

**SHELF LIFE EXTENSION OF PREFORMED PIZZA USING PULSED ULTRAVIOLET
LIGHT**

By
Tina Mohammadbeygy

**Department of Bioresource Engineering
Macdonald Campus, McGill University
Ste-Anne-de-Bellevue, Quebec, Canada**

**A thesis submitted to McGill University in partial fulfillment of the
requirements of the degree of
Master of Science
December, 2013**

© Tina Mohammadbeygy, 2013

DEDICATION

I dedicate this piece of work to my parents.

ABSTRACT

Mold is a common post-processing contaminant in pizza. Since contamination could occur in post-baking process, other safety barriers in addition to packaging are needed to ensure the continued safety of preformed pizza. The present research investigated the use of pulsed UV-light for the purpose of decontaminating *Penicillium roqueforti* (a common mold in bakery products) on the surfaces of agar media, bread and preformed pizza.

In the first step in the present study, critical process parameters were optimized to enhance the efficiency of pulsed UV-light treatments. The spatial distribution of pulsed UV-light was investigated. A bench-top pulsed UV system was used to produce up to 1,000 V (20 J). The Petri plates inoculated with *Penicillium roqueforti* were treated 10 min in the first three effective levels in the treatment chamber (5, 10 and 15 cm). 30, 75 and 90 percent of the surface area of the tray was exposed to pulsed light at 5, 10 and 15 cm from pulsed light lamp, respectively. Apple juice was also used as a simple substrate to characterize the efficacy of the pulsed light apparatus. Apple juice inoculated with *E. coli* was treated with pulsed UV-light at the same distances (5, 10 and 15 cm) from the light source, 3 voltage inputs (400, 750 and 1,000 V) and 3 treatment times (1, 5 and 10 min). The log reduction in *E. coli* population treated by pulsed UV-light varied from 1.4 to 2.05 log CFU ml⁻¹.

In the second phase of the study, the consistency of mold growth (*Penicillium roqueforti*) on the surface of flat bread was investigated using two different methods of inoculation; random spot and spread. The consistency trend over time was similar in both methods of inoculation. Two different inoculum populations (10² and 10³ CFU ml⁻¹) were also used to assess the effect of inoculum concentration on the distribution and consistency of mold growth. Samples inoculated with 10² CFU ml⁻¹ yielded more homogeneously distributed colonies. The efficacy of these two inoculation methods was also evaluated on pizza bread with and without toppings after treatment. Parameters of 400 V, 1,200 pulses and a distance of 5 cm from the UV strobe resulted in complete inactivation of *P. roqueforti* on the surface of bread in random spot and spread inoculated samples (by visual inspection).

In the third phase of the study, two methods, e.g., sensory and microbiological analyses, were used to evaluate the effectiveness of pulsed light treatment for shelf life extension of pizza and bread. Up to 40 days shelf-life extension was achieved for 8, 32, and 40 percent of samples with minimal, intermediate and maximal pulsed light treatment, respectively. Samples treated

with the more intense voltage (1,000 V) showed steady surviving population less than 10^2 CFU g^{-1} over the storage time in 21 days. However, 500 V treated breads and controls showed mold growth on every analysis day (more than 10^3 CFU g^{-1}).

In the fourth phase of the study, the effectiveness of a broad-spectrum pulsed ultraviolet light was evaluated for the decontamination of *Penicillium roqueforti* on the surface of solid agar. Process parameters evaluated were treatment time (1, 3, 5, 7 and 10 min) and voltage input (500, 750 and 1,000 V). The population of *Penicillium roqueforti* was reduced after 10 min of exposure to pulsed light by 3.74, 5.36 and 6.14 log CFU ml^{-1} , respectively for 500, 750 and 1,000 V. The inactivation data were used to construct models to estimate the inactivation. The log-linear and Weibull models were constructed. The results presented in this study indicate that first-order kinetics is not suitable for the estimation of *P. roqueforti* inactivation on solid agar with pulsed UV light. However, the inactivation kinetics was best described by the Weibull model with the smallest root mean squared error (RMSE) ($R^2 \geq 0.92$). Weibull model can be successfully used to estimate the inactivation and the concavity exhibited in the survival curves observed in this study.

Finally in the last phase of study, culture-dependent and independent methods were applied to study the ecology of preformed pizza produced in a pizzeria on the Island of Montreal. Classical microbiological analyses were performed to identify the main microbial groups present in the product. The average population of mesophilic aerobic bacteria (MAB), mesophilic anaerobic bacteria (MANB), lactic acid bacteria (LAB), molds and yeasts (M+Y) were 6.6 ± 0.5 , less than 2.4, 2.8 ± 0.6 and 5.4 ± 0.4 log CFU g^{-1} , respectively. Fungal diversity was analyzed by extracting DNA from naturally spoiled preformed pizza samples. Molecular methods incorporating conventional PCR targeting the 18S rRNA gene of fungi, TA cloning of PCR-amplified fragments and sequencing were carried out to detect spoilage fungi in naturally spoiled pre-formed pizza. The cloning approach enabled the putative identification of *Saccharomyces cerevisiae*, *Saccharomyces* sp. WW-W23, *Penicillium expansum*, *Penicillium freii*, *Penicillium* sp. HSL, *Penicillium* sp. ljl1, *Rhodotorula mucilaginosa*, *Monascus fuliginosus*, *Hordeum jubatum*, *Galactomyces geotrichum* strains as well as uncultured fungus and uncultured eukaryote clones.

Overall, pulsed UV-light was found to have a potential use for the decontamination of spoilage microorganisms on the surfaces of solid agar and bakery products. However, further

investigation using higher treatment voltages is necessary in order to achieve a higher target decontamination of *P. roqueforti*.

RÉSUMÉ

La pizza pré-cuisinée est couramment contaminée par la moisissure. Puisque la contamination pourrait se produire durant les procédés d'après cuisson, d'autres mesures, en plus de l'emballage, sont nécessaires pour assurer la sécurité permanente des pizzas pré-cuisinée. Cette recherche a examiné l'utilisation de la lumière UV pulsée pour décontaminer *Penicillium roqueforti* sur la surface de l'agar solide, du pain et de pizzas préformées.

Dans la première étape de la présente étude, les paramètres critiques du procédé ont été optimisés pour améliorer l'efficacité des traitements à la lumière pulsée. La répartition spatiale de la lumière UV pulsée a été étudiée. Un Samtech de paillasse à UV pulsée a été utilisé pour générer jusqu'à 1000 V (20 J). Les boîtes de Petri inoculées avec *Penicillium roqueforti* ont été traitées 10 min dans une chambre de traitement pour les trois premiers niveaux d'efficacité (5, 10 et 15 cm). Les résultats démontrent que 30, 75 et 90 pour cent de la surface du plateau a été exposée à la lumière pulsée à 5, 10 et 15 cm respectivement de la source lumineuse. Du jus de pomme a également été utilisé comme substrat simple pour caractériser l'efficacité du dispositif à lumière pulsée. Le jus de pomme inoculé avec *E. coli* a été traité avec la lumière pulsée aux mêmes distances (5, 10 et 15 cm) de la source lumineuse que précédemment, sous trois entrées de tension (400, 750 et 1000 V) et de temps de traitement (1, 5 et 10 min). La réduction en log de la population de *E. coli* traitée par la lumière UV pulsée varie de 1.4 à 2.05 log UFC ml⁻¹. Cette répartition a été obtenue avec des traitements de 120 impulsions à 400 V jusqu'à 1200 impulsions à 1000 V.

Dans la deuxième phase de l'étude, la cohérence de la croissance des moisissures (*Penicillium roqueforti*) sur la surface du pain plat a été étudiée en utilisant deux méthodes différentes d'inoculation : par inoculation ponctuelle aléatoire et par étalement. La cohérence de la croissance a respecté une tendance similaire dans le temps pour les deux méthodes d'inoculation. Deux populations d'inoculum différentes (10² et 10³ UFC ml⁻¹) ont également été utilisées pour évaluer l'effet de la densité de l'inoculum sur la distribution et la cohérence de la croissance de moisissures. Les échantillons inoculés avec 10² UFC ml⁻¹ ont donné une distribution des colonies plus homogène. L'efficacité de ces deux méthodes d'inoculation a également été évaluée sur le pain à pizza avec et sans garnitures après le traitement. Les paramètres de 400 V, 1200 impulsions et 5 cm de distance de l'impulsion d'UV ont abouti à une

inactivation complète de *P. roqueforti* sur la surface des échantillons de pain inoculés autant pour la méthode d'inoculation aléatoire que par étalement (par inspection visuelle).

À la troisième phase de cette étude, deux méthodes, soit l'analyse sensorielle et microbiologique, ont été utilisées pour évaluer l'efficacité du traitement à la lumière pulsée pour prolonger la durée de vie de la pizza et du pain. Jusqu'à 40 jours de prolongement de la durée de conservation a été obtenue pour 8, 32 et 40 pour cent des échantillons après un traitement à la lumière pulsée minimal, intermédiaire et maximal, respectivement. Les échantillons traités sous tension plus intense (1000 V) ont montré des populations survivantes régulières de moins de 10^2 UFC g^{-1} au cours de la durée d'entreposage. Toutefois, les pains et les contrôles traités à 500 V ont montré une croissance exponentielle pour chaque jour d'analyse (plus de 10^3 UFC g^{-1}).

Dans la quatrième phase de l'étude, l'efficacité d'une lumière UV pulsée à large spectre a été évaluée pour la décontamination de *Penicillium roqueforti* sur la surface de l'agar solide. Les paramètres du procédé évalués étaient le temps de traitement (1, 3, 5, 7 et 10 min) et la tension d'entrée (500, 750 et 1000 V). La population de *Penicillium roqueforti* a été réduite après 10 minutes d'exposition à la lumière pulsée par 3.74, 5.36 et 6.14 log UFC ml^{-1} respectivement pour 500, 750 et 1000 V. Les données d'inactivation ont été utilisées pour construire des modèles pour estimer l'inactivation. Les modèles log-linéaire et Weibull ont été construits. Les résultats présentés dans cette étude indiquent que la cinétique de premier ordre n'est pas appropriée pour l'estimation de l'inactivation de *P. roqueforti* sur l'agar solide avec la lumière pulsée. Cependant, la cinétique d'inactivation a été mieux décrite par le modèle de Weibull avec la plus petite erreur de moyenne quadratique (RMSE) ($R^2 \geq 0.92$). Le modèle de Weibull peut être utilisé avec succès pour estimer l'inactivation et la concavité présente dans les courbes de survie observées dans cette étude. L'augmentation de la température à l'intérieur de la chambre de traitement a également été suivie au cours de cette expérience.

Finalement, dans la dernière phase de l'étude, des méthodes dépendantes ou indépendantes des conditions de culture ont été appliquées pour étudier l'écologie des pizzas pré-cuisinées produites dans une pizzeria de l'île de Montréal. Des analyses microbiologiques classiques ont été effectuées pour identifier les principaux groupes microbiens présents dans le produit. La moyenne de la population des bactéries mésophiles aérobies (BMA), des bactéries mésophiles anaérobies (BMNA), des bactéries lactiques (BL), des moisissures et des levures

(M+L) étaient respectivement de 6.6 ± 0.5 , inférieur à 2.4, 2.8 ± 0.6 et 5.4 ± 0.4 log UFC g⁻¹. La diversité fongique a été analysée par extraction d'ADN à partir d'échantillons de pizzas pré-cuisinées naturellement contaminées. Les méthodes moléculaires incorporant la PCR conventionnelle ciblant le gène de l'ARNr 18S des champignons, le clonage TA de fragments amplifiés par PCR et le séquençage ont été réalisées pour détecter les champignons altérant les pizzas pré-cuisinées naturellement contaminées. L'approche du clonage a permis l'identification présumée de souches de *Saccharomyces cerevisiae*, *Saccharomyces* sp. WW- W23, *Penicillium expansum*, *Penicillium freii*, *Penicillium* sp. HSL, *Penicillium* sp. ljl1, *Rhodotorula mucilaginosa*, *Monascus fuliginosus*, *Hordeum jubatum*, *Geotrichum galactomyces* ainsi que des champignons et eucaryotes non cultivés.

Dans l'ensemble, la lumière UV pulsée a démontré avoir un potentiel d'utilisation pour la décontamination des microorganismes altérant les surfaces d'agar solide et les produits de boulangerie. Toutefois, une étude plus approfondie sur des tensions de traitement plus élevées est nécessaire pour parvenir à une décontamination satisfaisante de *P. roqueforti*.

ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my advisor, **Dr. Michael O. Ngadi**, for all his guidance, encouragement, and patience throughout my graduate program. In the same vein, I would like to appreciate my thesis co-supervisor, **Dr. Martin R. Chénier**, for his continuous and valuable guidance, support and motivation for successful completion of the research work. My special thanks go to **Mr. Devin Holman** for all the support and guidance extended to me during the course of this work. I am grateful to **Mr. Jamshid Rahimi**, **Mr. Chijioke Ejebe** and **Dr. Akinbode Adedeji** for their valuable help and support during this work.

My profound gratitude goes to **Les Aliment Rustica** in Montreal for having provided the pizzas for the research work. I would like to acknowledge the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ) for their financial support.

I would like to thank **Mrs. Susan Gregus**, **Mrs. Trish Singleton** and **Mrs. Abida Subhan** for their valuable help and support in all administrative aspects.

I express my deep sense of gratitude to my most affectionate and beloved parents, **Mrs. Eli Nader** and **Mr. Alex Beigi** for their great love, encouragement and advice during my studies.

CONTRIBUTION OF AUTHORS

This thesis research consists of five manuscripts authored by me. The manuscripts were corrected by Dr. Michael O. Ngadi, my supervisor, and by Dr. Martin R. Chénier, my co-supervisor. Dr. Michael O. Ngadi and Dr. Martin R. Chénier were on my direct advisory committee for designing the concepts, methodology and execution of the thesis research and reviewed the thesis report. Chapter 5 was presented at the 2013 of The Northeast Agricultural and Biological Engineering Conference (NABEC-2013, Altoona, PA, USA). A manuscript including Chapters 3 to 7 of this thesis will be submitted for publication in a scientific journal. The results from Chapter 6 have been submitted for oral presentation in the Annual Conference of the American Society of Agricultural Engineers (ASABE-2014, Montreal, QC, Canada)

TABLE OF CONTENT

ABSTRACT	ii
RÉSUMÉ	v
ACKNOWLEDGEMENTS	viii
CONTRIBUTION OF AUTHORS	ix
TABLE OF CONTENT	x
LIST OF TABLES.....	xiv
LIST OF FIGURES.....	xv
NOMENCLATURE	xvi
Greek	xvii
INTRODUCTION	1
OBJECTIVES	4
CHAPTER 2.....	5
LITERATURE REVIEW	5
2.1 Bakery products.....	5
2.2.1 Physical spoilage.....	7
2.2.2 Chemical spoilage.....	7
2.2.3 Microbial spoilage.....	7
2.3 Traditional preservation technologies for bakery product	9
2.3.1 Freezing.....	9
2.3.2 Modified atmosphere packaging (MAP).....	10
2.3.3 Microwave heating	10
2.4 Novel non-thermal processing technologies.....	11
2.4.1 High hydrostatic pressure processing	11
2.4.2 Oscillating magnetic field (OMF)	12
2.4.3 Ultraviolet.....	12
2.5 Pulsed light treatment.....	14
2.5.1 Pulse UV-light system	14
2.5.2 Pulsed-light mechanism of inactivation.....	15
2.5.3 Kinetics of pulsed UV-light in microbial inactivation.....	15
2.5.4 The efficiency of pulsed-light treatments	16
2.5.5 Light- substrate interaction.....	16
2.5.6 Combination of pulsed light and other technologies	17
2.5.7 Effects of pulsed light on food products	18
2.5.8 Various microorganism affected by pulsed UV-light.....	21
2.6 Identification and quantification of spoilage fungi	21
2.6.1 Methods of identification of fungi.....	22
2.7 REFERENCES	25

CHAPTER 3.....	31
CHARACTERIZATION OF PULSED UV-LIGHT APPARATUS	31
3.1 Abstract.....	31
3.2 Introduction.....	31
3.3 Materials and Methods.....	32
3.3.1 Pulsed UV- light system	32
3.3.2 Treatment chamber	33
3.3.3 Spatial distribution	34
3.3.4 Testing the equipment with the apple juice	35
3.3.5 Statistical analysis	37
3.4 Results and discussion	37
3.4.1 Spatial power distribution (12 plate experiment).....	37
3.4.2 Microbial analysis for apple juice	39
3.5 Conclusion	41
3.6 REFERENCES	41
CHAPTER 4.....	43
SELECTION OF MICROBIOLOGICAL PROTOCOLS FOR INOCULATION AND ENUMERATION OF MOLD ON BREAD SURFACE	43
4.1 Abstract.....	43
4.2 Introduction.....	43
4.3 Materials and Methods.....	44
4.3.1 Sampling	44
4.3.2 Fungal isolate and preparation of inoculum.....	45
4.3.3 Inoculation of bread and visual inspection of untreated bread	45
4.3.4 Inoculation, pulsed UV-light treatment and visual inspection of treated breads without tomato sauce topping.....	46
4.3.5 Inoculation, pulsed UV-light treatment and visual inspection of treated pizza bread with tomato sauce topping	46
4.4 Results and discussion	47
4.4.1 Inoculums size	47
4.4.2 Inoculation methods.....	47
4.4.3 Inspection of mold growth on bread with and without tomato sauce topping.....	49
4.5 Conclusion	51
4.6 REFERENCES	51
CHAPTER 5.....	52
SHELF LIFE EXTENTION OF PIZZA AND BREAD BY PULSED UV-LIGHT.....	52
5.1 Abstract.....	52
5.2 Introduction.....	52

5.3 Materials and Methods	54
5.3.1 Pizza bread with tomato sauce topping.....	54
5.3.2 Pizza bread without tomato sauce topping	54
5.3.3 Pulsed UV-light treatment for pizza samples	54
5.3.4 <i>Penicillium roqueforti</i> inoculum preparation (for bread samples).....	55
5.3.5 Pulsed UV- light treatment for bread samples	55
5.3.6 Analysis of pizza samples after pulsed UV treatments	55
5.3.7 Statistical analysis for bread	56
5.4 Results and Discussion	56
5.4.1 Pulsed UV-light on Pizza	56
5.4.2 Pulsed UV-light on Pizza	57
5.5 Conclusion	59
5.6 REFERENCES	59
CHAPTER 6	61
EFFECTIVENESS OF PULSED ULTRAVIOLET-LIGHT TREATMENT FOR INACTIVATION OF <i>PENICILLIUM ROQUEFORTI</i> ON AGAR SURFACE	61
6.1 Abstract	61
6.2 Introduction	62
6.3 Material and methods	63
6.3.1 Preparation of Inoculum	63
6.3.2 Treatments.....	64
6.3.3 Inactivation of <i>P. roqueforti</i>	64
6.3.4 Statistical analysis	64
6.3.5 Model development	64
6.4 Results and discussion	66
6.4.1 Pulsed UV light and microbiological analysis.....	66
6.4.2 Inactivation kinetics model	67
6.5 Conclusion	69
6.6 REFERENCES	70
CHAPTER 7	72
ANALYSIS OF FUNGAL DIVERSITY IN PRE-FORMED PIZZA BY SEQUENCING OF CLONED PCR- AMPLIFIED GENES ENCODING 18S rRNA	72
7.1 Abstract	72
7.2 Introduction	72
7.3 Materials and methods	73
7.3.1 Sampling	73
7.3.2 Preparation of pizza samples for microbiological analysis.....	74
7.3.3 Enumeration of Total Bacteria	74
7.3.4 Enumeration of Lactic Acid Bacteria.....	74
7.3.5 Identification of Lactic Acid Bacteria	74

