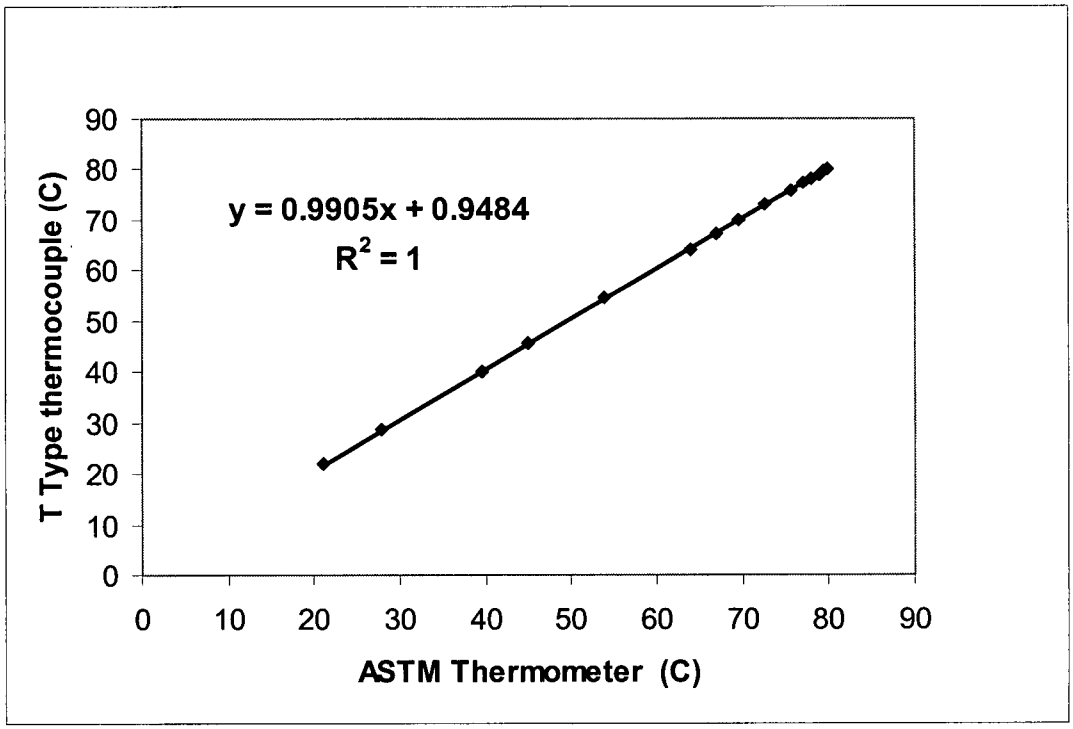

Figure 9.7. Regression equation for temperatures from the data logger versus recorded temperatures from the ASTM thermometer(ASTM 1c, -20/150 CP, VWRbrand, VWR), as a correction factor for T type thermocouple.