7.3.6	Enumeration of Molds and Yeasts.....	75
7.3.7	Extraction of Nucleic Acids	75
7.3.8	Quantification and purity of DNA.....	76
7.3.9	Verification of DNA quality	76
7.3.10	Polymerase Chain Reaction amplification	76
7.3.11	Temperature gradient PCR assay for primer pairs A and B	77
7.3.12	PCR on spoiled samples	78
7.3.13	Purification of PCR products	78
7.3.13	Cloning	78
7.3.14	Sequencing	81
7.4	Results and discussion	81
7.4.1	Enumeration of microbial population	81
7.4.2	Identification of LAB isolates.....	82
7.4.3	Concentration and purity of the isolated DNA after Extraction.....	83
7.4.4	Temperature gradient PCR	84
7.4.5	Conventional PCR on spoiled samples	85
7.4.6	Concentration and purity of PCR products	88
7.4.7	18S rDNA clone libraries.....	88
7.4.8	Diversity of fungal biota	91
7.5	Conclusion	94
7.6	REFERENCES	94
	CHAPTER 8.....	98
	GENERAL SUMMARY & CONCLUSION	98
	Future Recommendations	100

LIST OF TABLES

Table 2.1 Shelf-life of some bakery products.	6
Table 3.1 Spatial power distribution of pulsed UV light on treatment tray, at three different distances (5, 10 and 15 cm) from pulsed UV source (PUV- 01 system).....	38
Table 4.1 Incidence of mold growth on pizza bread (without tomato topping) surface after UV treatment. Ten random spots were applied to each dry bread surface (5 replicate bread slices per treatment)	49
Table 4.2 Incidence of mold growth on pizza surface with tomato sauce topping, after UV treatment. Five random spots were applied (4 replicated for each method).	50
Table 5.1 Conditions used for the pulsed UV-light treatments.....	56
Table 5.2 Percentage of non-moldy pizza before 10, 15, 20, 30, 40 days.....	57
Table 6.1 Goodness-of-fit parameters of the first order kinetics models estimating reductions of <i>Penicillium roqueforti</i> on solid agar after treatment with pulsed UV light.....	69
Table 6.2 Goodness-of-fit parameters of Weibull models estimating reductions of <i>Penicillium roqueforti</i> on solid agar after treatment with pulsed UV light	69
Table 7.1 Overview of the oligonucleotide sequences used in the study.....	77
Table 7.2 Cloning conditions	80
Table 7.3 Microbia populations in spoiled pre-formed pizza. Data are the results of the microbiological analysis of 10 samples.....	82
Table 7.4 Identification of LAB by biomedical analysis. Biomedical profile was compared with the closest relatives in the APIWEB database.....	83
Table 7.5 Concentration and purity of extracted DNA from spoiled samples.	84
Table 7.6 Concentration and purity of the purified PCR products spoiled samples... ..	88
Table 7.7 Tentative identification of clones by sequencing and BLAST analysis. Sequences were aligned with the closest relatives in the GenBank database with BLAST	93

LIST OF FIGURES

Figure 3.1 Schematic diagram of pulsed UV-light treatment system.....	33
Figure 3.2 Spectral output from the light source at operating voltages of 400, 700 and 1,000 V	34
Figure 3.3 Position of Petri plates on pulsed UV-light chamber treatment tray	35
Figure 3.4 Distribution of pulsed UV-light at varying distances (5, 10 and 15 cm) from the pulsed UV source (1,000 V, 5 min)	38
Figure 3.5 Log reduction in population of <i>Escherichia coli</i> treated by pulsed UV- light in apple juice at different distances, voltage inputs and treatment times	40
Figure 5.1 Surviving mold (<i>P. roqueforti</i>) population over storage time in each analysis day when time and distance are fixed at 5 cm and 7 minutes	58
Figure 6.1 Inactivation of <i>Penicillium roqueforti</i> with pulsed UV light fitted with the firstorder and Weibull kinetics models.	68
Figure 7.1 Temperature Gradient PCR for by primer set A (A), and primer set B (B). Two replicates per temperature are used.....	85
Figure 7.2 PCR performed on spoiled samples using primer set B with 58°C annealing temperature. Two replicates per temperature are used. Ladder shows 100 to 1,000 bp.	87
Figure 7.3 Four images show PCR performed after cloning using primer set C. The amplicons with insert of the expected size are equal to 1,400 bp	89

NOMENCLATURE

°C	Degree Celsius
A_{260/230}	Absorbance at 260 nm / Absorbance at 230 nm
A_{260/280}	Absorbance at 260 nm / Absorbance at 280 nm
ANOVA	Analysis of Variance
API	Analytical Profile Index
ATCC	American Type Culture Collection
a_w	Water Activity
BLAST	Basic Local Alignment Sequence Tool
CFSAN	Center for Food Safety and Applied Nutrition
CFU	Colony Forming Unit
CM	Centimeter
CO₂	Carbon Dioxide
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxyribo Nucleotide TriPhosphate
D-value	Time necessary for a 1 log reduction
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
HHP	High Hydrostatic Pressure
IPTG	Isopropyl b-D-Thio Galactoside
ITS	Internal Transcribed Spacers
J	Joule
LAB	Lactic Acid Bacteria
LB Broth	Luria-Bertani Broth
M+Y	Molds and Yeasts
MAB	Mesophilic Aerobic Bacteria
MANB	Mesophilic Anaerobic Bacteria
MAP	Modified Atmosphere Packaging
MHz	Mega Hertz
MPa	Mega Pascals
N	Number of Microorganisms
N₀	Initial Number of Microorganisms
N₂	Nitrogen
O₂	Oxygen

OMF	Oscillating Magnetic Fields
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pH	Hydrogen Ion Concentration
PL	Pulsed Light
Pps	Pulse Per Second
PS	Polystyrene
PUV	Pulsed UV
R₂	R-squared
rDNA	Ribosomal DNA
RFLP	Fragment Length Polymorphism
RMSE	Smallest Root Mean Squared Error
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
SAS	Statistical Analysis Software
TA cloning	Adenine and Thymine cloning
	Tris-Acetate-EDTA
TAE buffer	buffer
<i>Taq</i>	Thermus Aquaticus Polymerase
t_R	Reliable life
UHP	Ultra High Pressure
UV	Ultraviolet
V	Voltage
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Greek

μl	Micro liter
μM	Micro Molar
t	Time
α	Wavelength
β	Characteristic Time
λ	Concavity

INTRODUCTION

Pizza is among the world's most widely consumed convenience or fast food. It is included in more than \$2.3 billion industrial bread and bakery product in Canada and its consumption is rapidly increasing (AAFC, 2011). In particular, pizza in its various forms is an important part of the bakery industry. Beside the very large quantities that are prepared and consumed at restaurants and homes, increasing numbers are produced as preformed pizza dough that are ready to be finish-baked by consumers. The increasing popularity of pizza can be attributed to its ability to address consumer demand for convenience products. Preformed pizzas are sometimes sold frozen while others are sold as shelf stable products. Frozen pizza must be kept frozen at all times until it is prepared for consumption. This requires significant energy demand for processing. However, there is no need for refrigeration for shelf stable pizza products as they are available in forms that can be stored at ambient temperature. Hence, the major difficulty with these products is that they have very short shelf lives (Smith et al., 2004).

Despite their increasing popularity, the products are characterized by highly variable and sometime unsatisfactory quality (Coppola et al., 1998). Spoilage problem of preformed pizza can be grouped into 3 main sources namely: (1) physical spoilage (moisture loss, hardening); (2) chemical spoilage (staling, rancidity); and (3) microbiological spoilage (yeast, mold, bacterial growth). Spoilage due to microbial growth is by far the major factor limiting shelf life of preformed pizza (Singh et al., 2011). Apart from health concerns, microbial spoilage presents serious economic concern to the bakery industry accounting for about 1 to 5% of product losses. Some of the common molds that have been identified on different bakery products include *Wallemia sebi*, *Penicillium spp.*, *Cladosporium spp.*, *Eurotium (Aspergillus) glaucus* group, and other *Aspergilli* (Smith et al., 2004). Though, there has not been any study to identify and characterize mold in preformed pizza samples.

Preformed pizza products are generally free of viable microorganisms after baking. However, contamination mostly occurs during the cooling and packaging processes (Pfeiffer et al., 1999; Singh et al., 2010). Preventing contamination and growth of microbes in a manufacturing environment is hardly achievable simply by adoption of aseptic conditions during packaging. Therefore, most producers normally use chemical preservatives such as sodium propionate, sorbic acid or other methods to prevent growth on the surface of the products

(Rodriguez et al., 2003). The use of chemical additives and thermal treatment of the products are methods that are largely available to the industry to decontaminate, maintain the quality and extend the shelf life of preformed pizza products. However, with more consumer desire for high quality and no-chemical added products, it has become essential to search for novel non-thermal methods of extending shelf life of the products.

Several non-thermal processing and preservation techniques have been used to minimize these undesirable changes in bakery products. This includes traditional technologies such as freezing and modified atmosphere packaging (MAP), as well as novel technologies such as high hydrostatic pressure (HHP) processing, oscillating magnetic field (OMF), microwave heating, and ultraviolet processing (Singh et al., 2010; Singh et al., 2011; Singh and Goyal, 2011; Roriguez et al., 2003). However, there is always a need to investigate novel technologies as an alternative to existing preservation methods to improve efficiency, minimize cost, and yield minimal quality changes. One such technique is pulsed light treatment. Pulsed UV- light is a method of decontamination when the microbial contamination is largely on the surface of a product. This method of food preservation that involves the use of intense and short duration pulses of broad-spectrum "white light", where each pulse, or flash, of light lasts a fraction of a second and the intensity of each flash is approximately 20,000 times the intensity of sunlight at sea level (Elmnasser et al., 2007). UV-light inactivates microorganisms by damaging the DNA via the formation of thymine dimers, which prevents transcription thus inhibiting the replication of the cell, which leads to death (Miller et al., 1999; Wang et al., 2005). The use of pulsed UV-light is preferred to continuous UV-light, because it can provide a greater amount of instantaneous energy which can result in a shorter application time.

Pulsed UV-light has been shown to successfully inactivate food pathogens on the surfaces of some foods (Hillegas and Demirci, 2003; Jun, 2003; Sharma and Demirci, 2003b). Literature reports on successful applications of pulsed light have been growing although important gaps still remain with respect to actual application of the technology in the industry (Ozer and Demirci, 2005; Elmnasser et al., 2007). Specifically, there is very limited information on its application on baked products such as preformed pizza. Pulsed light is typically generated by systems that accumulate electrical energy in a capacitor and subsequently dissipate the energy within a very short time (nanoseconds) thus multiplying the power available several folds (Dunn et al., 1995; MacGregor et al., 1998). Rowan et al. (1999) described a pulsed power device that

was evaluated for inactivation of microorganisms namely *Listeria monocytogenes*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus* on the surface of solid media. The authors reported inactivation up to 6 log reduction.

A vital aspect of characterizing novel technologies is the study of kinetic models to determine the relationship between applied dose and inactivation levels. There have been limited studies on the kinetics of pulsed UV inactivation of microorganisms on surfaces such as preformed pizza. Unlike thermal processing, pulsed light treatment typically presents non-linear inactivation curves (Izquier and Gomez-Lopez, 2011; Luksiene et al., 2007; Geeraerd et al., 2000). These curves may consist of a shoulder; a log-linear inactivation part and a tail. Literatures report that these features are well described by the Weibull model. This model has been used to describe pulsed light inactivation of *E. coli* O157:H7 and *Salmonella enterica* on fruit surfaces (Bialka et al., 2008). Pulsed light inactivation kinetics of pure bacterial cultures inoculated into model liquids or fruit juices has also recently been characterized (Uesugi et al., 2007; Sauer and Moraru, 2009). The inactivation by pulsed light of microorganisms in solid food substrates is however a more complex issue. A study on pulsed light application in the baked product industry will contribute to the knowledge that will be required to implement industrial applications of the technology. The possibility of using the technology will be a major industrial break through. Adoption of the technology will give the industry an advantage that can be used to produce and market superior and shelf stable products. Another novelty of the study is the assessment and characterization of the microbial flora in the preformed pizza. The data generated by the study will be critical for identifying the sources of contamination. The proposed study is designed to be relevant to the bakery industry, particularly the preformed pizza sector.

OBJECTIVES

The objectives of this research were to:

- (1) evaluate the effectiveness of pulsed light to inactivate molds and other microorganisms on the surface of preformed pizza;
- (2) determine the effect of pulse parameters and pulse durations on microbial inactivation;
- (3) develop a method to study and model kinetics of inactivation of microorganisms on a flat surface due to pulsed UV-light;
- (4) to analyze the diversity and composition of the fungal community in pre-formed pizza

CHAPTER 2

LITERATURE REVIEW

2.1 Bakery products

Bakery products have been a significant part of a balanced diet for thousands of years. These products that are widely available and consumed include sweet goods (pancakes, doughnuts, waffles and cookies), unsweetened goods (bread, rolls, buns, crumpets, muffins and bagels), and filled goods (sausage rolls, fruit and meat pies, sandwiches, pastries cream cakes, pizza and quiche) (Smith et al., 2004). Bakery products are an essential source of calories, and an important supply of nutrients such as protein, iron, calcium and several vitamins as well as a good target for fiber enrichment (Saranraj and Geetha, 2012). Nowadays, the bakery products production has evolved from a primeval, small house industry into a extensive, modern manufacturing, creating thousands of employments and generating revenue in billions of dollars. As these products are becoming a major part of the international food market, baking industry struggles to satisfy the healthy eating trends, safety issues and the consumer demands for fresh products (Byrne, 2000; Kohn, 2000). Deteriorations such as moulding and staling result in the decrease of consumer acceptance for bakery products and in great economic losses (Kotsiani et al., 2002). In the U.S. only, for example, it is estimated that losses due to microbiological spoilage alone, are 1 to 3% or over 90 million kg of products each year (Oraikul et al., 1991). Such concerns have made shelf life extension of bakery products an immense issue to be discussed in the industry.

It is recognized that defining the shelf life of a food is a difficult task and is an area of intense research for food product development scientists. Extending of shelf life is application specific. Factors influencing shelf life of the bakery products are processing condition, production properties (moisture content, physic-chemical properties), packaging materials (barrier water vapor, O₂, CO₂, aroma, mechanical characteristics) and storage condition (temperature, humidity, light) (Galić et al., 2009).

Table 2.1 Shelf-life of some bakery products

Product	Shelf life (average)
Fresh bakery products	2 days (bread) 7 days (cake)
Breakfast cereals	6–18 months
Pasta	Pasta with egg solids 9–36 months; Macaroni and spaghetti 24–48 months

(Labuza and Szybist, 1999)

Extending the shelf life of the bakery products not only is a powerful preventing measurement against outbreaks of food borne illnesses, but also can be a major economic help to the bakery industry and consequently help to better feed increasing population in the world. Therefore modern bakery plants and advanced food technologies should develop a suitable method to ensure the necessary shelf life for bakery products.

2.2 Classification and spoilage of bakery products

Bakery products on the basis of their pH are classified into three groups, (i) high acid bakery products with $\text{pH} < 4.6$, such as Sourdough bread, (ii) low acid bakery products with $\text{pH} > 4.6$ but < 7 like white or whole wheat bread and (iii) non-acid or alkaline bakery products with $\text{pH} > 7$ such as carrot muffin. Other important classification of bakery products is on the basis of their water activity (a_w) as (i) low moisture bakery products with $a_w < 0.6$, (ii) intermediate moisture bakery products with a_w between 0.6 and 0.85, and (iii) high moisture bakery products with $a_w > 0.85$ and generally between 0.95 and 0.99 (Galić et al., 2009). The inter-related factors that cause the spoilage problems in baked products are: storage temperature, relative humidity, level of preservatives, pH, packaging material and gaseous environment surrounding the product and most importantly, the moisture content and a_w (Smith et al., 2004). In this respect pre-cooked pizzas dough (the main product in the present study) produced with cereal, is an example of intermediate moisture bakery product. This designation makes it an ideal substrate for fungal development, due to contamination occurring after baking during packaging and storage (Pinho and Furlong, 2000).

2.2.1 Physical spoilage

Spoilage generally limits the shelf life of low and intermediate moisture bakery products. Physical spoilage involves textural changes and may encourage chemical and microbiological spoilage. This type of spoilage can be caused by moisture loss or gain as well as staling (Rodríguez et al., 2003). The problem with the moisture balance of bakery products can be prevented by packaging in materials with selective moisture and gas barrier properties, such as low or high density polyethylene. However, the more serious physical spoilage problem in bakery product, staling occurs specially in bread during the post baking period, making it less acceptable to the consumer (Kulp and Zobel, 1996). Such changes can be sensory (loss of aroma, mouth feel) or physical (loss of crumb softness, development of crumbliness). Reformulation with lipids and shortenings, and more recently, adding an anti-staling enzyme are the methods that has been commercially used to delay staling in many bakery products (Boyle et al., 1990). Moreover, the addition of chemical additives, the use of CO₂ enriched atmospheres has also been investigated as an effective means of retarding staling (Knorr et al., 1985).

2.2.2 Chemical spoilage

Low and intermediate moisture bakery products (especially those with a high fat content) are also subject to chemical spoilage or rancidity which causes off-odor and off-flavor as a result of lipid degradation. Two types of rancidity problems can occur; oxidative and hydrolytic (Saranraj et al., 2012). The former leads to formation of free radicals and peroxides during lipid oxidation that causes detrimental effects on food quality. Concerning present project's product oxidative rancidity and consequently free radicals might have bleaching effects on pigments e.g. lycopene in tomato paste in pizza (Smith et al., 2004). However the latter result in hydrolysis of triglycerides and the subsequent release of glycerol and malodorous fatty acids. Chemical spoilage is usually prevented by the addition of antioxidants or displacement of atmospheric O₂ by gas packaging in 100% N₂ where microbiological problems are not a concern (Smith et al., 2004).

2.2.3 Microbial spoilage

The major factor limiting the shelf life of high and intermediate moisture bakery products is microbiological spoilage by bacteria and fungi. It has been estimated that in the USA alone,

losses due to microbiological spoilage are 1 - 3% or over 90 million kg of product each year (Betchell et al., 1953). The most important factor influencing the microbiological spoilage of bakery products is the a_w . Basically, products with a a_w higher than 0.85 are considered as high moisture products, Preformed pizza with has a water activity of about 0.96 to 0.99. In high moisture products (a_w 0.94–0.99), almost all bacteria, yeasts and molds are capable of growth (Smith and Simpson, 1995).

2.2.3.1 Bacterial spoilage

Bacteria have a potential to contaminate baked products; however, this type of spoilage is mostly a concern in bakery products with high moisture content, since most bacteria require high a_w and low pH for growth (Saranraj and Geetha, 2012). There are several cases of foodborne illnesses associated with bakery products involving bacteria such as *Salmonella spp.*, *Listeria monocytogenes*, *Bacillus cereus*, and even *Clostridium*. *Bacillus subtilis* is reported to be the major bacteria causing spoilage in bakery products. This spore forming bacteria is typically present in raw ingredients (e.g., flour, sugar, and yeast) and it causes “rope” in bread. Ropy bread is characterized by discoloration from brown to black, the release of a rotten fruit odor and having an extremely moist and stringy bread crumb (Rosenkvist et al., 1995).