9.6 Appendix VI: Survival curves for *E. coli* plated on PCA and PCA-BS

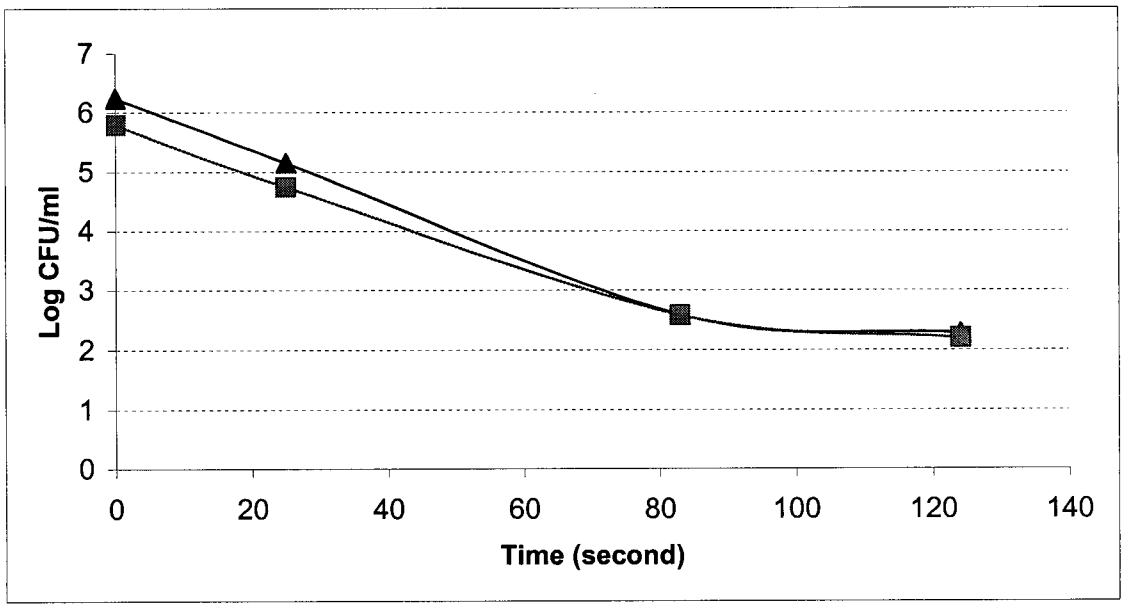

Figure 9.8. Differential counts of *E. coli* on PCA —▲—and PCA-BS —■— during vacuum microwave (711W) at 58.43°C.

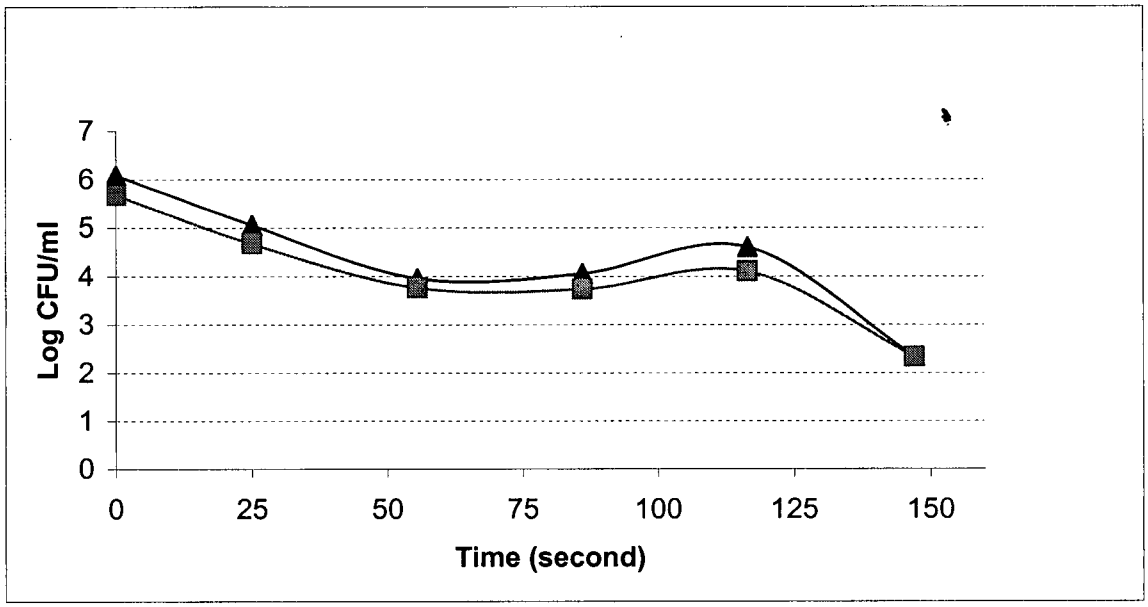

Figure 9.9. Differential counts of *E. coli* on PCA —▲—and PCA-BS —■— during vacuum microwave (510W) at 58.19°C.