2.2.3.2 Yeast spoilage

Yeast problems occur frequently in bakery products. This spoilage in bakery products could be categorized in two broad types; (i) visible yeasts growth on the surface of the products in white or pinkish patches, which is affiliated with products of high a_w and short shelf life (ii) fermentative spoilage that could be recognized by alcoholic, essence odors and/or evidence of gas production which is usually associated with low a_w and long shelf life products. *Pichia burtonii*, also known as chalk mold, is the main yeast which cause of surface spoilage in bread. Other yeasts namely *Candida guilliermondii*, *Hansenula anomala* and *Debaromyces hansenii* also account for a lesser extent of spoilage (Saranaj and Geetha, 2012).

2.2.3.3 Mold spoilage

A perennial problem of intermediate and high moisture bakery products is mold growth. Many molds prefer a_w values of > 0.8 to grow, while a few xerophilic molds are capable of growing at a_w values as low as 0.65. Mold spoilage mostly results from post-baking contamination, during cooling and packaging by mold spores from the air, bakery surfaces and equipment (Black et al., 1993). According to Hickey (1998) losses due to mold spoilage in the bakery industry average about 200 million pounds of product each year. Jarvis (2001) found that mold spoilage caused undesirable odors and is often found on the surface of the product. The most common mold species found in bakery products are: *Penicillium sp.*, *Rhizopus sp.*, *Aspergillus sp.*, *Monilia sp.*, *Mucor sp.*

2.3 Traditional preservation technologies for bakery product

2.3.1 Freezing

Most of the bakery products are marketed fresh and stored at ambient temperature. However, microbial growth particularly pathogens and spoilage fungi are of a concern in a number of baked products such as cream, fruit, and meat filled products; cakes and prepackaged pizzas. These products are stored under frozen storage conditions to achieve a longer shelf life. Freezing can be used to prevent mold growth, if applied directly after packaging (Matz, 1992). The most important factors which determine shelf-life of frozen bakery products are storage temperature, the original quality of raw material, the processing methods and the packaging materials (Kotsianis et al., 2002). Chapatties (an indian bakery product) stored at ambient temperature showed higher retrogradation enthalpy than samples kept at -18°C . The study revealed that frozen storage could extend the shelf life and eating quality of chapatties (Gujral and Singh et al., 2008). Despite the advantages of frozen bakery products such as rapid preparation time and affordable prices, quality limitations of this type of product should be carefully taken into consideration. For instance, Banwart et al. (2004) reported, that slow freezing large crystals can disrupt membranes and internal cellular structures of bread leading to thawing losses and poor quality of the product.

2.3.2 Modified atmosphere packaging (MAP)

Increasing energy costs associated with traditional methods of food storage, such as freezing, have led to the growth of more economical and less energy consuming methods of preservation such as modified atmosphere packaging (MAP). It was estimated that MAP can reduce energy consumption by 18 – 20% as compared to freezing for the shelf life extension of bakery products (Aboagye et al., 1986). Nowadays, more than 150 European bakery firms are using gas packaging technology to extend the shelf life of rolls, cakes, pizza, baguettes, and sliced bread (Altekruse et al., 1998). Crumpet produced by Forecrest Foods in Calgary was reported to have a mold-free shelf life of 4 days prior to gas packaging; however, shelf life of the product was extended for 1 month at ambient temperature through product reformulation and gas packaging in 60% CO₂. It is also reported that the vacuum packaging method is used for soft products, such as bread and rolls, while for products such as cakes, doughnuts, pies and pizzas gas packaging method is more functional (Smith et al., 1995).

2.3.3 Microwave heating

Microwave energy causes food molecules with a dipole or charge to oscillate when placed in an electromagnetic field, creating an intermolecular friction which is noticeable as heat. In fact, microwave energy make molecules to resonate or vibrate when placed in an electromagnetic field, and the energy is conveyed to molecular structure that is manifested as heat (Smith et al., 2004). Electromagnetic energy in the microwave region (223 to 100 GHz and 34500 MHz) has been studied as an alternative energy source for sterilization (Lakins et al., 2008). In one of the earliest usage of high frequency microwave on prepackaged bakery product the experimental treatment was shown to extend the mold free shelf life of bread (energy was applied for 45 to 60 secs) (Pomeranz, 1969). Microwave heating is rapid and may increase the shelf-life of fresh bread for 21 days (Smith, 1993). Lakins et al. (2008) reported directional microwaves can be used to extend the shelf life of white enriched bread up to 2 months with minimal mold growth. However, a disadvantage of this technology is that microwave heating might adversely affect the appearance of the product due to the heat generating nature of the technology (Cauvain et al., 2007). Therefore, despite the fact that the packaging films with selective heat resistance are prevalent, microwave heating of pre-packaged products is not widely used in the bakery industry.

2.4 Novel non-thermal processing technologies

Market demands for high-quality foods have led to the introduction of non-thermal treatments which allow the processing of foods without applying temperature. Unlike thermal processing, the expectation of non-thermal technology is that undesirable microorganisms and enzymes are inactivated without damage to essential nutrients, vitamins and sensory properties of the treated product. Foods can be non-thermally processed by technologies such as irradiation, high hydrostatic pressure, antimicrobials, ultrasound, and electrical methods such as pulsed electric fields, light pulses, and oscillating magnetic fields. The principles and applications of a few novel non-thermal preservation methods are briefly described.

2.4.1 High hydrostatic pressure processing

High-pressure processing (HPP) also known as ultra-high pressure (UHP) is a novel non-thermal technology capable of inactivating and eliminating pathogenic and food spoilage microorganisms. Some of the potentials of this technology in the food industry are controlling food spoilage and extending product shelf life while retaining the food's inherent colour, flavour, nutrients and texture (Morris et al., 2007). High hydrostatic pressure processing applies instantaneously and uniformly on liquid and solid foods with or without packaging. In this process, foods independent of size, shape and their compositions are subjected to pressure, generally in the range of 100–600 MPa (1000 – 6000 atm) at below 0 to 100°C, from a few secs to over 20 mins. Nevertheless, HPP is not generally applied to all food types, such as dairy, animal products and shelf-stable low-acid foods. A number of changes in biological systems like denaturation of enzymes and proteins as well as breakdown of biological membrane of microorganisms occur under HPP processing. HPP is commercially used for entrees, guacamole, salsa and fruit juices. EI-Khoury (1999) determines the effect of high pressures on the shelf life of pita bread. This study showed that in all samples mold growth appeared after only 4 and 7 days in bread treated with low pressures (5–10 MPa). However, at higher pressure (30 –70 MPa) mold growth did not occur until day 14, while mold growth was completely inhibited in all products subjected to pressures higher than 200 MPa. Noteworthy, the samples were inoculated with spores of *A. niger* and *P. notataum* packaged in a high gas barrier film.

2.4.2 Oscillating magnetic field (OMF)

Oscillating magnetic fields (OMF) is a technology in which magnetic wave intensity is applied at a non-constant strength at alternate amplitudes over time (Morris et al., 2007). This technology has the capability to inactivate vegetative and spores of spoilage microorganisms (Pothakamury et al., 1993). With OMF technology, sealed food in a plastic bag can receive 1-100 pulses at a frequency of 5-500 kHz between 25 to 100 ms total exposure time. OMF has been shown to inactivate microorganisms in bread roll dough, juice, milk and yogurt (FDA/CFSAN, 2006). In one study, packaged chopped bread dough inoculated with mold spores with 3000 spores/cm³ concentration was subjected to 1 pulse of 8.5 KHz oscillating magnetic field. After treatment, the samples show a mold spore concentration of only about 1 spore per cm³ (Hafmann et al., 1985). Advantages of the technology beside its convenient application on flexible film packaged foods are less energy consumption as well as minimal thermal denaturation of treated products. However, factors involved in magnetic fields are not fully understood yet.

2.4.3 Ultraviolet

Ultraviolet (UV) light has been used as a bactericidal agent from as early as 1928 (Xenon, 2003). In UV light processing, radiation is obtained from the UV region of the electromagnetic spectrum. Ultraviolet light is classified into four wavelength ranges: UV-A (315-400 nm), UV-B (280 - 315 nm), UV-C (200 - 280 nm), and Vacuum-UV (100 - 200 nm) (International Ultraviolet Association, 2009). While the effect of each of these ranges is not fully clarified it is reported that, UV-C specifically is responsible for 50% of the disinfections, and it has capability to cause damage to the nucleic acid of microorganisms such as bacteria, viruses, and fungi, thus destroying their ability to multiply and cause disease and spoilage (Miller et al., 1999; Xenon, 2006). Critical factors with UV light processing include the transmissivity of the product, the power, wavelength and physical arrangement of the UV source(s), the product flow profile and the radiation path length (FDA, 2006).

2.4.3.1 Continuous UV

The use of constant ultra-violet light source as a means of inactivation of bacteria and fungi vegetative cells and spores goes back to a time before World War II (Dennington, 1942). In most UV-based decontamination systems, mercury lamps have been the classical sources of radiation. Lyon et al. (2007) evaluated the efficacy of UV light treatment at 254 nm on *L. monocytogenes* on broiler breast fillets. About 2 log CFUg⁻¹ reduction was achieved after 24 h at 4°C overnight storage. In a similar study, Sommers et al. (2009) reported 1.31, 1.49, and 1.93 log CFU g⁻¹ reductions of *L. monocytogenes* as a result of UV irradiation with the doses of 1, 2 and 4 J cm⁻², respectively on surfaces of frankfurters. Ultra-violet light is also used in bakeries to prevent yeast and mold growth on the surface of freshly baked products. UV light is reported to be non-ionizing and is absorbed by proteins and nucleic acids which may lead to cell death (Jay, 1996). However, it is not very effective in extending the mold free shelf-life because it does not penetrate the product and molds spores can still grow in the product. Limitation with UV technology is considered to be its cost, its potential effect on packaging material, and effect on workers' eyes (Kyzlink, 1990).

2.4.3.2 Pulsed UV-light

Pulsed UV-Light is a unique nonthermal method for food preservation that involves the use of intense, short duration pulses of a broad spectrum. This technology was first developed by Pure Pulse Technologies in San Diego, California in the 1990s (Barbosa-Canovas et al., 2000; Dunn et al., 1997). The light generated by pulsed UV lamps consists of a continuum broadband spectrum from ultraviolet (200 nm) to the infrared (1,000 nm). The system offers sterilization technique that provides a high degree of penetration as compared to continuous UV. Germicidal properties of pulsed light can almost be attributed to the UV part of the spectrum. The 250 to 260 nm range is considered to be capable of destroying most microorganisms due to the alteration of DNA (Bintsis et al., 2000). Application of pulsed light as a method to sterilize or partially decontaminate microbial loads on the surface of packaging materials, transparent pharmaceutical products, surfaces, and transmissive materials, including water, air, and many solutions is well published (Dunn et al., 1997b). U.S. Food and Drug Administration (FDA) has approved the use of pulsed light method after evaluating it for both safety and effectiveness (Dunn et al., 1997).

2.4.3.3 Continuous UV vs. Pulsed UV-light

UV-light can be applied in two modes namely continuous mode and pulsed mode. In continuous mode, energy UV-light is released continuously in a monochromatic or polychromatic wavelength. In the pulsed mode, the electrical energy is stored in a capacitor over a short period of time and released as very short duration pulses (Krishnamurthy and Demirci, 2008). Pulsed UV has a higher penetration depth and may be more effective than continuous UV light (Krishnamurthy, 2006; Jun et al., 2003). Oxidative reactions could effectively be restricted by pulsed UV-light rather than continuous UV light because of the short pulse duration, typically 300 ns to 1 ms (Fine and Gervais, 2004). Pulsed light system by generating a high energy density is designed for the maximum conversion and collection efficiency of UV radiation. When products are exposed to continuous UV, temperature increases over time and damages food attributes. However, in pulsed light, due to short duration of pulses, the temperature increase is lower and limited to a small area in a thinner surface layer. McDonald et al. (2000) reported inactivation of pulsed UV-light treated *Bacillus Subtilis* spores by nearly three orders of magnitude of enhancement over continuous UV-light treated spores. However, it is reported that continuous UV treatment resulted in a larger amount of DNA damage in *Saccharomyces cerevisiae* than pulsed light; except that pulsed light induced a greater level of structural damage i.e., greatly expanded vacuoles and compromised cell membranes.

2.5 Pulsed light treatment

2.5.1 Pulsed UV-light system

Pulsed light is a system that magnifies power many times by accumulating electrical energy in a capacitor over relatively short period of time (fractions of a second) and produce pulses of very short duration (millionths or thousandths of a second) (Vicente and Gómez-López, 2007). The main components in pulsed light systems are high-voltage power supply, capacitor, pulse-forming network, trigger, flash lamp and reflectors (Koutchma et al., 2002). Pulsed light technology has a great potential to be used on a commercial scale as a result of its cost efficiency features. An in-depth look at pulsed light treatment at 4 J cm^{-2} showed that usage cost was estimated to 1 - 4 cents m^{-2} of treated surface area (including amortization of the capital expenditure, lamp replacement, maintenance, and electrical costs) (Dunn et al., 1997).

2.5.2 Pulsed UV-light mechanism of inactivation

Although the exact mechanisms by which UV light causes cellular inactivation are not yet fully understood, it is generally accepted that the radiation affect conjugated carbon double bonds in proteins and nucleic acids, which result in DNA structural changes as well as irregular ion flow that leads to increased permeability and depolarization of the cell membrane (Anderson et al., 2000; Woodling et al., 2005). The main mechanism of the microbial inactivation by pulsed UV-light is explained by the photochemical, photothermal and photophysical effects. The photochemical effect causes transformation of pyrimidine (cytosine and thymine) and formation of dimers in DNA of microorganisms. Such bonds prevent DNA strands from becoming unzipped for replication. The second mentioned effect is due to a significant temperature increase during longer duration pulsed UV light treatment. Essentially, the difference between the temperature of the bacteria cells and the surrounding media leads to cell disruption and death. However, short-duration treatments (< 10 s) may have a negligible increase in the temperature and consequently less significant photothermal effects. The third, photophysical effect, is due to constant disturbance and structural damages to microbial cell caused by the high-energy pulses. Krishnamurthy et al. (2008) suggested photophysical effects of Pulsed UV-light on *Staphylococcus aureus* cell including wall damage, membrane shrinkage, leakage of cellular content and mesosome disintegration.

2.5.3 Kinetics of pulsed UV-light in microbial inactivation

The quantitative characterization of kinetics is crucial for application of every microbial-inactivation technique, including pulsed light. The traditional first-order-kinetics approach does not apply for pulsed-light treatment because it shows an obvious non-log-linear approach (Woodling and Moraru, 2005, 2007; Uesugi et al., 2007). Therefore, nonlinear models have been mostly suggested in pulsed light studies to describe the variation of survivor ratio as a function of treatment intensity.

The Weibull distribution $\log\left(\frac{N}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^\beta$ is being used to a greater extent to describe microbial inactivation, and is based on the engineering principle of failure. Instead of a structural or mechanical failure, the failure is that of the microorganism (Peleg, 2006). This model has been used to describe the survival of *Listeria innocua* after exposure to pulsed light

(Uesugi et al., 2007). Additionally, the inactivation of *E. coli* O157:H7 and *Salmonella* by pulsed UV-light were accurately predicted by the Weibull model rather than by first-order kinetics (Bialka et al., 2008). Weibull, models were used to describe the inactivation of *Escherichia coli* K-12, *E. coli* O157:H7 and *Listeria innocua* treated with UV light in liquid egg white. However, there are some limitations towards Weibull model including its accuracy restraint to predict microbial inactivation in product with various substrate properties. The Weibull model resulted in significant overestimation of pulsed UV-light effectiveness for the stainless steel substrates, where the influence of various substrate properties and inoculum level on inactivation was considerable.

2.5.4 The efficiency of pulsed-light treatments

Pulsed-light treatment efficiency depends on several of factors; the distance from light source to the sample, pulsed-light dose, spectral distribution, type of microorganism, interaction between light and the microorganisms as well as the interaction between the light and the treated substrate (Elmnasser et al., 2007; Levy et al., 2012). Distance clearly affect energy incidence and consequently the inactivation efficacy of pulsed UV-light; the more the distance between the sample and the lamp is, the lower the lethality of the process (Gómez-López et al., 2005). However, some studies demonstrated that a group of food pieces placed very close to the lamp was not efficiently decontaminated. When the treatment is applied to surfaces, other factors such as inoculums size have strong influence on the efficiency of pulsed UV-light (Uesugi et al., 2007). Elmnaser et al. (2010) showed decreasing trend in the bacterial inactivation effect of pulsed UV light by increasing the inoculums size on agar surfaces and liquid medium. Hillegas and Demirci (2003) reported that increasing the number of pulses from 135 to 540 raised the percentage of microbial reduction from 8 to 89.4% in pulsed UV-light treated milk samples.

2.5.5 Light- substrate interaction

Depending on the composition of the substrates and the wavelength of the light, when a beam of light reach the target, it can be reflected, refracted, scattered or absorbed in various degrees (Demirci & Krishnamurthy, 2011). This effect is crucial for pulsed-light treatment, as reflection by the substrate could significantly decrease the efficiency of the treatment. When surface roughness size is larger than the wavelength of the incident light, scattering effects are

low; however, when roughness size is similar, scattering is considerable (Guenther et al., 1993). Based on the surface topography and potential hiding possibility of microorganism it could be expected that the rough surfaces show a lower level of reduction of microorganism as compare to the smooth surfaces. However, the results occasionally are contradictory in experiments. Therefore, the roughness is not the only surface property that affect microbial inactivation, a number of factors such as surface hydrophobicity and the reflective nature are also effective (Woodling and Moraru, 2005). High level of pulsed UV-light photons absorption by molecules of substrates result in reduction in the intensity of light traversing the products layers (in food products mainly due to complex structure of proteins). Such absorption can decline significantly the amount of light that reaches the microbial cells. In one study, significant inactivation of yeast *S. cerevisiae* cells was obtained in uncolored samples of sugar syrup treated with pulsed UV-light. This was achieved in only less than 10-mm-thick sugar syrup (Chaine et al., 2012). In a similar study inoculated alfalfa seeds with *E. coli* O157:H7 were treated by pulsed UV-light. Sharma and Demirci (2003) showed significantly less microbial reduction when the thickness of the seed layer increased.

2.5.6 Combination of pulsed light and other technologies

Some studies show improvement in the effectiveness of the UV light by combining it with thermal treatments. In one study, combination of pulsed UV-light with mild heat treatment was used to inactivate spores of *Botrytis cinerea* and *Monilia fructigena*. Although in this experiment, complete inactivation of conidia was not observed, it suggested a possible inhibition of DNA repair mechanism by thermal treating of the product after the damage caused by pulsed UV-light (Marquenie, 2003). Another way to improve the efficiency of pulsed UV-light to develop the product's shelf life is to expose the product to infrared source immediately before the processing take place (Krishnamurthy et al., 2008). Ngadi et al. (2004) showed a higher inactivation of *E. coli* O157:H7 in poultry chiller water, when combination of UV light irradiation, pulsed electric field and ozone were applied. The effect was essentially a summation of the different inactivation levels obtained separately for each of these technologies.

2.5.7 Effects of pulsed light on food products

2.5.7.1 Liquid food

Since the efficacy of UV light may be limited by its low degree of penetration, transparent media such as water would be excellent examples of food to be treated by pulsed UV-light. Nevertheless, other liquids, such as sugar solutions and wines, which exhibit more limited transparency, are not proper samples for being treated by pulsed UV-light. Essentially, in an aqueous solution, low transparency level reduces the intensity of UV penetration (Shama, 1999; Bintsis et al., 2000). A proportional relationship between vitamin C content and the absorption coefficient of clear apple juices which reduces pulsed light efficacy was observed by (Koutchma, 2002). Krishnamurthy et al. (2007) demonstrated milk pasteurization has been efficiently performed by pulsed UV-light. In this study milk samples were exposed to pulsed UV-light at a minimum dose of 12.6 J cm^{-2} delivered in 56 s resulted in complete inactivation of *Staphylococcus aureus*. Pulsed UV-light processing has been proved to achieve the same level of microbial reduction in clear liquids, regardless of the level of contamination. In comparison with thermal pasteurization, less adverse effect of pulsed UV-light treatment on protein and lipid components of milk were observed (Elmnasser et al., 2007). Ngadi et al. (2003) investigated the effectiveness of pulsed UV-light on peptone water, apple juice, and egg white inoculated with *E. coli* O157:H7. UV doses between 0 and 23.4 J cm^{-2} were evaluated and reductions of 6.0, 4.2, and $4.2 \text{ log CFU ml}^{-1}$ were achieved in peptone water, apple juice, and egg white. The researchers noted that the pH of the medium did not influence the degree of inactivation.

2.5.7.2 Vegetable and fruits

Log reductions between 0.56 and 2.04 were obtained in mesophilic aerobic bacteria after treating spinach, celeriac, radicchio, iceberg lettuce, white cabbage, carrots, green bell pepper, and soybean sprouts with 2,700 pulses (Gómez-López et al., 2005). Two log reductions caused by using only 2 pulses of 0.15 J cm^{-2} per flash, extended the shelf-life of cut vegetables by four additional days (stored at 7°C) (Jun et al., 2003). The respiration rate of lettuce was enhanced by 80% after being treated by pulsed UV-light, while the respiratory rate of cabbage was not affected (Gómez-López et al., 2005). Pulsed UV-light has also been used for surface decontamination of fresh fruits. Most fungi, except *Aspergillus niger*, were controlled with less

than 0.5 J cm^{-2} on fruit surfaces (Lagunas-Solar et al., 2006; Bialka and Demirci, 2007; 2008). It has been observed that the quality of fruits such as apples, oranges, lemons, peaches, raspberries, and grapes could be damaged above UV fluence of 2 J cm^{-2} . The effects of pulsed UV-light on the sensory properties of vegetables like color have been studied by Fine and Gervais (2004). According to this study, the main cause of color changes of black pepper and wheat flour was attributed to overheating combined with oxidation, which happened evidently more rapidly for black pepper than for wheat flour. That is because dark products absorb more light energy than lighter products. Another study showed that untreated potatoes began to brown rapidly as compared to samples treated by flashes of pulsed UV-light at a fluence of 3 J cm^{-2} . They retained their color after a prolonged storage (Dunn et al., 1989).

2.5.7.3 Meat

Although pulsed UV-light treatments achieved high levels of microbial inactivation on relatively simple surfaces, it generally shows only 1 to 3 log reductions on complex surfaces such as meats. That's because proteins and lipids absorb light effectively and decreasing pulsed light dose in order to inactivate microorganism (Gómez-López et al., 2005). Dunn et al. (1995) demonstrated 2 log reductions in microbial counts of beef steaks samples treated with pulsed UV-light using 5 J cm^{-2} to each side and stored 3 days at $4 - 5^{\circ}\text{C}$. *Listeria innocua* was reported to be reduced by 2 log cycles on hot dogs after pulsed UV-light treatment (Dunn et al., 1997).

2.5.7.4 Baked goods

Baked goods are generally free of viable microorganisms after baking. However, contamination occurs during the post baking process such as cooling and packaging. Contamination sources could be bakery atmosphere and equipment, such as slicing machines, and bakery personnel. In one study, bread loaves were treated by pulsed light through polyethylene bags. Untreated samples showed mold growth after 5-11 days at room temperature, while samples treated with 3 pulsed light flashes at 1 J cm^{-2} per flash were mold-free for the duration of storage (Pollock et al., 2007). In a similar study, breadsticks packaged in low-density polyethylene bags and free of any preservative were treated with eight flashes of pulsed light at 0.5 J cm^{-2} . These products were placed at three positions along the long axis of each of them with

1200 radial rotation in between treatment positions. Mold appeared on approximately 17% of the untreated samples by day 6 and 100% were moldy by day 7. However, mold growth happened on only less than 4% of the treated breadsticks by day 13 and no further visible signs of mold were shown for the storage duration of 26 days (Dunn et al., 1995).

In a study chocolate cupcakes were subjected to the pulsed light treatment at various angles to the incident light and exposed to eight flashes at 0.5 J cm^{-2} per flash. Approximately 14 cm distances from pulsed light source. 8% showed mold growth by day 15, 18% by day 19 and 58% by day 23. On the other hand a different trend was observed for control samples. Approximately 6% of untreated samples had mold growth by day 8, 53% by day 12, 82% by day 15, and 94% by day 19.

The test showed that pulsed light treatment contributed to an approximate 10-day extension in cupcake's shelf life (Dunn et al., 1997). Pulsed light extend shelf life of bread more than two weeks at ambient temperature as compared to untreated samples which were visibly spoiled with mold growth after one week. Overall, pulsed light technology shows promising results as a novel method to extend the mold free shelf life of pre-packaged bakery products (Dunn et al., 1995; and 1997).

2.5.7.5 Pizza

High gas barrier pouch is often used to package commercially pre-baked pizza crusts. Although, it is a very effective preservation method against aerobic molds, this means of packaging is very costly. In one study, pizza samples were placed in polyethylene pouches under atmospheric conditions and treated with pulsed light with complete exposure on both sides (Dunn et al., 1995). UV treated samples showed no mold growth as compared to untreated samples. This suggests that integrating pulsed light treatment in the packaging process may allow for less-expensive packaging systems. In another study by Dunn et al., (1997), pre-baked pizza with an approximate composition of 60% dough, 18% tomato sauce, and 18% cheese were inoculated by environmental exposure for 15 mins on either side, and then treated through the pouch with an exposure of 12 flashes of pulsed light at 0.5 J cm^{-2} . Visual detection showed mold growth in more than 80% untreated pizzas, while only one of the pulsed light treated samples became moldy after 30 days storage at 7°C (Pollock et al., 2007).

2.5.8 Various microorganism affected by pulsed UV-light

Pulsed UV-light for inactivation of pathogenic microorganisms has gained interest because of the very short treatment time. Unlike chemical biocides, UV does not introduce toxins or residues and does not alter the chemical composition, taste, odor or PH of the product. (Lamont et al., 2007) showed that the application of pulsed UV-light treatment for the inactivation of poliovirus and adenovirus is significantly effective (Lamont et al., 2007). Although both viruses showed vulnerability to the treatment, adenoviruses were more resistant to pulsed UV-light than polioviruses. The same number of pulses resulted in approximately 1 log reduction of adenovirus as compared to log reduction of 4 CFU ml⁻¹ in poliovirus. At an energy level of 31.12 J cm⁻² (64 pulses), inactivation reached 0.7 and 2.93 log reductions in *Saccharomyces cerevisiae* cells inoculated into wheat flour and black pepper, respectively. Jun et al. (2003) showed an optimization of 3.12 log reduction, when pulsed UV-light system was used to inactivate fungal spores of *Aspergillus niger* in corn meal. In another study, the effect of Ultraviolet irradiation (254 nm, UV-C) on some fungi such as *Aspergillus flavus*, *Aspergillus niger*, *Penicillium corylophilum* and *Eurotium rubrum* was investigated. These experiments reported that pulsed light can effectively inactivate spores of *A. flavus*, *Penicillium corylophilum*, *Empetrum rubrum* and *A. niger* but the efficacy of UV-C radiation against fungal spores depends on the genera and the method of exposure to the irradiation (Begum et al., 2009). There is little information on the microflora of preformed pizza or the influence of tomato sauce on the microbiological quality and safety of flat bread and other end products.

2.6 Identification and quantification of spoilage fungi

Microbiological analysis of a food product could be performed for a variety of different reasons. The most important reason is confirm the presence and estimate the quantity of pathogenic microorganisms in foods which may jeopardize public health. However, other reasons must be considered from the industrial point of view, such as the estimation of the products' shelf life, the evaluation of the quality of raw materials, the contamination route tracing in the processing lines, etc (Loureiro and Querol, 2000). For more than 50 years several methods of classification on the basis of morphological, biochemical and physiological properties have been proposed for yeasts and molds. However, there is not a single standardized

method for many of these tests and their results are often dependent on the applied techniques. In addition, misidentification is very frequent due to variable results of the tests for different strains of the same species (Loureiro and Querol, 2000).

2.6.1 Methods of identification of fungi

2.6.1.1 Classical microbiology

Culturing, standard plate counts and measuring individual microorganisms growth is the traditional method of detection of viable bacteria and fungi. This method has been performed by hundreds of commonly used microbiological media in the food industry (Harrigan et al., 1998). Quantification of the growth of filamentous fungi is more complicated in comparison with bacteria or yeasts, since no primary mold enumeration standard exists (such as cell numbers used for yeasts and bacteria). Hyphae of mold in vegetative growth do not survive blending completely and because of their poor detachment to the substrate, the estimation of fungal growth or biomass is complicated. Nevertheless, most food laboratories continue to rely on viable counting (dilution plating) for detecting and quantifying fungal growth in foods. Besides dilution plating, recently a new method called direct plating has been developed for estimating fungal numbers and growth in foods (Pitt and Hocking, 2009).

2.6.1.2 Molecular microbiology

In recent years, a new generation of innovative methods and technologies for the enumeration, detection, identification and characterization of microorganisms in contaminated foods has attracted a lot of attention. Molecular approaches that imply near-time or real-time detection are considered an indispensable tool that allows the accurate description of the microbial ecology of food (Iacumin et al., 2009). For instance, recent progress in molecular biology has led to the development of new techniques such as fragment length polymorphism (RFLP) of mitochondrial DNA, chromosomal DNA electrophoresis and ribosomal DNA restriction analysis that facilitated identification and characterization of yeast. Esteve-Zarzoso et al. (1999) proposed a new easy and rapid method of yeast identification based on the restriction analysis of internal transcribed spacers (ITS). This method enables identification of yeast from isolated colonies or directly from food samples. Dlauchy et al. (1999) assembled a database of

restriction fragment patterns to categorize 128 species associated mostly with food, wine, beer, and soft drinks. Baleiras et al. (1969) used a type of PCR technique to identify strains of *Z. Bailli* and *Zygosaccharomyces bisporus* present in mayonnaise and salad dressings. Pulsed electrophoresis techniques have made possible the separation of the chromosomes of *Saccharomyces cerevisiae* and other yeasts.

2.6.1.2.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a powerful genetic technique for copying a piece of DNA (sequences of interest) a billion-fold. The PCR technique, by amplifying specific gene fragments, allows fast and selective identification and detection of microorganisms in different matrices by amplifying specific gene fragments (Varga, 2004). This method consists of reaction cycle with the following steps: (1) denaturation of the double-stranded DNA (2) annealing of primers to single DNA strands, and (3) extension of the primers with a thermostable DNA polymerase (Rapley, 2007). Consequently, repetitive cycles of the mentioned procedure results in an exponential increase of the number of copies of the target DNA that increase the possibility of detection (Ercolini, 2004). PCR is considered to be a useful method to determine the microflora of fermented foods and to identify significant microbial communities associated with specific characteristics of the final product (Settanni and Corsetti, 2007). PCR techniques are simple, fast and sensitive (Feret et al., 1994). These features have made PCR-techniques popular for food analysis (Meyer and Candrian, 1996). Thus, the detection and sequence determination of nucleic acids via PCR-techniques is currently being extensively applied to the detection of adulteration in foods (Javier Senorans et al. 2003). Amplification and direct sequencing of ribosomal DNA (rDNA) was one of the first applications of PCR within mycology (White et al., 1990). Several PCR-based techniques permit determination of taxonomic status of a fungal isolate which PCR-cloning is one of them. This technique is mainly used when less diversity in micro-flora of the examined sample is expected.

2.6. Conclusion

Contamination by yeast and mold in bakery products is a serious concern in the industry from a health and economic perspective. These microorganisms continue to be a problem as a post packaging contaminant of preformed pizza, primarily due to post processing contamination in the manufacturing facility. Several conventional and novel decontamination methods are utilized by the industry to inactivate fungi effectively; however, the challenge posed in the preservation of food quality limits their application. Therefore, there is always a need to optimize the existing processing method and/or identify a new inactivation method while preserving the quality of the food.

UV-light has been used as a bactericidal agent for over a century, because it is effective for inactivation of pathogens on a solid surface and in clear liquid. Pulsed UV-light technology is the application of broadband UV-light in a pulsed mode, wherein the instantaneous intensity of the UV-light is increased significantly. Increased UV-light intensity and the shocking effect of pulses may aid in enhancing the effectiveness of UV-light on microbial inactivation. Optimization of pulsed UV-light and a proper equipment design may result in a disinfection process for food products for which the surface contamination is the major concern, such as preformed pizza. Pulsed UV-light may provide a cheaper alternative to existing decontamination methods as the capital and operational costs are comparatively less than existing technologies.

As the literature review substantiates, there was not much research work done in optimization of pulsed UV-light inactivation of fungi in bakery products. In this study, *P.roqueforti* (a dominant spoilage mold in bakery products) inoculated on solid agar, bread and pizza, was treated with pulsed UV-light and the degree of *P.roqueforti* inactivation were determined. Additional research was conducted to gain a better insight into the technologies through inactivation modeling and characterization studies. Furthermore, classical and molecular detection methods were performed to identify microbial flora in preformed pizza.

2.7 REFERENCES

- Alvarez, I., Raso, J., Palpo, A., and Sala, F.J. 2000. Influence of different factors on the inactivation of *Salmonella senftenberg* by pulsed electric fields. *International Journal of Food Microbiology* 55: 143–146.
- Anderson, J. G., Rowan, N. J., Macgregor, S. J., Fouracre, R. A., Farish, O., & Member, S. (2000). Inactivation of Food-Borne Enteropathogenic Bacteria and Spoilage Fungi Using Pulsed-Light, 28(1), 83–88.
- Anon, 1995; <http://www.cfsan.fda.gov/~comm/ift-xray.html> [accessed 26 January 2006].
- Baleiras Couto, M., van der Vossen, M., Hofstra, H. Veld, H. (1994). RAPD analysis: a rapid technique for differentiation of spoilage yeasts. *Int J Food Microbiol* 24, 249-260.
- Banwart, G. J. 2004. *Basic Food Microbiology*. A VI Publ. Inc., Westport. pp. 505-544
- Begum, M., Hocking, A. D., & Miskelly, D. (2009). Inactivation of food spoilage fungi by ultra violet (UVC) irradiation. *International journal of food microbiology*, 129(1), 74–7.
- Betchel, W.G. and Meisner, D.F. and Bradley, W.B. 1953. The effect of the crust on the staling of bread. *Cereal Chemistry*. 16: 160-168.
- Berry D., 2004 “Keeping foods fresh”. *Food product design.*; <http://www.foodproductdesign.com/archive/2004/0104De.html> [accessed 22 February 2006].
- Bialka, K.L., Demirci, A., Puri, V.M., 2008. Modeling the inactivation of *Escherichia coli* O157:H7 and *Salmonella enterica* on raspberries and strawberries resulting from exposure to ozone or pulsed UV-light. *Journal of Food Engineering* 85, 444–449.
- Bialka, K. L., & Demirci, A. (2007). Decontamination of *Escherichia coli* O157:H7 and *Salmonella enterica* on blueberries using ozone and pulsed UV-light. *Journal of Food Science*, 72(9), 391–396.
- Bintsis, T., Litopoulou-Tzanetaki, E., Robinson, R.K., 2000. Existing and potential application of ultraviolet light in the food industry - a critical review. *Journal of the Science of Food and Agriculture* 90, 637–645.
- Boyle, P.J. and Hebeda, R.E. 1990. Anti-staling enzyme for baked goods. *Food Technol.*, 44:129.
- Boynton BB, Welt BA, Sims CA et al. Effects of low-dose electron beam irradiation on respiration, microbiology, texture, color, and sensory characteristics of fresh-cut cantaloupe stored in modified-atmosphere packages. *J. Food Sci.* 2006; 71(2): S149–S155.
- Byrne, M. (2000). Barilla: investing for the future. *Food Engineering and Ingredients*, 25, 18–23.

- Chaine, A., Levy, C., Lacour, B., Riedel, C., & Carlin, F. (2012). Decontamination of sugar syrup by pulsed light. *Journal of food protection*, 75(5), 913–7.
- Corsetti A & Settanni L (2007) Lactobacilli in sourdough fermentation. *Food Res Int* 40: 539–558.
- Demirci, A., & Krishnamurthy, K. (2011). Chapter 18 Pulsed Ultraviolet Light, 249–261.
- Dunn, J. (1996). Pulsed light and pulsed electric field for foods and eggs. *Poultry Science*, 75(9), 1133–1136.
- Dunn, J., Bushnell, A., Ott, T., & Clark, W. (1997). Pulsed white light food processing. *Cereal Foods World*, 42, 510–515.
- Dunn, J. E., Clark, R. W., Asmus, J. F., Pearlman, J. S., Boyer, K., Painchaud, F., et al. (1989). Methods for preservation of food- stuffs. US Patent number 4871559.
- Dunn, J. E., Clark, R. W., Asmus, J. F., Pearlman, J. S., Boyer, K., Painchaud, F., et al. (1991). Methods for preservation of food- stuffs. US patent number 5034235.
- Dunn, J., Ott, T., & Clark, W. (1995). Pulsed light treatment of food and packaging. *Food Technologist*, 49(9), 95–98.
- Elmnasser, N., Guillou, S., Leroi, F., Orange, N., Bakhrouf, a, & Federighi, M. (2007). Pulsed-light system as a novel food decontamination technology: a review. *Canadian journal of microbiology*, 53(7), 813–21.
- Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of microbiological methods*, 56(3), 297–314.
- Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and A. Querol (1999). Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* 49, 329–337.
- Fine, F. and Gervais, P. 2004. Efficiency of pulsed UV light for microbial decontamination of food powders. *Journal of Food Protection* 67(4):787–792.
- Favier Senorans, Elena Ibanez Cifuentes, A. 2003. New Trends in Food Processing. *Ministerio de ciencia y tecnologia, spain* 507-526
- Galić, K., Curić, D., & Gabrić, D. (2009). Shelf life of packaged bakery goods--a review. *Critical reviews in food science and nutrition*, 49(5), 405–26.
- Gómez-López, V M, Devlieghere, F., Bonduelle, V., & Debevere, J. (2005). Intense light pulses decontamination of minimally processed vegetables and their shelf-life. *International journal of food microbiology*, 103(1), 79–89.