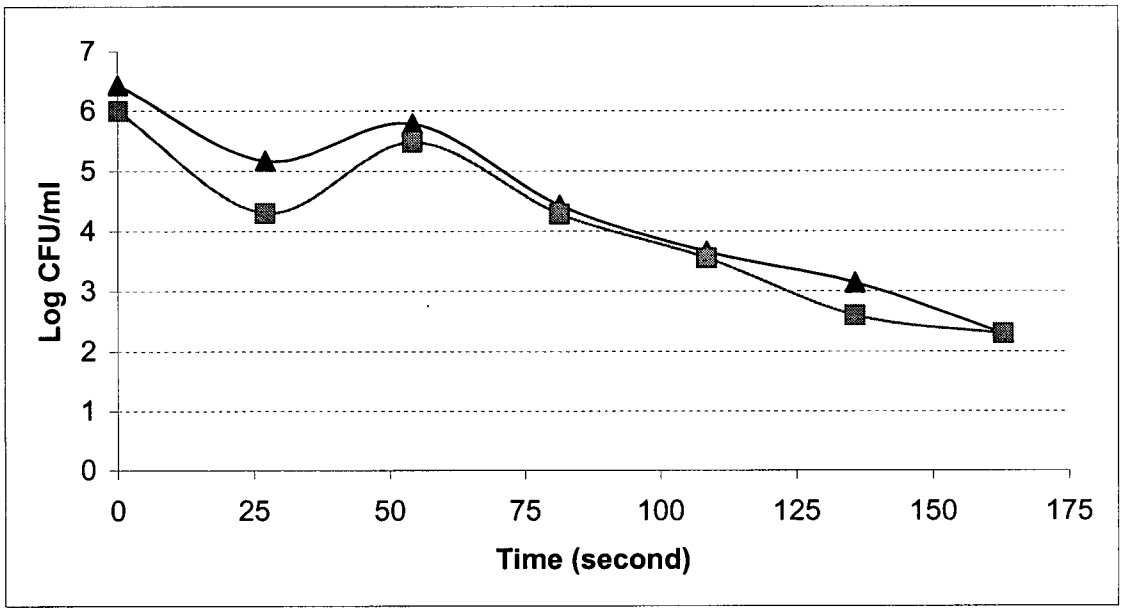

Figure 9.10. Differential counts of *E. coli* on PCA —▲—and PCA-BS —■— during water bath treatment under vacuum at 58.62˚C.

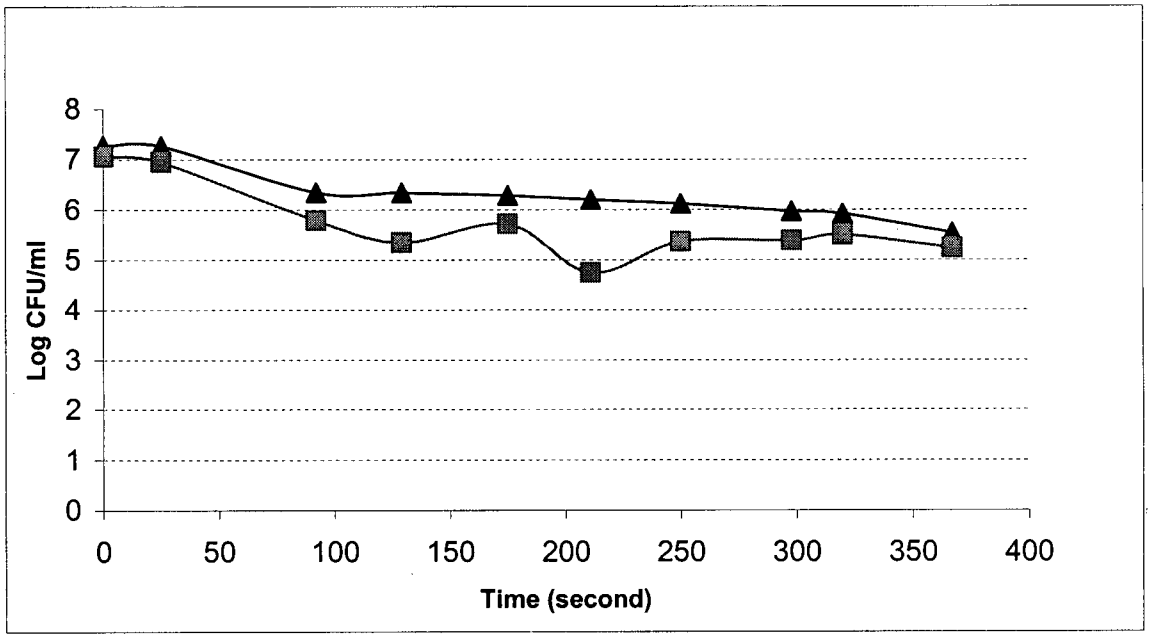

Figure 9.11. Differential counts of *E. coli* on PCA —▲—and PCA-BS —■— during vacuum microwave (711W) at 51.84°C.