- Gómez-López, Vicente M., Ragaert, P., Debevere, J., & Devlieghere, F. (2007). Pulsed light for food decontamination: a review. *Trends in Food Science & Technology*, 18(9), 464–473.
- Hickey, C.S. 1998. Sorbate spray application for protecting yeast-raised bakery products. *Baker's Digest*, 54: 4-7.
- Hillegas, S. L., & Demirci, A. (2003). Inactivation of *Clostridium sporogenes* in clover honey by pulsed UV-light treatment. *Agricultural Engineering International*, V. Manuscript FP03009.
- Iacumin, L., Cecchini, F., Manzano, M., Osualdini, M., Boscolo, D., Orlic, S., & Comi, G. (2009). Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food microbiology*, 26(2), 128–35.
- Jarvis, B. 2001. Mould spoilage of food. *Process Biochemistry*, 7:11-14. 25.
- Jun, S., Irudayaraj, J., Demirci, A., & Geiser, D. (2003). Pulsed UV-light treatment of corn meal for inactivation of *Aspergillus niger* spores, , 883–888.
- Kohn, S. (2000). An update of the US baking industry. *Cereal Foods World*, 45, 94–97.
- Kotsianis, I. ., Giannou, V., & Tzia, C. (2002). Production and packaging of bakery products using MAP technology. *Trends in Food Science & Technology*, 13(9-10), 319–324.
- Koutchma, T. (2002). UV Light for Processing Foods, 10(4), 24–29.
- Knorr, D. and Tomlins, R.I. 1985. Effect of carbon dioxide modified atmosphere on the compressibility of stored baked goods. *Journal of Food Science*. 50: 1172-1173, 1176.
- Krishnamurthy, K. (2006). decontamination of milk and water by pulsed UV-light and infrared heating. *Applied and Environmental Microbiology* 7:11-14. 25
- Krishnamurthy, K., Tewari, J. C., Irudayaraj, J., & Demirci, A. (2008). Microscopic and Spectroscopic Evaluation of Inactivation of *Staphylococcus aureus* by Pulsed UV Light and Infrared Heating. *Food and Bioprocess Technology*, 3(1), 93–104.
- Kulp, K. and Zobel, H.P (1996). The Staling Mechanism. In: *Baked Goods Freshness: Technology, Evaluation, and Inhibition of Staling* (ed. Hebeda, E.R. and Zobel, HF) Marcel Dekker, Inc., New York, NY 10016. pp. 1-60
- Labuza and Szybist F. (1999). *Advancing Knowledge About Processing, Distribution, Sales, and Food Service*, (March)
- Lagunas-Solar, M. C., Piña, C., MacDonald, J. D., & Bolkan, L. (2006). Development of pulsed UV light processes for surface fungal disinfection of fresh fruits. *Journal of Food Protection*, 69 (2), 376–384.

- Lakins, D. G., Echeverry, a, Alvarado, C. Z., Brooks, J. C., Brashears, M. T., & Brashears, M. M. (2008). Quality of and mold growth on white enriched bread for military rations following directional microwave treatment. *Journal of food science*, 73(3), M99–103.
- Lamont, Y., Rzezutka, A., Anderson, J. G., MacGregor, S. J., Given, M. J., Deppe, C., et al. (2007). Pulsed UV-light inactivation of poliovirus and adenovirus. *Letters in Applied Microbiology*, 54 (5), 564–567.
- Levy, C., Aubert, X., Lacour, B., & Carlin, F. (2012). Relevant factors affecting microbial surface decontamination by pulsed light. *International journal of food microbiology*, 152(3), 168–74.
- Liew, E. Y., Guo, L., Ranghoo, V. M., & Hyde, K. D. (1998). Molecular approaches to assessing fungal diversity in the natural environment, 1–17.
- Loureiro, V. Ã., & Querol, A. (2000). The prevalence and control of spoilage yeasts in foods and beverages, 10(1999), 356–365.
- Matz, S. A. (1992). Formulas and processes. In *bakery technology and engineering* AVI Publishing (3rd ed., pp.443–460).
- Marquenie, D. (2003). Combinations of pulsed white light and UV-C or mild heat treatment to inactivate conidia of *Botrytis cinerea* and *Monilia fructigena*. *International Journal of Food Microbiology*, 85(1-2), 185–196.
- Meyer, R.; Candrian, U.; Lüthy, J. Detection of pork in heated meat products by the polymerase chain reaction. *J. AOAC Int.* 1994, 77, 617-622.
- Mcdonald, K. F., Curry, R. D., Clevenger, T. E., Unklesbay, K., Eisenstark, A., Golden, J., ... Morgan, R. D. (2000). A Comparison of Pulsed and Continuous Ultraviolet Light Sources for the Decontamination of Surfaces, 28(5), 1581–1587.
- Michael Ngadi, Xue Jun 1, J. S. and G. S. V. R. (2004). Inactivation of *Escherichia coli* O157:H7 in Poultry Chiller Water Using Combined Ultraviolet Light, Pulsed Electric Field and Ozone Treatments. *International Journal of Poultry Science*, 3(11), 733–737.
- Morris, B. C., Brody, A. L., & Wicker, L. (2007). *Non-Thermal Food Processing / Preservation Technologies: A Review with Packaging Implications # and Science*, (July), 275–286.
- Ngadi, M., Smith, J., Cayouette, B., 2003. Kinetics of ultraviolet light inactivation of *Escherichia coli* O157:H7 in liquid foods. *Journal of the Science of Food and Agriculture* 83,1551e1555.
- Oraikul, B. (1991). Modified atmosphere packaging of bakery Pro- ducts. In B. Oraikul, M. E. Stiles (Eds.), *Modified atmosphere packaging of food* (pp. 38–114).

- Peleg, M. (2006). *Advanced quantitative microbiology for foods and biosystems*. Boca Raton, FL: CRC Press.
- Pinho, B. H., & Furlong, E. B. (2000). The occurrence of molds, yeasts and mycotoxins in pre-cooked pizza dough sold in Southern Rio Grande do Sul. *Brazilian Journal of Microbiology*, 31(2), 99–102.
- Pitt, J. I., & Hocking, A. D. (2009). *Fungi and Food Spoilage* (pp. 19–52). Boston, MA: Springer US.
- Pollock, A. M. (2007). *characterization of pulsed light treatment on the shelf life of and safety of vacuum packaged cold smoked salmon as a thesis fulfillment at McGill University*.
- Qin, B.L., Chang, F., Barbosa-Cánovas, G.V., and Swanson, B.G. 1995. Nonthermal inactivation of *S. cerevisiae* in apple juice using pulsed electric fields. *Lebensmittel-Wissenschaft und-Technologie—Food Science and Technology* 28: 564–568.
- Rapley, R. (n.d.). *Molecular Analysis and Amplification Techniques*.
- Rosenkvist, H. and Hansen A. (1995) Contamination profiles and characterization of *Bacillus* species in wheat bread and raw materials for bread production. *International Journal of Food Microbiology* 26, 353–363.
- Rodríguez, V., Medina, L., & Jordano, R. (2003). Influence of modified atmosphere packaging on the shelf life of prebaked pizza dough with and without preservative added. *Die Nahrung*, 47(2), 122–5.
- Woodling and Carmen I. (2005). Food Microbiology and Safety Influence of Surface Topography on the Effectiveness of Pulsed Light Treatment for the Inactivation of *Listeria innocua* on Stainless-steel Surfaces, 70(7).
- Saranraj, P., & Geetha, M. (2012). Microbial Spoilage of Bakery Products and Its Control by Preservatives, 3(1), 38–48.
- Seiler, D.A.L. 1968. Prolonging the shelf life of cake. *Brit. Baker*, 156:25.
- Settanni, L., Valmorri, S., Van Sinderen, D., Suzzi, G., Paparella, A., Corsetti, A., 2006. Combination of multiplex PCR and PCR-Denaturing Gradient Gel Electrophoresis for monitoring common sourdough-associated *Lactobacillus* species. *Appl. Environ. Microbiol.* 72, 3739–3796
- Shama, G. 1999. Ultraviolet light. In: *Encyclopedia of Food Microbiology*, edited by Robinson, R.K., Batt, C., and Patel, P. San Diego, CA: Academic Press.

- Sharma, R., and Demirci, A. 2003. Inactivation of *Escherichia coli* O157:H7 on inoculated alfalfa seeds with pulsed ultraviolet light and response surface modeling. *J. Food Sci.*, 68(4): 1448-1453.
- Smith, J. P., Daifas, D. P., El-Khoury, W., Koukoutsis, J., & El-Khoury, A. (2004). Shelf life and safety concerns of bakery products--a review. *Critical reviews in food science and nutrition*, 44(1), 19–55.
- Smith, J.P. and Simpson, B.K. 1995. Modified atmosphere packaging of bakery and pasta products . in: *Principles of Modified atmosphere packaging and Sous- Vide Processing* (eds. I. Farber and K.L. Dodds). Technomic Publ. Co. , hncester, PA. U.S.A. pp. 207- 242
- Uesugi, A. R., Woodling, S. E. & Moraru, C. I. (2007). Inactivation kinetics and factors of variability in the pulsed light treatment of *Listeria innocua* cells. *Journal of Food Protection* 70, 2518-2525.
- Varga, J. (2004). *Molecular Detection Of Fungi In Foods And Feeds*.
- Vega-Mercado, H. 1996. Inactivation of *Escherichia coli* by combining pH, ionic strength and pulsed electric fi elds. *Food Research International* 29: 117.
- White, T.J., Burns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomalRNAgenes for phylogenetics.
- Wekhof, A., Trompeter, F.J., Franken, O., 2001. Pulsed UV Disintegration (PUVD): a new sterilisation mechanism for packaging and broad medical-hospital applications. *The First International Conference onUltraviolet Technologies, WashingtonD.C*, p. 15.
- Woodling, S.E., Moraru, C.I., 2005. Influence of surface topography on the effectiveness of pulsed light treatment for the inactivation of *Listeria innocua* on stainless-steel surfaces. *J. Food Sci.* 70 (7), M345–M351.

CHAPTER 3

CHARACTERIZATION OF PULSED UV-LIGHT APPARATUS

3.1 Abstract

Pulsed Ultraviolet (UV) light treatment has been proven effective for killing a wide variety of microorganisms on foods and food contact materials. However, critical process parameters need to be optimized for a better efficiency of pulsed UV-light treatments. Since the light dissipation and exposure area to the pulsed light source is different for various distances from the light source, in this chapter the spatial distribution of pulsed UV-light was investigated. A bench-top pulsed UV was used to produce up to 1,000 V (20 J). Potato Dextrose Agar Petri plates inoculated with *Penicillium roqueforti* ATCC 10010 were treated 10 min in the first three effective levels in the treatment chamber (5, 10 and 15 cm). The result illustrated showed that 30, 75 and 90 % of the surface area of the tray was exposed to pulsed light at 5, 10 and 15 cm distance from pulsed light lamp, respectively. Apple juice was also used as a simple substrate to characterize the efficacy of the pulsed light apparatus. Apple juice inoculated with *E. coli* (pathogenic microorganism frequently associated with apple juice) was treated with pulsed UV-light at the same distances (5, 10 and 15 cm) from the light source, 3 voltage inputs (400, 750 and 1,000 V) and treatment time (1, 5 and 10 min). A multiple-way ANOVA method was used for designing and analyzing the experiments. The log reduction in *E. coli* population treated by pulsed UV-light varied from 1.4 to 2.05 log CFU ml⁻¹. This range was obtained after 120 pulse treatment with 400 V to 1,200 pulses with 1,000 V.

3.2 Introduction

High intensity light pulses, also known as pulsed UV-light (PL), is a novel non-thermal technology which involves the use of intense and short duration pulses of broad-spectrum white light. The intensity of these light pulses is approximately 20,000 times greater than that of sunlight on the Earth's surface (Dunn, 1996). This technology is applicable mainly in decontaminating or reducing microbial populations on the surface of packaging materials, transparent liquids, or other surfaces (Dunn et al., 1997b). In addition, it has negligible effect on product organoleptic quality as there is no substantial increase in temperature during the

treatment (Ross et al., 2003). Prior to challenge studies to assess microbial inactivation capability of pulsed UV-light and inactivation kinetics of the technology on targeted microbes on bakery products, it was necessary to characterize the pulsed UV-light system and treatment conditions. High intensity pulsed UV-light treatment's efficacy depends on several factors related to the equipment, process, product and target microorganism (Pollock, 2007). The intensity of a light and its effect also depends on its initial power and distance of the target from the light source. In order to standardize the factors mentioned above, prior to extensive use of equipment in the experiments, the spatial power distribution in the pulsed UV-light apparatus were characterized. This chapter investigates the evaluation and characterization of the spatial power distribution in the pulsed UV-light apparatus using inactivation of *Penicillium roqueforti* subjected to the pulsed UV-light at different distances. Apple juice was used to study effectiveness of the pulsed UV-light apparatus to inactivate a target microorganism in a simple substrate before further studies on bakery products with variable porosity.

The specific objectives of this study were:

- 1-To optimize the pulsed UV-light unit with vegetative mold cells (*Penicillium roqueforti*) and
- 2-To evaluate the efficacy of pulsed UV-light on decontamination of apple juice as a non-complex and pulsed UV- light penetrable substrate.

3.3 Materials and methods

3.3.1 Pulsed UV- light system

A bench-top pulsed UV- light system fitted with a low-pressure xenon flash lamp (Model PUV-01, Magnavolt Technologies Inc., Plattsburgh, NY, USA) was used in the entire experiment (Figure 3.1). This device provides a compact and portable source of intense pulsed UV- light, with a significant biocidal component in the UV. The output intensity can be varied by changing the operating voltage. The pulse repetition rate is continuously variable between single shot and 5 pulse per second (pps) at 1,000 V. At lower voltages, the pulse repetition rate can be increased up to a maximum of 10 pps. The emitted spectrum from the flash lamp is shown in Figure 3.2. The light source produces a broad spectrum of white light with peak spectral emissions at 250 nm. On the panel control, the system has a timer for multiple automatic and manual pulsing and a switch for adjusting the voltage level. Each pulse is delivered in 250 μ s and corresponds to ultra violet light energy from 3 to 20 J depending on the delivered voltage. The

machine specification is as follows; Input Voltage (400 to 1,000 V), Pulse Frequency (0.1-10 pps).

3.3.2 Treatment chamber

The apparatus consists of a metal housing surrounding a treatment chamber made of polished stainless steel (20 cm wide×14 cm deep×12 cm high) and equipped with an upper xenon lamp. The sample holder plate in the treatment chamber is divided into five different zones which determine the distance from the PL source (5-35 cm, with 5 cm increments) (Figure 3.1). However the first three effective distances (5, 10 and 15 cm) were used during the experiment because of light dissipation that occurs in longer distances from the light source.

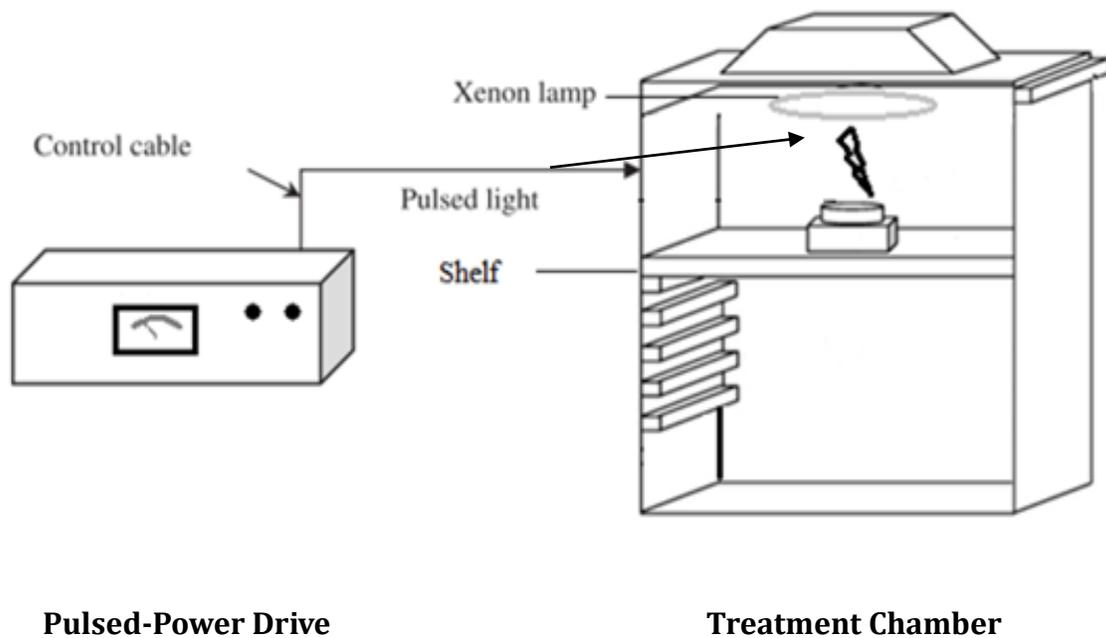


Figure 3.1 Schematic diagram of pulsed UV- light treatment system.

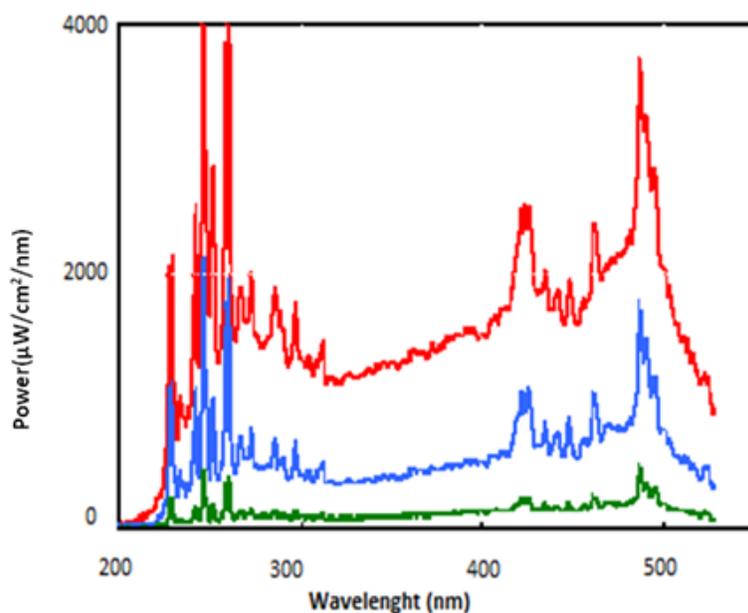


Figure 3.2 Spectral output from the light source at operating voltages of 400, 700 and 1,000 V (bottom to the top)

3.3.3 Spatial distribution

In this study, it was important to determine the exposed treatment area at each distance due to various level of light distribution in the chamber at different distances from the pulsed UV source.

3.3.3.1 *Penicillium roqueforti* inoculum preparation

A stock-culture of *Penicillium roqueforti* ATCC 10010 (Cedarlane Corporation, Burlington, ON, Canada) was inoculated on Potato Dextrose Agar (PDA, Difco, Sparks, MD, USA) in order to obtain a pure culture. Plates were incubated for 3-7 days at 25°C in the dark, after which colonies were transferred to fresh Potato Dextrose Broth (PDB, Difco, Sparks, MD, USA) and re-incubated for 5-7 days at 180 rpm and 24°C to generate the inoculum which used to inoculate Petri plates.

3.3.3.2 Inoculation of solid medium

Potato Dextrose Agar (Difco) Petri plates were inoculated by spreading 0.1 ml of a 1×10^5 CFU ml⁻¹ suspension of *P. roqueforti* at room temperature. Inoculum was evenly spread onto each plate using a sterile hockey stick, to give a final inoculum level of 1.59×10^3 CFU cm⁻¹. Control Potato Dextrose Agar Petri plates were prepared by spreading 0.1 ml of sterile 0.1 % peptone water.

3.3.3.3 Spatial power distribution (12 plate experiment)

Twelve inoculated plates were placed on the treatment tray as illustrated in Figure 3.3 with their lids removed and subjected to a series of pulsed UV- light treatments for 10 mins at 4 pps frequency with the following conditions: 1,000 V; at 5, 10 or 15 cm distance from the pulsed UV source.

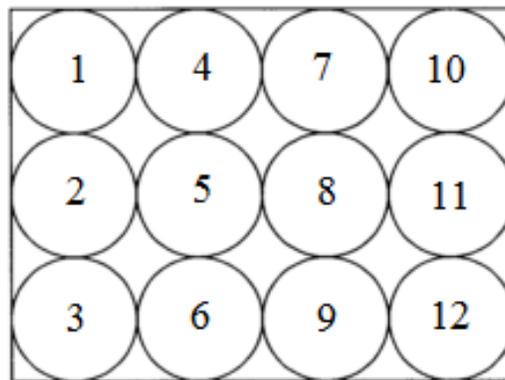


Figure 3.3 Position of Petri plates on pulsed UV-light chamber treatment tray

3.3.4 Testing the equipment with the apple juice

To characterize the efficacy of the pulsed light apparatus, in this study apple juice was inoculated by *E. coli* and treated by various parameters of the system.

3.3.4.1 Apple juice preparation

Apple juice Tropicana (PepsiCo Beverages Canada, Peterborough, ON, Canada) was obtained from a local grocery store (Ste-Anne-de-Bellevue, QC, Montreal). The product was

prepared by autoclaving at 120 °C for 15 mins by Electric Sterilmatic Sterilizer, (Market Forge Industries Inc., Everett, MS, USA). The sterilization was done to avoid potential interference in microbial counts between the spiked microorganism and the natural microflora.

3.3.4.2 *E. coli* inoculums preparation

To evaluate pulsed UV-Light effectiveness on decontamination of apple juice, the experiment was performed using *E. coli* K12 DSM 1607 (ATCC) as surrogate for their pathogenic counter-parts that mostly occurs in apple juice. Stock cultures were stored in 30% glycerol at -20 °C. The strain was grown overnight in LB Broth (Luria-Bertani, Difco, spark, MD, USA) at 37 °C and 180 rpm to generate inocula.

3.3.4.3 Pulsed UV- light treatment of apple Juice

Samples of apple juice were inoculated with over-night culture *E. coli* (to the proportion of 10:1) and initial population of approximately 1×10^{10} CFU ml⁻¹ was achieved. For the purpose of pulsed UV-light processing, 2 ml of the inoculated apple juice were pipetted into empty Petri dish (50 mm diameter) to ensure that the entire dish surface was covered with sample to a depth of 1 mm. A period of 20 mins following inoculation was allowed for adaptation of the cultures to the product environment prior to the pulsed UV-light treatment. Inoculated apple juice samples were exposed to two different input voltage (400 and 1,000 V), treatment time (1 and 2 min) and distance from the light source (5 and 15 cm) at 1 and 10 pps frequencies.

3.3.4.3 Enumeration of *E. coli*

Ten-fold dilution series of UV-treated apple juice samples were prepared using Buffered Peptone Water (Don Whitley Scientific, WY, UK) and 0.1 ml aliquots of relevant dilutions plated out on solid LB medium. Plates were incubated at 37 °C for 48 h and survivors (CFU ml⁻¹) were enumerated. In order to establish the background microflora, fresh uninoculated juice were also plated on LB agar and incubated at 37 °C for 48 h to determine total bacterial populations. Treated and untreated samples were enumerated immediately after UV treatment.

3.3.5 Statistical analysis

Statistical analysis of the data was performed using the SAS software 9.2. A Multiple-way ANOVA was used to compare the experimental treatments and a Student's t-test was used to determine significant differences between the means. All microbiological experiments were performed in triplicate. All significant differences were reported at 95% ($P < 0.05$) confidence intervals. In addition qualitative analysis was performed to facilitate analysis of pulsed UV- light spatial power distribution.

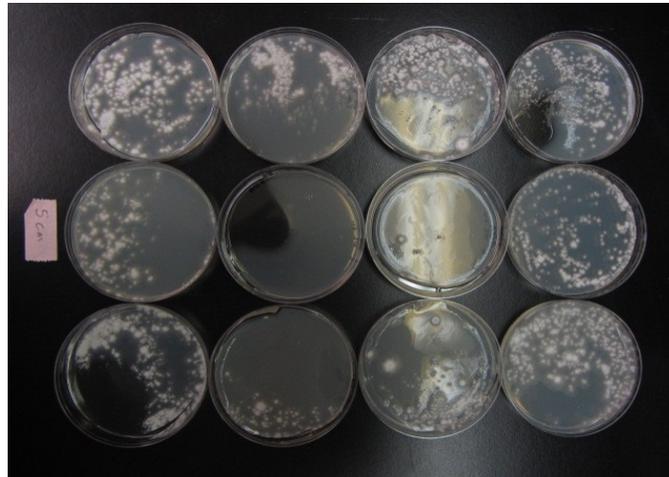
3.4 Results and discussion

3.4.1 Spatial power distribution (12 plate experiment)

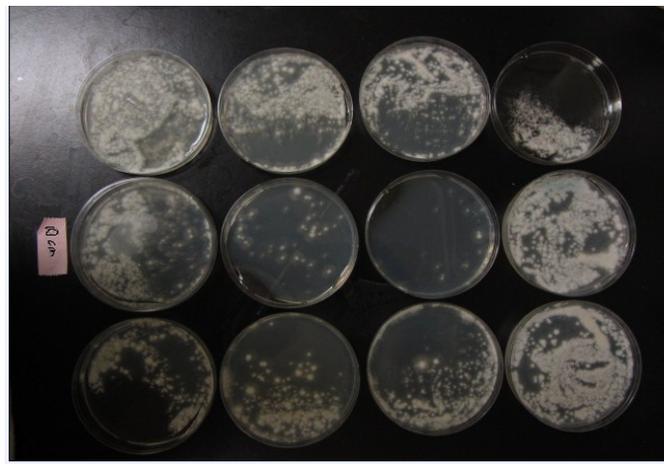
Figure 3.4 presents photographs showing the distribution of pulsed UV-light at varying distances from the pulsed UV source at 1,000 V. The percentage of exposed surface area was calculated by dividing Petri plate surface area by exposed surface area (the decontaminated area). As it is illustrated in the images the exposed surface percentage increased as the distance from the pulsed UV source is increased (Table 3.1). The affected surface area on Petri plates expands by increasing the distance; however, the intensity of pulsed UV-light decline and consequently fewer molds were eliminated from these areas. This might be due to the increase in energy dissipation as the light pulse travel from the xenon lamp to the Petri dishes.

Ben Saïd et al. (2010) also indicates that the inactivation of bacterial populations on the agar surface reduced significantly ($p < 0.05$) when the distance between the plate and the xenon lamp increased. At the same number of pulses and at the same intensity of energy, a single pulse with 162 J of energy achieved >7 , 5–6, and 3 log reductions at shelf height of 4, 9, and 12 cm, respectively. Similarly, study of inactivation of *Aspergillus niger* spores on corn meal by pulsed UV-light showed log reduction of fungal spores increased significantly with respect to a decrease in the distance from 8 to 13 cm (Jun et al., 2003). In another study honey inoculated with *Clostridium sporogenes* spores was treated with pulsed UV-light. The distance between the honey and the lamp, were investigated (Hillegas and Demirci, 2003). The results showed that reducing the distance between the honey surface and the UV lamp enabled improved spore reduction.

5cm



10cm



15cm

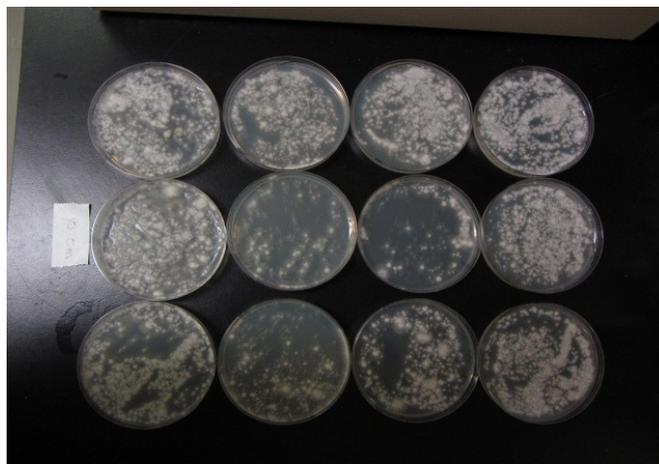


Figure 3.4 Distribution of pulsed UV- light at varying distances (5, 10 and 15 cm) from the pulsed UV source (1,000 V, 5 min)

Table 3.1 Spatial power distribution of pulsed UV- light on treatment tray, in three different distances (5, 10 and 15 cm) from pulsed UV source (PUV- 01 system).

Treatment voltage (V)	Distance from pulsed UV source (cm)	Surface area exposed to pulsed UV-light treatment (%)
1,000	5	30
	10	75
	15	90

3.4.2 Microbial analysis for apple juice

Analysis of variance (ANOVA) was performed and the log reductions were plotted as a function of different number of pulses and voltage input for two different distances from pulsed UV source (Figure 3.5).

All of the treatment times had a significant impact on *E. coli* populations ($p < 0.05$). Even so, the study showed no significant differences in *E. coli* inactivation of samples when the treatment time increased from 60 to 120 and then to 600 pps ($p > 0.05$). This might be due to low intensity of the treatment, for the reason that significantly greater reductions in *E. coli* populations were observed in samples exposed to pulsed UV-light for 2 minutes at a frequency of 10 pps (1,200 pulses)($p < 0.05$). No significant differences in counts were observed when samples exposed to pulsed UV- light from 5 cm distance compare to 15cm distance from UV strobe ($p > 0.05$). Although statistical analysis showed no significant differences ($p > 0.05$), figure 3.5 illustrates more inactivation when higher voltage input and less distance between sample and lamp is used in the treatment.

Literature review on the effectiveness of pulsed UV-light have shown that this technology may result in significant reductions in levels of microbial pathogens, moulds and yeasts (Demirci and Krishnamurthy, 2011; Takeshita, 2003; Turtoi and Nicolau, 2007). In a study by (Ngadi et al., 2003) apple juice inoculated with *E. coli* O157:H7 were treated with UV-light. UV doses between 0 and 23.4 J cm⁻² was evaluated and reductions of 4.2 log CFU ml⁻¹ were achieved. In another study evaluating the effectiveness of pulsed UV- light treatments for

the control of *Escherichia coli* and *Listeria innocua* in apple juice, a reduction of 4.7 log CFU ml⁻¹ for *E. coli* was obtained after 8 seconds when total energy doses of 28 J cm⁻² were imposed to the samples (Palgan et al., 2011). In disagreement with the mentioned studies, the current study on apple juice showed that at the most a reduction of 2 log CFU ml⁻¹ for *E. coli* was obtained after 2 min treatment (1,200 pulses) at 1,000 V and 5 cm. The lower inactivation observed in the present study was as a result of low energy dose which is generated by 1,000 V input (20 J).

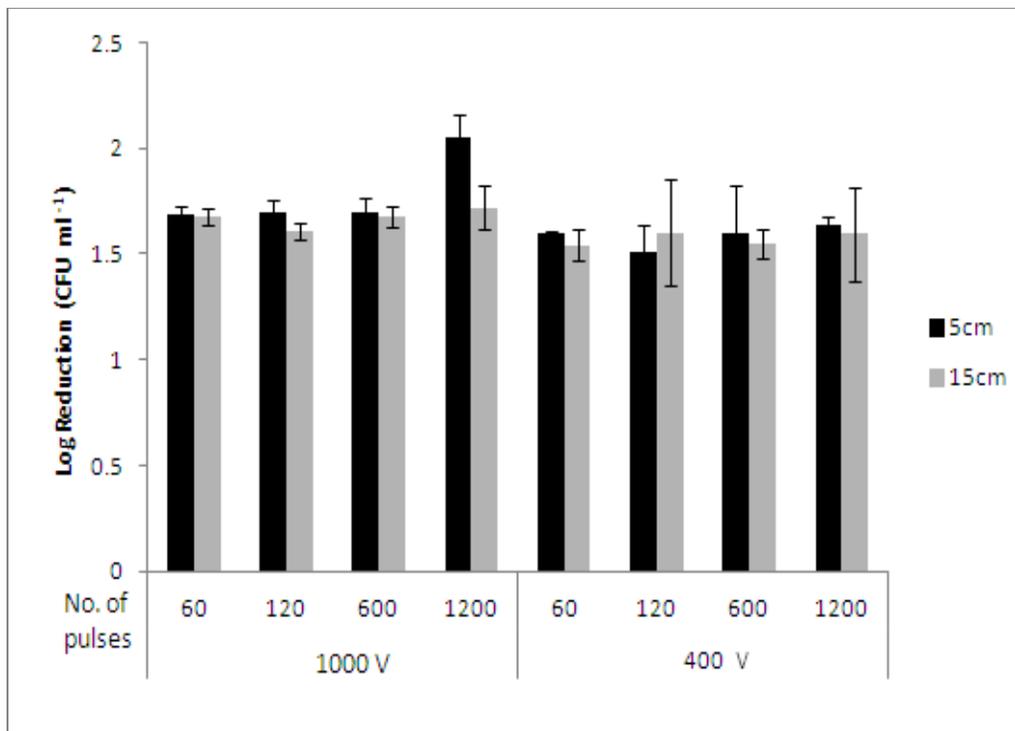


Figure 3.5 The log reduction in population of *Escherichia coli* in apple juice treated by pulsed UV- light at different distances, voltage inputs and treatment times.

3.5 Conclusion

The result of the effect of voltage and distance from the pulsed UV-light source on inactivation of *P. roqueforti* showed mold inactivation is increased as treatment time increased. When the distance from the pulsed UV-light source is increased (from 5 to 15 cm), the inactivation of *P. roqueforti* is decreased due to decline in the intensity of the pulsed UV-light. Thus, the findings show, it is crucial when choosing pulsed UV-light parameters (for the Magnavolt PUV-01 in this study), to consider the sample exposed surface area to pulsed UV-light. Based on the results achieved from bacterial inactivation in apple juice, it is concluded, in the next experiments more treatment time needs to be considered in order to maximize microbial destruction of highly resistant microorganism on the surface of more complex food substrates than apple juice.

3.6 REFERENCES

- Ben Saïd, N. E., Federighi, M., Bakhrouf, A., & Orange, N. (2010). Effectiveness of pulsed ultraviolet-light treatment for bacterial inactivation on agar surface and liquid medium. *Foodborne pathogens and disease*, 7(11), 1401–6.
- Demirci, A., & Krishnamurthy, K. (2011). Chapter 18 Pulsed Ultraviolet Light, 249–261.
- Dunn, J.E., Clark, R.W., Asmus, J.F., Pearlman, J.S., Boyerr, K., Painchaud, F., et al., 1989. Methods for preservation of foodstuffs. Vol. US Patent 4871559.
- Dunn, J., 1996 Sep. Pulsed light and pulsed electric field for foods and eggs. *Poultry Science* 75 (9), 1133e1136.
- Gómez-López, V. M., Ragaert, P., Debevere, J., & Devlieghere, F. (2007). Pulsed light for food decontamination: a review. *Trends in Food Science & Technology*, 18(9), 464–473.
- Hillegas, S.L., and Demirci, A. 2003. Inactivation of *Clostridium sporogenes* in clover honey by pulsed UV-light treatment. In *Agriculture Engineering International: the CIGR Journal of Scientific Research and Development*. Vol. V. Manuscript FP 03 009
- Jun, S., Irudayaraj, J., Demirci, A., & Geiser, D. (2003). Pulsed UV-light treatment of corn meal for inactivation of *Aspergillus niger* spores, (2000), 883–888.
- Ngadi, M., Smith, J., Cayouette, B., 2003. Kinetics of ultraviolet light inactivation of *Escherichia coli* O157:H7 in liquid foods. *Journal of the Science of Food and Agriculture* 83, 1551e1555.

- Palgan, I., Caminiti, I. M., Muñoz, a, Noci, F., Whyte, P., Morgan, D. J., ... Lyng, J. G. (2011). Effectiveness of High Intensity Light Pulses (HILP) treatments for the control of *Escherichia coli* and *Listeria innocua* in apple juice, orange juice and milk. *Food microbiology*, 28(1), 14–20.
- Pollock, A. M. (2007). characterizaton of pulsed light treatment on the shelf life of and safety of vacuum packaged cold smoked salmon.
- Ross, A.I.V., Griffiths, M.W., Mittal, G.S., Deeth, H.C., 2003. Combining nonthermal technologies to control foodborne microorganisms. *International Journal of Food Microbiology* 89, 125e138.
- Takeshita, K. (2003). Damage of yeast cells induced by pulsed light irradiation. *International Journal of Food Microbiology*, 85(1-2), 151–158.
- Turtoi, M., & Nicolau, a. (2007). Intense light pulse treatment as alternative method for mould spores destruction on paper–polyethylene packaging material. *Journal of Food Engineering*, 83(1), 47–53.

CHAPTER 4

SELECTION OF MICROBIOLOGICAL PROTOCOLS FOR INOCULATION AND ENUMERATION OF MOLD ON BREAD SURFACE

4.1 Abstract

Microbial contamination on the surface of food and food processing equipment is a major concern for the bakery industry. In the current study, the distribution consistency of mold growth (*Penicillium roqueforti* ATCC 10100) was investigated using different methods of inoculation. Random spot and spread inoculation methods were employed on the upper surface of flat bread. The consistency of *P. roqueforti* growth over time was similar for both methods of inoculation. Two different inoculum concentrations (10^2 and 10^3 CFU ml⁻¹) were also used to assess the effect of inoculum size on the distribution and consistency of mold growth after incubation of bread for 7 days at room temperature. Samples inoculated with 10^2 CFU ml⁻¹ yielded a more homogenous distribution of colonies across the upper surface of flat bread. The efficacy of these two inoculation methods was also evaluated on pizza dough with and without toppings after treatment of 400V, 60 to 1,200 pulses, 5 and 25 cm from UV source. Parameters of 400 V, 1,200 pulses and a distance of 5 cm from the UV strobe resulted in complete inactivation of *P. roqueforti* on the surface of some of the random spot and spread inoculated pizza breads (by visual inspection).

4.2 Introduction

One of the major challenges in designing an experiment for the present study is to inoculate and enumerate mold populations on the surface of pre-formed pizza samples. One of the critical concerns was to achieve a better pulsed UV-light penetration and treatment homogeneity. This depicts the importance of inoculation techniques which could assure an even distribution of inoculums over the surface of treated substrate. Therefore choosing an appropriate method of inoculation can result in better dispersion inoculum and treatment homogeneity. As a result, the inoculua distribution would not only be more similar to the natural distribution of contaminants but would also simplify the detection of individual colonies for enumeration. A literature review reveals numerous methods of propagation of bacteria and fungi on the surface

and inside various food products, depending on the nature of the samples and the treatment method. For instance, an inoculum of 0.1 ml was spread on salmon skin and muscle tissue to evaluate the inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on raw salmon fillets by pulsed UV-light treatment (Ozer & Demirci, 2006). The spray inoculation of spores on polystyrene (PS), glass, or aluminum was performed with an airbrush connected to a compressor (delivering compressed air at 2.5×10^5 Pa) to study microbial surface decontamination by pulsed UV-light (Levy and Bornard, 2011). In a previous study, pita bread was inoculated with a total of 100 μ l of mold suspension at random spots on the bread surface to give a final inoculum of 10^3 spores g^{-1} (El-khoury et al., 1999). In another study which investigated the inhibition of fungal growth on wheat and rye bread by Modified Atmosphere Packaging (MAP), samples were inoculated by inserting inoculums at 3 points. Among studies that assess the efficiency of possible inoculation methods (spread, random spot, spray and central spot), Levy et al. (2011) showed that spray was an inefficient method of inoculation due to the fact that not more than 20 % of the sprayed volume comes into contact with the target surface (the agar plate or the Petri dish). The central spot method was also identified as a limited method since the accumulation of inoculum in only one spot can affect pulsed UV-light effectiveness and yield a low level of inactivation (Bialka, 2007).