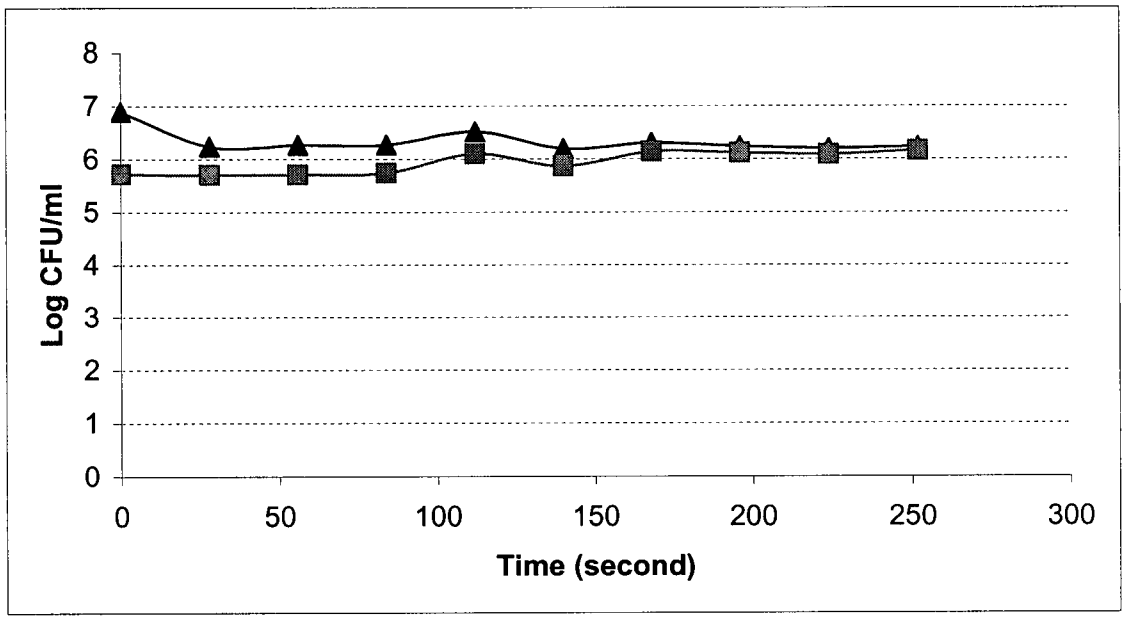

Figure 9.12. Differential counts of *E. coli* on PCA —▲—and PCA-BS —■— during vacuum microwave (510W) at 50.21°C.