The objectives of the present study were:

- 1- To compare the reproducibility of two different methods of inoculation (random spot and spread) on untreated flat bread.
- 2- To conduct a preliminary study of the efficacy of pulsed UV-light on mold inactivation of randomly spot inoculated pizza bread without tomato topping.
- 3- To conduct a preliminary study on the efficacy of pulsed UV-light on mold inactivation on pizza bread with topping, using two methods of inoculation (spread and random spot).

4.3 Materials and Methods

4.3.1 Sampling

Pita bread (Selection, QC, Canada) with approximate radius of 10 cm was purchased from a local grocery store in the region of Montreal Island. Pita bread was autoclaved in Electric Sterilmatic Sterilizer (Market Forge Industries Inc., Everett, MS, USA) for 20 mins at 121°C

prior to inoculation in order to reduce the native microflora. Pizza bread, with and without tomato sauce topping, were obtained from a pizzeria company (Les aliments Rustica, Montreal QC, Canada) and were sterilized as above.

4.3.2 Fungal isolate and preparation of inoculum

A stock-culture of *Penicillium roqueforti* ATCC 10010 (Cedarlane Corporation, Burlington, ON, Canada) was inoculated on Potato Dextrose Agar (PDA, Difco, Spark, MD, USA) in order to obtain a pure culture. Plates were incubated for 3-7 days at 25 °C in the dark, after which colonies were transferred to fresh Potato Dextrose Broth (Difco, Sparks, MD, USA) and re-incubated for 5-7 days at 180 rpm and 24°C to generate the inoculums. Suspensions of vegetative cells were made by diluting the inoculum with peptone water (10^2 and 10^3 CFU ml⁻¹). Afterwards 100 µl suspensions were used for inoculating each piece of bread. Controls were prepared using the same method. The inoculum was not diluted more than 3 folds as the initial concentration of inoculum was estimated to be 10^5 CFU ml⁻¹ and four subsequent serial tenfold dilutions yielded counts that were lower than the detection level. The first tenfold dilution produced mold counts that were greater than detection level.

4.3.3 Inoculation of bread and visual inspection of untreated bread

For the random spot method, pita bread samples were inoculated by pipetting 0.1 ml of the inoculum suspension and depositing the suspension on five randomly chosen spots of the bread's surface. For the spread method, an equal amount 0.1 of inoculum was introduced on the surface of the bread and spread over the entire surface with a sterile hockey stick. The inoculated samples were dried for 10 minutes under laminar flow in a biological safety cabinet (Labconco Corporation, Kansas City, MO, USA) before packaging in aluminum foil to facilitate the absorption of the liquid inoculum by the bread. Each experiment was done in quadruplicate for each inoculation method and repeated 15 times with an independently prepared vegetative cell suspension. Mold growth on the surface of the bread was enumerated at different time periods: time 1 (0 - 8 days), time 2 (9 to 16 days) and time 3 (>16 days). The enumerations were done almost every day, however as there were no significant difference in daily mold growth counts, the results were reported in time periods.

4.3.4 Inoculation, pulsed UV-light treatment and visual inspection of treated breads without tomato sauce topping

4.3.4.1 Inoculum preparation

The inoculum consisted of vegetative cell suspension of *Penicillium roqueforti* and was prepared as detailed above.

4.3.4.2 Inoculation of bread

Samples were inoculated by pipetting 0.1 ml of *P. roqueforti* suspension in 10 drops (approximately same volume) onto 10 randomly chosen spots on the surface of each piece of bread. Controls (uninoculated breads) were prepared in the same fashion. In this experiment, the random spot method has been used to create a feasible approach to detecting surviving spots of inoculum after pulsed UV treatment. Only the top surface was inoculated due to the fact that the pulsed UV-light chamber was equipped with only one lamp, at the top. The samples were kept under laminar flow in a biological hood for 10 min before treatment to facilitate absorption of the liquid inoculum by the bread.

4.3.4.3 Pulsed UV-light treatment

Two UV treatments were investigated: a minimum treatment (400 V, 300 pulses and 25 cm from UV strobe) and a maximum treatment (400 V, 600 pulses and 5 cm from UV strobe). Mold growth on the bread was enumerated after 12 days of incubation at room temperature.

4.3.5 Inoculation, pulsed UV-light treatment and visual inspection of treated pizza bread with tomato sauce topping

4.3.5.1 Inoculum preparation

The inoculum consisted of vegetative *Penicillium roqueforti* and was prepared as above.

4.3.5.2 Inoculation of bread

For the random spot method, pizza bread samples were inoculated by pipetting 0.1 ml of *P. roqueforti* suspension in 5 drops (approximately same volume) onto 5 randomly chosen spots on the surface of each piece of pizza bread. In this part of the experiment, the number of random spots was reduced to prevent the spots of inoculum from coalescing since sample surface area is limited. For the spread method, an equal amount of inoculum was introduced onto the entire surface of the bread. The deposited suspension was dried for 10 minutes under a laminar flow hood before treatment to facilitate the attachment of microbial cells to sample surfaces.

4.3.5.3 Pulsed UV-light treatment

Three UV treatments were investigated: minimum treatment (400 V, 60 pulses and 25 cm from UV strobe), intermediate treatment (400 V, 120 pulses and 5 cm from UV strobe), and maximum treatment (400 V, 1,200 pulses and 5 cm from UV strobe). Mold growth was enumerated after 12 days of incubation at room temperature.

4.4 Results and discussion

4.4.1 Inoculum size

In this study, the effect of inoculum size on the growth of vegetative fungi cells was evaluated. As expected, when bread was inoculated with 10^2 CFU ml⁻¹, less mold growth was visible on the surface of the bread in comparison with bread inoculated with 10^3 CFU ml⁻¹ (Figure 4.1). Visual inspection also revealed that a lower initial fungal concentration was easier to enumerate. However, mold growth (in number and size) increased on all the breads over time. This can be seen on the bread inoculated with 10^3 CFU ml⁻¹. A non-homogeneous dispersion is visible compared to those inoculated with 10^2 CFU ml⁻¹.

4.4.2 Inoculation methods

In this study, the consistency between the numbers of mold colonies grown during the incubation time, with each inoculation method was determined in 15 experimental replications.

The enumeration scatters obtained using the spread and random spot methods are shown in Figure 4.1.

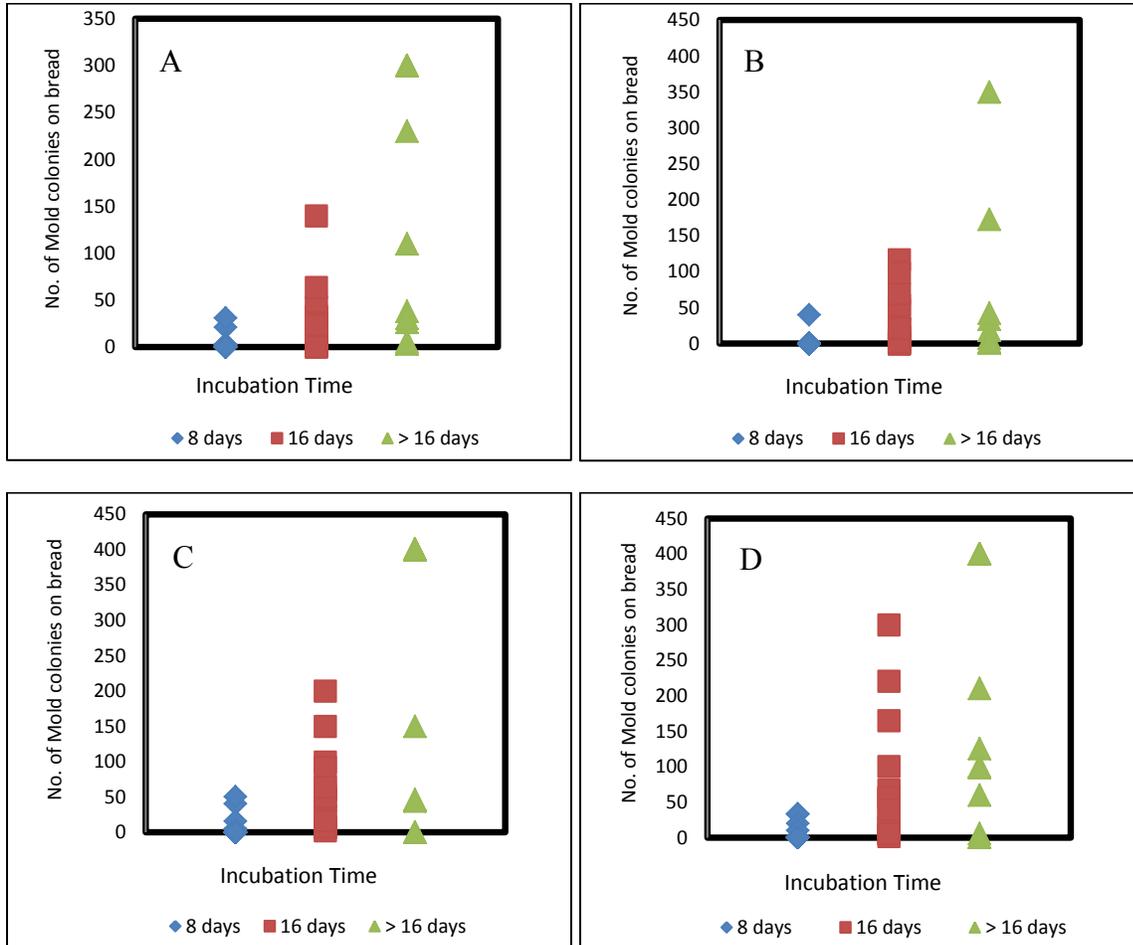


Figure 4.1 Enumeration scatter of mold count on pizza bread surface after inoculation with 10^2 and 10^3 CFU ml⁻¹ of *P. roqueforti*. Figure A (upper left) = 102 random spot, B (upper right): 102 spread, C (lower left): 103 random spot, D (lower right): 103 spread. Mold growth on the surface of the bread was enumerated at different time periods: time 1 (0 - 8 days), time 2 (9 to 16 days) and time 3 (>16 days).

A comparison was carried out between the consistency of mold growth obtained from two different inoculation methods, random spot and spread (Figure 4.1). During the first 8 days of incubation, mold growth on the bread was less than 50 colonies per bread in most of the experimental replicates by using both inoculation methods and both inocula concentrations. For 10^2 CFU ml⁻¹ inoculum size, colony numbers were less than 150 colonies per bread until day 16.

However, the majority were still under 100 colonies per bread. In the third time period, mold growth increased to 300 and 350 per bread. Bread inoculated with an inoculum size of 10^3 CFU ml^{-1} followed the same pattern as those inoculated with 10^2 CFU ml^{-1} . Nonetheless, these breads had more colonies over each period of time because of a higher level of starting inoculum. Despite the similarities between the two inoculation methods, visual observation of colonies formed during incubation showed a more homogeneous dispersion of cells when they were spread on the surface as compared to those randomly spotted on it. In the random spot method there is also some underestimation due to colonies which had never been detected because they were in the shade of other colonies (Levy et al., 2011). However, it was noticed that the mold grouping was easier to differentiate when random spot was used. Therefore, both inoculation methods can be selected as an ideal method of inoculating mold onto the sample surface depending on the feasibility of each method with regards to the purpose of each experiment.

4.4.3 Inspection of mold growth on bread with and without tomato sauce topping

This part of the experiment was conducted using the concept of minimum and maximum treatments, as described in Table 4.1 Random spot was applied as inoculation method on pizza bread without topping.

Table 4.1 Incidence of mold growth on pizza bread (without tomato topping) surface after UV treatment. Ten random spots were applied to each bread surface (5 replicates per treatment).

Treatments	Replicates				
	1*	2	3	4	5
Control (no treatment)	+++++ +++	+++++ +++++	+++++ +-----	++++- -----	++++- -----
Minimum UV treatment (400 V, 300 pulses and 25 cm from UV strobe)	++- - - -----	+ - - - - -----	+++++ ++++-	+++ - - -----	+ - - - - -----
Maximum UV treatment (400 V, 600 pulses and 5 cm from UV strobe)	- - - - - -----	- - - - - -----	++- - - -----	- - - - - -----	- - - - - -----

* + represents growth; - represents no growth.

This part of the experiment was also conducted using the concept of minimum, intermediate and maximum treatments as described in Table 4.1 the inoculation was performed using random spot and spread methods. Two inoculation methods were examined on bread with topping to determine the experimental efficacy of both methods on bread with topping. Results are shown in Table 4.2.

Table 4.2 Incidence of mold growth on pizza surface with tomato sauce topping, after UV treatment. Five random spots were applied (4 replicated for each treatment).

Replications	Spread Method				Random Method			
	1	2	3	4	1	2	3	4
Control (no UV treatment)	+*	+	+	+	-	+	+	+
Minimum UV treatment (400V, 60 pulses and 25 cm from UV strobe)	+	-	-	+	+	+	+	-
Intermediate UV treatment (400V, 120 pulses and 5 cm from UV strobe)	-	-	-	-	-	+	-	+
Maximum UV treatment 400V, 1200 pulses and 5 cm from UV strobe)	-	-	+	-	-	-	-	-

* + represents growth; - represents no growth

Although the results varied between replicates of the same treatment, trends of mold inactivation were considerably the same for both inoculation methods and bread with or without tomato topping (Table 4.1 and 4.2). *Penicillium roqueforti* decontamination was improved by decreasing the distance between the sample surface and the UV-lamp as well as by increasing voltage input and the number of pulses (which also means increasing the treatment time).

4.5 Conclusion

The results showed that the scatter obtained by both random spot and spread methods are in the same order of magnitude and both could be used to inoculate the surfaces. However, it was noticed that the mold grouping was easier to differentiate when random spot was used, while colonies' scatter is more homogenous on the bread when spread inoculation method was applied. Therefore, the random spot and spread methods will be selected as a preferred method of inoculating mold onto the sample surface for the remaining part of study depending on the feasibility of each method with regards to the purpose of each experiment. The effect of pulsed UV-light was found to be more efficient to inactivate 10^2 CFU ml⁻¹ on some of the bread samples. Considering that in practice the degree of cooked food contamination is much lower, this achievement could lead to an effective reduction of molds (vegetative cells), and even to obtain a total decontamination of the surface after packaging.

4.6 REFERENCES

- Bialka, K. L. (2007). The Pennsylvania State University The Graduate School Department of Agricultural and Biological Engineering decontamination of berries with ozone and pulsed uv-light (thesis), 223–7.
- KARIN I. SUHR. (2005). M;Food Microbiology and Safety Inhibition of Fungal Growth on Wheat and Rye Bread by Modified Atmosphere Packaging and Active Packaging Using, 70(1).
- Levy, C., Bornard, I., & Carlin, F. (2011). Deposition of *Bacillus subtilis* spores using an airbrush-spray or spots to study surface decontamination by pulsed light. *Journal of microbiological methods*, 84(2), 223–7.
- Ozer, N. P., & Demirci, A. (2006). Inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* inoculated on raw salmon fillets by pulsed UV-light treatment. *International Journal of Food Science and Technology*, 41(4), 354–360.

CHAPTER 5

SHELF LIFE EXTENTION OF PIZZA AND BREAD BY PULSED UV-LIGHT

5.1 Abstract

Intense light pulse is one of the emerging non-thermal technologies considered as an alternative to traditional thermal treatment due to its microbial destruction effects without deteriorative impact on food quality. The aim of this study was to evaluate the possibility of using pulsed UV-light treatment for effective inactivation of molds on pizza and bread surfaces. The pizza samples had tomato sauce topping. The bread samples were plain pizza bread with no topping. For pizza samples, three sets of pulsed UV light treatments were applied namely 500 V, 1 min (minimum treatment); 750 V, 5 min (intermediate treatment) and 1,000 V, 10 min (maximum treatment). The treated samples were visually examined for mold growth within 40 days storage. The results showed that 8, 32, and 40% of pizza samples were mold free after the 40 days storage with minimal, intermediate and maximal pulsed UV-light treatment, respectively. The bread samples were inoculated with *Penicillium roqueforti*, and treated with different pulsed UV input voltage (500 and 1,000 V), treatment time (2 and 7 min) and distance from the light source (5 and 15 cm). Treated bread samples were examined microbiologically after 7, 14, 21 and 28 days storage. UV input voltage significantly influenced inactivation of *P. roqueforti* on the surface of bread. There was a significant effect of voltage on the reduction of mold population on the samples ($P < 0.05$). Samples treated with the more intense voltage (1,000 V) showed steady surviving population less than 10^2 CFU g^{-1} over the storage time. However, 500 V treated breads and controls showed an exponential growth in every analysis day (more than 10^3 CFU g^{-1}). The results indicate that pulsed UV-light can be considered as a potential decontamination method for bakery products.

5.2 Introduction

Cereal-based products, especially bakery products, are important part of a balanced diet. Although microbial, chemical and physical spoilage all occur in bakery and pasta products, mold spoilage is often the main shelf life limiting factor (Rodriguez et al., 2003). In fact, microbial

spoilage is the major cause of economic loss in the bakery industry (Ooraikul and Stiles, 1991). Pulsed UV-light is a non-thermal technology which can be considered as an effective technique to minimize these problems while at the same time extending their shelf life (Hierro et al., 2011). Pulsed light has been investigated as a mean of extending the shelf life of baked goods, seafood, meat products, and fruits and vegetables (Dunn, 1997). Changes in consumer preferences in recent years have led to the development of a wide variety of ready-to-eat foods. Among these foods, the bakery industry offers pre-cooked pizza products which can be kept at room temperature. However, these products are presumed to be contaminated in post-cooking procedures due to further manipulation such as cutting, slicing in handling and packaging sections. Concerning food safety, preformed pizza have been reported to be contaminated by mold such as *Penicillium roqueforti* (Saranraj and Geetha, 2012). In this regard pulsed UV-light is a techniques which would aid in preventing the growth of spoilage or pathogenic bacteria, while maintaining the sensory quality of the final product. The ability of pulsed UV light to kill several fungus spores is well documented. Investigations showed a 60–99% reduction of viable spores of *Aspergillus flavus*, *Aspergillus niger*, *Penicillium corylophilum* and *Eurotium rubrum* on the surface of agar plates after a 15 s exposure to pulsed UV light (Begum et al., 2009). Pulsed light extends shelf life of bread more than two weeks at ambient temperature as compare to untreated samples which were visibly spoiled with mold growth after one week. Overall, pulsed light technology shows promising results as a novel method to extend the mold free shelf life of pre-packaged bakery products (Dunn et al., 1995;1997). Pulsed UV-light effect on bakery products was investigated by treating breadstick with eight flashes at 0.5 J cm^{-2} . Approximately 17% of the control breadsticks showed visible molding by day 6 while 100% were moldy by day 7. However, less than 4% of the treated breadsticks showed visible signs of mold by day 13, and no further mold growth occurred throughout the 26 days of storage (Dunn et al., 1997b). Application of pulsed UV-light treatment on disinfection of hard crusted white bread rolls are described in the US patent of Dunn et al. (1989). Conventional UV exposure produced about 2.5 to 4.5 log reduction in CFU of *Aspergillus niger* (a common bread mold) during the first 3-10 seconds of treatment (Cerny, 1977). In another study, a few flashes of pulsed UV-light applied in a fraction of a second resulted in 7 log CFU cm^{-2} reduction of *A. niger* in packaged bread (Dunn et al., 1997). The objectives of this study were; to evaluate the efficacy of pulsed UV-light

intensity on the shelf life of pizza bread with topping (non-inoculated samples) and to enumerate the mold surviving population on treated pizza without topping (inoculated samples) over time.