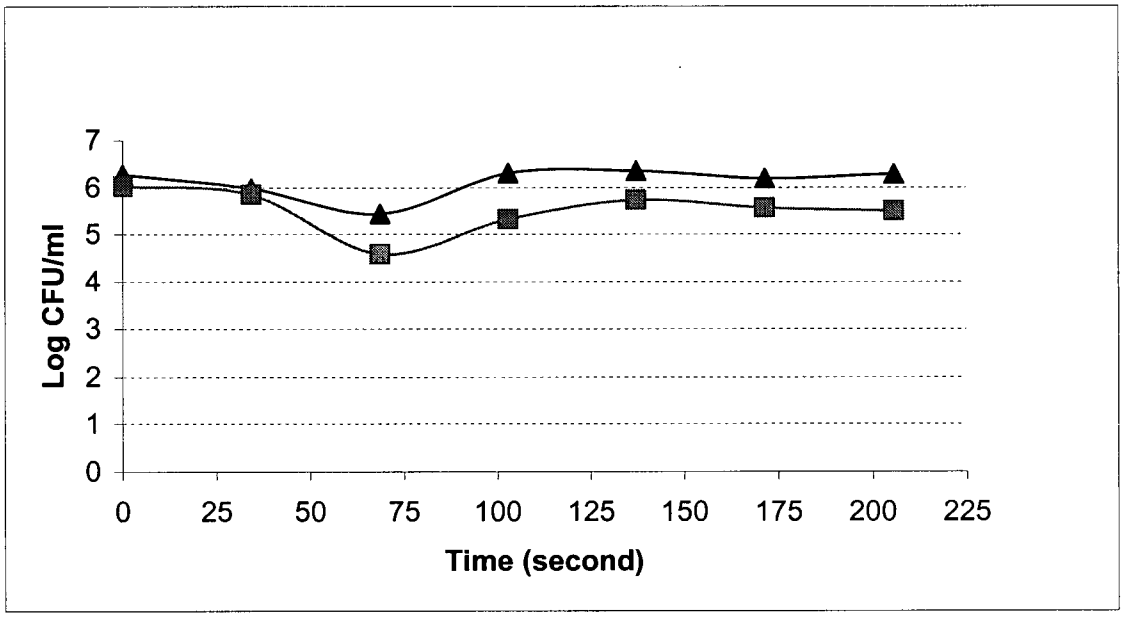

Figure 9.13. Differential counts of *E. coli* on PCA —▲—and PCA-BS —■— during water bath treatment under vacuum at 50.5°C.

9.7 Appendix VII: Genes altered less than two fold between late-log and mid-stationary cells ($p<0.05$).

Table 9.4. Genes up-regulated less than 2 fold in mid-stationary phase cells compared to late-log phase *E. coli* cells ($p<0.05$).

Gene	b no.	Fold change	Call	
			stationary phase	log phase
citC	b0618	1.40	P[3]	A[4]
csgF	b1038	1.42	P	P
cvpA	b2313	1.40	P	P
dmsA	b0894	1.42	P	P
gapC_2	b1416	1.44	P	A
glcB	b2976	1.73	P	A
glgS	b3049	1.76	P	P
pheL	b2598	1.72	P	P
pheV	b2967	1.38	P	P
phoH	b1020	1.47	P	A
rhaR	b3906	1.59	P	A
rhoL	b3782	1.35	P	P
rspA	b1581	1.54	P	P
slp	b3506	1.59	P	P
soxS	b4062	1.93	P	A
syd	b2793	1.37	P	P
t150	b3558	1.65	P	A
tehA	b1429	1.31	P	A
ubiX	b2311	1.50	P	P
wcaD	b2056	1.46	P	P
xylF	b3566	1.57	P	A
yadQ	b0155	1.51	P	A
yadT	b0158	1.36	P	P
yaeE	b0198	1.65	P	P
ybcI	b0527	1.54	P	P
ycdQ	b1022	1.18	P	A
yceE	b1053	1.30	P	P
ycfQ	b1111	1.23	P	P
ycjZ	b1328	1.63	P	P
ydjZ	b1752	1.58	P	A
yebF	b1847	1.37	P	P
yeeT	b2003	1.43	P	A
yfaA	b2230	1.66	P	A
yfcC	b2298	1.84	P	A
yffB	b2471	1.24	P	P

Table 9.4. Continued.

Gene	b no.	Fold change	Call	
			stationary phase	log phase
yfiP	b2583	1.30	P	P
ygaC	b2671	1.59	P	A
yghK	b2975	1.63	P	P
yhdM	b3292	1.51	P	P
yhdU	b3263	1.55	P	P
yhd	b326	1.28	P	P
yhdY	b3270	1.45	P	A
yheL	b3343	1.35	P	P
yiaG	b3555	1.48	P	P
yicE	b3654	1.51	P	P
yicO	b3664	1.66	P	P
yidB	b3698	1.19	P	P
yihG	b3862	1.57	P	P
yjhR	b4308	1.75	P	P
yjjP	b4364	1.42	P	P
ynfL	b1595	1.48	P	P
yohJ	b2141	1.17	P	A
yqgD	b2941	1.53	P	P
yrbL	b3207	1.52	P	P
yrfG	b3399	1.43	P	P
ytfH	b4212	1.90	P	P
	b1680	1.84	P	A
	b2596	1.75	P	P
	b3050	1.73	P	P
	b1675	1.73	P	A
	b2372	1.64	P	A
	b0832	1.61	P	P
	b3051	1.41	P	P
	b2375	1.41	P	P
	b0919	1.39	P	P
	b2666	1.39	P	P
	b0539	1.27	P	P
	b0964	1.26	P	A

[3]P= Present (gene was detected)
[4]A= Absent (gene was not detected)

Table 9.5. Genes down-regulated less than two fold in mid-stationary phase cells compared to late-log phase *E. coli* cells ($p<0.05$).

Gene	b no.	Fold change	Call	
			stationary phase	log phase
accB	b3255	1.73	P[3]	P[4]
accD	b2316	1.77	P	P
argC	b3958	1.86	A	P
aroB	b3389	1.85	P	P
artQ	b0862	1.51	P	P
atpI	b3739	1.59	P	P
basS	b4112	1.68	A	P
bcp	b2480	1.86	P	P
bisC	b3551	1.25	A	P
carB	b0033	1.94	P	P
cbl	b1987	1.47	P	P
ccmH	b2194	1.73	A	P
crp	b3357	1.88	P	P
crr	b2417	1.88	P	P
damX	b3388	1.95	P	P
dcp	b1538	1.75	A	P
dctA	b3528	1.65	A	P
deoR	b0840	1.42	P	P
dksA	b0145	1.95	P	P
dld	b2133	1.88	A	P
dppD	b3541	1.61	A	P
dppF	b3540	1.34	P	P
dsbA	b3860	1.45	P	P
edd	b1851	1.40	P	P
efp	b4147	1.90	P	P
fdoI	b3892	1.77	P	P
fimF	b4318	1.77	A	P
fnr	b1334	1.77	A	P
focA	b0904	1.58	P	P
ftsL	b0083	1.89	P	P
fumA	b1612	1.86	P	P
gcpE	b2515	1.82	P	P
glgP	b3428	1.86	P	P
glnL	b3869	1.54	P	P
gloB	b0212	1.59	P	P
glyS	b3559	1.71	P	P
greA	b3181	1.75	P	P

Table 9.5. Continued.

Gene	b no.	Fold change	Call	
			stationary phase	log phase
guaA	b2507	1.69	A	P
guaC	b0104	1.84	P	P
hemC	b3805	1.80	P	P
hemE	b3997	1.63	P	P
hemX	b3803	1.42	P	P
himD	b0912	1.81	P	P
hisA	b2024	1.71	P	P
hisF	b2025	1.83	P	P
hybA	b2996	1.77	P	P
hybG	b2990	1.47	P	P
ilvC	b3774	1.56	P	P
imp	b0054	1.79	P	P
kdsA	b1215	1.87	P	P
kdsB	b0918	1.48	P	P
lexA	b4043	1.66	P	P
manY	b1818	1.41	P	P
map	b0168	1.54	P	P
mdh	b3236	1.48	P	P
mltA	b2813	1.64	P	P
mltB	b2701	1.37	A	P
modB	b0764	1.54	A	P
motB	b1889	1.73	A	P
mraY	b0087	1.81	P	P
mrcA	b3396	1.27	P	P
mreC	b3250	1.79	P	P
msbB	b1855	1.74	A	P
mtlA	b3599	1.24	A	P
murA	b3189	1.67	A	P
nagA	b0677	1.55	P	P
nagE	b0679	1.90	A	P
nemA	b1650	1.57	A	P
nrdA	b2234	1.72	A	P
nrdB	b2235	1.82	P	P
nrfB	b4071	1.70	A	P
nuoK	b2279	1.78	P	P
nusG	b3982	1.76	P	P
ogrK	b2082	1.43	A	P
oppC	b1245	1.66	P	P
panD	b0131	1.60	P	P
parE	b3030	1.79	A	P

Table 9.5. Continued.

Gene	b no.	Fold change	Call	
			stationary phase	log phase
pepB	b2523	1.57	P	P
pepD	b0237	1.94	P	P
pepQ	b3847	1.54	A	P
pgk	b2926	1.76	P	P
pgpA	b0418	1.20	P	P
pheS	b1714	1.54	P	P
phnE	b4104	1.31	P	P
pinO	b3322	1.13	A	P
pitA	b3493	1.53	P	P
plsB	b4041	1.67	A	P
pncB	b0931	1.90	P	P
pntB	b1602	1.75	P	P
ppa	b4226	1.62	P	P
ppsA	b1702	1.74	P	P
proB	b0242	1.47	P	P
pta	b2297	1.65	P	P
ptsA	b3947	1.28	P	P
ptsH	b2415	1.89	P	P
putA	b1014	1.88	A	P
pyrE	b3642	1.55	P	P
recC	b2822	1.12	P	P
rfaC	b3621	1.29	P	P
rffG	b3788	1.62	P	P
rhlB	b3780	1.40	P	P
rho	b3783	1.71	P	P
rnpA	b3704	1.93	P	P
sbcB	b2011	1.67	P	P
sdhB	b0724	1.92	P	P
sdhC	b0721	1.85	P	P
sdhD	b0722	1.92	P	P
secE	b3981	1.85	P	P
serA	b2913	1.79	A	P
slpA	b0028	1.81	A	P
slyA	b1642	1.83	P	P
slyD	b3349	1.43	P	P
speB	b2937	1.52	P	P
spoT	b3650	1.70	P	P
sseB	b2522	1.80	P	P
suhB	b2533	1.34	P	P
tbpA	b0068	1.71	A	P
thiG	b3991	1.74	P	P

Table 9.5. Continued.

Gene	b no.	Fold change	Call	
			stationary phase	log phase
tldD	b3244	1.41	P	P
torA	b0997	1.79	P	P
ubiA	b4040	1.70	P	P
uspA	b3495	1.38	P	P
uvrC	b1913	1.63	A	P
wecB	b3786	1.47	P	P
xthA	b1749	1.43	P	P
yadG	b0127	1.68	A	P
yaeQ	b0190	1.36	A	P
ybaS	b0485	1.27	P	P
ybeA	b0636	1.62	A	P
ybiC	b0801	1.33	P	P
ybiS	b0819	1.62	P	P
ybiT	b0820	1.70	A	P
ybjT	b0869	1.61	A	P
ycbE	b0933	1.42	P	P
ycfN	b1106	1.66	P	P
ycgR	b1194	1.48	P	P
ychH	b1205	1.32	P	P
ychN	b1219	1.47	P	P
yehT	b2125	1.36	P	P
yeiO	b2170	1.43	A	P
yeiP	b2171	1.49	A	P
yfaO	b2251	1.86	A	P
yfcE	b2300	1.83	A	P
yfhJ	b2524	1.71	P	P
yfhO	b2530	1.66	P	P
ygdD	b2807	1.73	A	P
ygdH	b2795	1.68	A	P
ygfQ	b2884	1.62	P	P
yhbC	b3170	1.82	P	P
yhbG	b3201	1.92	P	P
yhbZ	b3183	1.74	A	P
yhcG	b3220	1.65	A	P
yhjH	b3525	1.38	A	P
yi5A	b3557	1.63	P	P
yiaF	b3554	1.75	P	P
yijC	b3963	1.45	P	P
yjcE	b4065	1.34	A	P

Table 9.5. Continued.

Gene	b no.	Fold change	Call	
			stationary phase	log phase
yjdF	b4121	1.32	P	P
yjeQ	b4161	1.38	P	P
yjfG	b4233	1.53	P	P
yjgF	b4243	1.63	P	P
yjgQ	b4262	1.32	A	P
yjjK	b4391	1.59	A	P
yjjU	b4377	1.37	P	P
ykgF	b0307	1.84	A	P
yliJ	b0838	1.57	P	P
yqiB	b3033	1.42	P	P
yrbI	b3198	1.18	A	P
ytfM	b4220	1.34	P	P
	b0955	1.85	P	P
	b2340	1.76	A	P
	b1832	1.76	A	P
	b1840	1.76	P	P
	b3838	1.72	P	P
	b2290	1.64	P	P
	b2511	1.58	P	P
	b2875	1.49	P	P
	b2817	1.39	P	P
	b2899	1.37	P	P
	b1007	1.23	P	P
	b0762	1.21	P	P
	b0105	1.37	P	P
	b1448	1.35	P	P
	b1809	1.68	A	P
	b1647	1.34	A	P

[3]P= Present (gene was detected)
[4]A= Absent (gene was not detected)