5.3 Materials and Methods

5.3.1 Pizza bread with tomato sauce topping

Samples of pizza bread with tomato sauce topping were obtained from both a nearby factory (Les Aliments Rustica) and local grocery stores in the region of Montreal Island. The samples were stored at room temperature for two days prior to pulsed UV irradiation. Following treatment, the pizza samples were stored at room temperature until 40 days for visual inspections. 40 days storage was decided as last visual inspection day because it is two times longer than the product's normal expiry date. After this day, physical deterioration such as stalling will happen and this condition without considering microbiological spoilage would nonetheless make the product unacceptable.

5.3.2 Pizza bread without tomato sauce topping

Samples of pizza bread without tomato sauce topping were also obtained from a pizza producing factory (Les Aliments Rustica) and from grocery stores in the region of Montreal. Each piece of bread (approximate radius of 10 cm) was divided into 4 equal size pieces. Pizza bread slices were autoclaved for 20 mins at 121°C in Electric Sterilmatic Sterilizer (Market Forge Industries Inc., Everett, MS, USA,) in order to reduce their native microflora prior to the inoculation with test mold. Each piece was inoculated with 0.1 ml of inoculum which was spread on the sample surface while ensuring that there was no spill off the edges of the sample. Since pulsed UV-light is largely a surface treatment, the reduction of inoculums on the surface as a result of inoculum spill off the edges, could be considered as a decontamination effect of pulsed light. Visual and microbiological analyses were performed 7, 14 and 21 days after pulsed UV treatment throughout the storage time.

5.3.3 Pulsed UV-light treatment for pizza samples

3 treatment combinations were considered for the experiments (Table 5.1). 25 samples were treated by each treatment intensity to determine the efficacy of individual parameters on the

shelf life of the pizzas. The treated and untreated samples were then kept at room temperature, the same storage conditions where the pizzas would be stored in the industrial setting. Visual inspection was performed in anticipation of 40 days after treatment.

5.3.4 *Penicillium roqueforti* inoculum preparation (for bread samples)

A stock-culture of *Penicillium roqueforti* ATCC 10010 (Cedarlane Corporation, Burlington, ON, Canada) was inoculated on Potato Dextrose Agar (PDA, Difco, Spark, MD, USA) in order to obtain a pure culture. Plates were incubated for 3-7 days at 25°C in the dark, after which colonies were transferred to fresh Potato Dextrose Broth (Difco, Sparks, MD, USA) and re-incubated for 5-7 days at 24°C to generate the inoculum. The inoculum was homogenized in a Stomacher 400 Lab Blender (Seward medical, London, UK) for 2 min and a population of about 1×10^5 CFU mL⁻¹ was obtained in the resulting inoculum solution. An equal amount of inoculum was introduced onto the entire surface of the bread. The deposited suspension was dried for 10 minutes under a laminar flow hood before pulsed UV treatment to facilitate the attachment of microbial cells to sample surfaces.

5.3.5 Pulsed UV- light treatment for bread samples

Characterization of pulsed UV-light system used in the present study is describes in chapter 1. Each inoculated bread was centered individually on the adjustable stainless-steel shelf in the pulsed UV unit. The experiments were performed by positioning the shelf at 5 and 15 cm beneath the Xenon lamp. At each distance, the effect of treatment time (2 and 7 min) and input voltage (500 and 1,000 V) was investigated. Treated breads were consequently wrapped with aluminum foil immediately after flashing and kept at room temperature up to 21 days. Untreated slices were analyzed as controls. Each set of parameters were performed in duplicate.

5.3.6 Analysis of pizza samples after pulsed UV treatments

To assess the efficacy of pulsed UV-light on shelf life extension of samples, molds were enumerated immediately after treatment (day 0) and after storage for 7, 14, 21 days at room temperature.

On each day, one slice was aseptically removed from the package, transferred into an individual sterile stomacher bag, weighted and then mixed with buffered peptone water (in the proportion

with bread samples at 9:1). The mixture was homogenized in a Stomacher 400 Lab Blender (Seward medical, London, UK) for 2 min. Serial decimal dilutions were conducted and the dilutions were spread-plated onto PDA (Potato Dextrose Agar) plates. The plates were incubated at 24°C for 3 days, and then colonies were counted using a manual colony counter (Reichert technologies, Depew, NY, USA).

5.3.7 Statistical analysis for bread

All experiments were performed in duplicate for the same treatment at the same time for all two trials. Statistically significant differences were tested by repeated measurement analysis of variance using SAS 9.1 software.

5.4 Results and Discussion

5.4.1 Pulsed UV-light on Pizza

The result shown in Table 5.1 indicates the average time of mold spoilage on the samples for the selected treatment intensities. 25 samples were treated by each treatment intensity.

Table 5.1 Conditions used for the pulsed UV-light treatments. 25 replicate per each treatment intensity.

Treatment Intensity	Voltage(volts)	Time(min)	Frequency(Hz)	NO. of pulses	Time for mold spoilage (days)
Control	0	0	0	0	17.56 ± 6.7
Minimum	500	1	4	240	15.52 ± 15.5
Intermediate	750	5	4	1200	26 ± 12.7
Maximum	1,000	10	4	2400	30.8 ± 9.6

The overall trend showed, by increasing the intensity of treatment from minimum to maximum, the shelf life of samples were increased by 10 to 40 percent (Table 5.2). The companies' specified expiry date for the product of our study is 21 days. According to this information, 20, 48 and 60 percent of the samples were still visually proper to use after minimum, intermediate and maximum pulsed light treatment respectively. Intermediate and maximum treatment showed an increase in shelf life, as the number of non-moldy pizza was still high and above 50% at 20 days. However, during the same period, mold spoilage occurred on more than 70% of the minimally treated pizzas. This percentage was even higher than that of

control (untreated) samples which was around 68% until the inspection day of 20. This can be attributed to a phenomenon, known as photoreactivation which is a DNA repairing mechanism based on the activity of DNA photolyases. This enzyme uses the UV-A component of light for repairing the damaged nitrogenous bases (Cook, 1970). Although all the pizzas were kept in the same condition at room temperature, it is possible that photoreactivation took place on some of the lower intensity treated products. Therefore, some injured fungi cells could have been repaired, which reduced the samples' shelf life to the level of untreated pizzas. Photoreactivation can occur when the UV-C injured cells are exposed to wavelengths higher than 330 nm (Guerrero-Beltrn, 2004; Hierro et al., 2009). It should be noted that the wavelength of the pulsed UV- light system used in the present experiment has a peak spectral emissions at the wavelength 250 nm.

Table 5.2 Percentage of non-moldy pizza after 10, 15, 20, 30, 40 days.

Inspection time (days)	Percentage of non-moldy pizza			
	Control	Minimum PL treatment	Intermediate PL treatment	Maximum PL treatment
10	88	56	92	100
15	48	28	60	88
20	32	28	52	76
30	4	20	48	60
40	0	8	32	40

5.4.2 Pulsed UV-light on Pizza

5.4.2.3 Microbiological analysis

The effectiveness of pulsed UV-light was evaluated by determining the surviving population of *P. roqueforti* on bread surfaces. While the UV treatment time and distance showed no significant influence on mold population over time ($P > 0.05$), there was a significant effect of voltage on the reduction of mold population on the samples ($P < 0.05$). Within each analysis day, there were also a significant difference between surviving population obtained by 500 volts and 1,000 volts ($P < 0.05$). However, other parameters including distance and treatment time were not significantly different on their effect on surviving population in all of the analysis days. This

disagreement with the result from non-inoculated pizza treatment could be due to non-homogeneous inocula introduced on the surface of samples.

Samples treated with the more intense voltage (1,000 V) showed a steady surviving population of less than 10^2 CFU g^{-1} over the storage time (Figure 5.1). However 500 V treated breads and controls showed an exponential growth in every analysis day to the level of more than 10^3 CFU g^{-1} in day 21. UV-light damages the DNA of microorganisms by primarily forming thymine dimers and thus the DNA cannot be replicated. Hence, microorganisms lose their ability to reproduce (Oms-Oliu et al., 2008; Demirci and Krishnamurthy, 2011). The exponential growth of *P. roqueforti* in 500 V treated breads could be attributed to the fact that mold cell's DNA was not vigorously affected by pulsed UV in a low intensity.

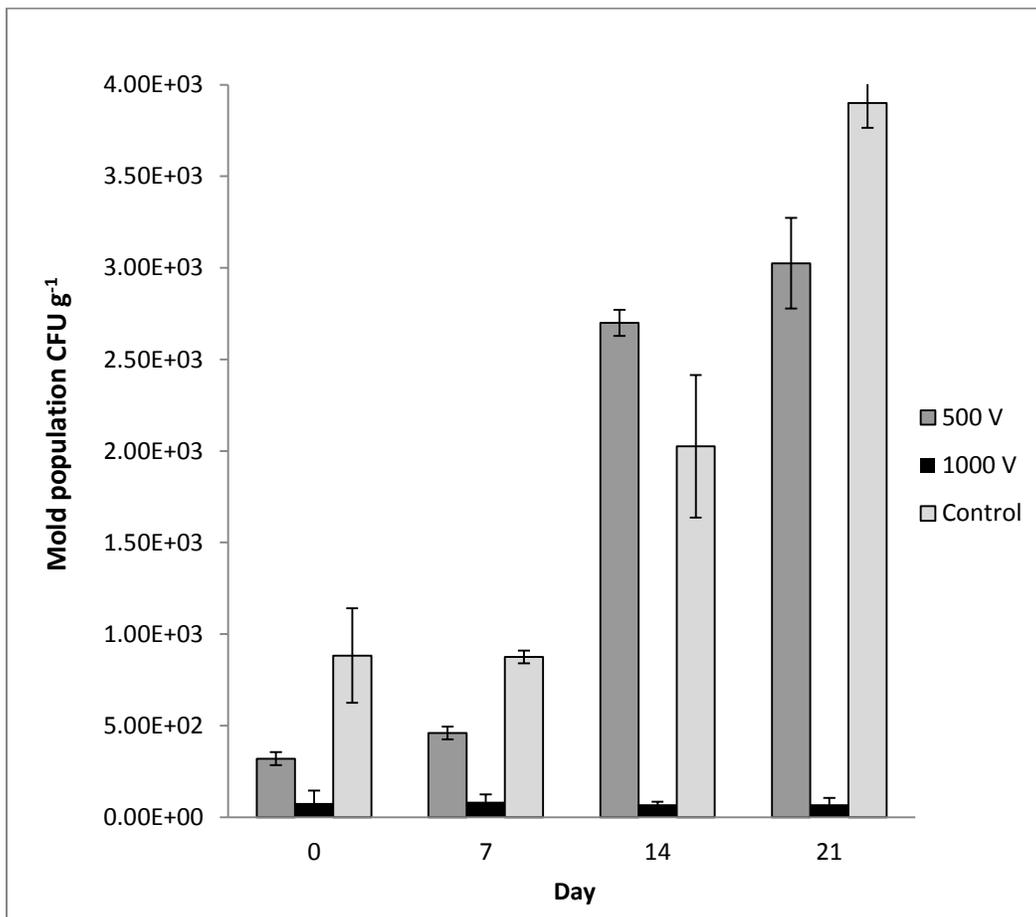


Figure 5.1 Surviving mold (*P. roqueforti*) population over storage time in each analysis day when time and distance are fixed at 5 cm and 7 mins.

5.5 Conclusion

In non-inoculated pizza breads with topping, pulsed light treatment of 1,000 V for 10 min at 10 cm distance from pulsed UV source extended shelf-life of 40% of the maximally treated pizzas up to 40 days. Considering this result, pulsed light appears as a promising technique for extending the shelf life of product in bakery industry applications. In bread without topping, there was a significant effect of voltage on the reduction of the mold population on the samples ($P < 0.05$). In addition, samples treated with the more intense voltage (1,000 V) showed steady a surviving population of less than 10^2 over the storage time. However, many irregularities and unpredictability in the result had been seen especially when treatments with lower level of voltage input were performed. This inconsistency might be because of the non-homogenized nature of vegetative cell inoculum. Therefore, more homogenized source of inoculum such as spore suspension has to be applied in the future experiments. The homogeneity in the inocula helps to observe a more consistence and reliable inactivation results corresponding to the level of pulsed light intensity even at lower levels.

5.6 REFERENCES

- Cerny, G. (1977). Sterilization of packaging materials for aseptic packaging. *Ver pack*, 28(10): 77.
- Cook, J.S., 1970. Photoreactivation in animal cells. *Photophysiology* 5, 191–233.
- Demirci, A., and Krishnamurthy, K. (2011). Chapter 18 Pulsed Ultraviolet Light, *Nonthermal Processing Technologies for Food*, 249–261.
- Dunn, J., Bushnell, A., Ott, T., & Clark, W. (1997). Pulsed white light food processing. *Cereal Foods World*, 42, 510e515.
- Fine, F. and P. Gervais (2004). "Efficiency of pulsed UV light for microbial decontamination of food powders." *Journal of Food Protection* 67: 787-792.
- Fine, F. and P. Gervais (2004). "Efficiency of pulsed UV light for microbial decontamination of food powders." *Journal of Food Protection* 67: 787-792.
- Guerrero-Beltrn, J. a. (2004). Advantages and Limitations on Processing Foods by UV Light. *Food Science and Technology International*, 10(3), 137–147.
- Hierro, E., Barroso, E., la Hoz, L. De, Ordóñez, J. a., Manzano, S., & Fernández, M. (2011). Efficacy of pulsed light for shelf-life extension and inactivation of *Listeria monocytogenes*

- on ready-to-eat cooked meat products. *Innovative Food Science & Emerging Technologies*, 12(3), 275–281.
- Hierro, E., Manzano, S., Ordóñez, J. a, de la Hoz, L., & Fernández, M. (2009). Inactivation of *Salmonella enterica* serovar Enteritidis on shell eggs by pulsed light technology. *International journal of food microbiology*, 135(2), 125–30.
- O'Connor-Shaw, R. E., Reyes, V. G., in: Robinson, R. J., Batt, C. A., Patel, P. D. (Eds.) (2000), *Encyclopedia of Food Microbiology*, Academic Press, pp. 410–416.
- Oms-Oliu, G., Martín-Belloso, O., & Soliva-Fortuny, R. (2008). Pulsed Light Treatments for Food Preservation. A Review. *Food and Bioprocess Technology*, 3(1), 13–23.
- Ooraikul, B., & Stiles, M. E. (1991). Review of the development of modified atmosphere packaging, 1–17.
- Pollock, A. M. (2007). characterization of pulsed light treatment on the shelf life of and safety of vacuum packaged cold smoked salmon. Thesis
- Rodríguez, V., Medina, L., & Jordano, R. (2003). Influence of modified atmosphere packaging on the shelf life of prebaked pizza dough with and without preservative added. *Die Nahrung*, 47(2), 122–5.
- Saranraj, P., & Geetha, M. (2012). Microbial Spoilage of Bakery Products and its Control by Preservatives, *International Journal of Pharmaceutical & Biological Archives* 2012; 3(1):38-48 3.

CHAPTER 6

EFFECTIVENESS OF PULSED UV-LIGHT TREATMENT FOR INACTIVATION OF *PENICILLIUM ROQUEFORTI* ON AGAR SURFACE

6.1 Abstract

In the present study, the effectiveness of a broad-spectrum pulsed ultraviolet light was evaluated for the decontamination of *Penicillium roqueforti* on the surface of solid agar as a representative of a flat food surface. The process parameters evaluated were treatment time (1, 3, 5, 7 and 10 min), voltage input (500, 750 and 1,000V). The results demonstrated that an increase of the input voltage of the lamps (which also increases the UV-C intensity) and the number of pulses resulted in a higher inactivation. The population of *Penicillium roqueforti* was reduced after 10 min of exposure to pulsed light by 3.74, 5.36 and 6.14 log CFU ml⁻¹, respectively at 500, 750 and 1,000 V. Overall, these observations suggest that pulsed UV-light can effectively inactivate spores of *Penicillium roqueforti* on the surface of a solid agar medium. Additionally, the inactivation data obtained for each voltage were correlated by using Weibull (2 parameters), log-linear (1 parameter, based on first-order kinetics) models. The results presented in this study indicate that first-order kinetics is not suitable for the estimation of *P. roqueforti* inactivation on solid agar with pulsed UV-light. However, the inactivation kinetics was best described by the Weibull model with the smallest root mean squared error (RMSE) ($R^2 \geq 0.92$). Weibull model can be successfully used to estimate the inactivation and the concavity exhibited in the survival curves observed in this study. Temperature increase inside the treatment chamber was also monitored during this experiment.

6.2 Introduction

Pulsed UV-light is a unique emerging technology mostly used for surface decontamination. This non-thermal sterilization technology uses intense flashes of broad-spectrum radiation ranging from UV light (200 nm) to infrared radiation (1,000 nm) (McDonald et al., 2000). This novel technique is capable of reducing microbial populations of both vegetative cells and spores of bacteria, mold and yeast (Ben Saïd and Orange, 2010). Pulsed UV-light has been investigated as a means of extending the shelf life of baked goods, seafood, meat products, and fruits and vegetables (Dunn, 1997). Nevertheless, due to lack of penetration, the technology is increasingly proposed as an alternative non-thermal technology especially for disinfection of transparent liquid foods and decontamination of solid food surfaces (Unluturk and Atilgan, 2010; Baysal et al., 2010). Post-baking fungal contamination of bakery products is a concern for the bakery industry. *Penicillium roqueforti* is one of the dominant molds that cause spoilage on bread and other baked products.

The ability of pulsed UV light to kill several fungus spores is well documented. Investigations showed a 60–99% reduction of viable spores of *Aspergillus flavus*, *Aspergillus niger*, *Penicillium corylophilum* and *Eurotium rubrum* on the surface of agar plates after a 15 s exposure to pulsed UV-light (Begum et al., 2009). In addition, 50, 40 and 20 J cm⁻² energy were used successfully to inactivate *S. cerevisiae* at the populations of 5.4×10⁶, 7×10⁵, 5×10⁴ and 4×10³ CFU ml⁻¹, respectively in non-alcoholic beer (Hosseini et al., 2011). Marquenie et al., (2003) reported a maximal inactivation of 3 and 4 log units for conidia of fungi *B. cinerea* and *M. fructigena* in vitro. In a study by Jun et al. (2003) on corn meal, a 100-s treatment time, 3 cm of distance from the UV strobe, and with 3,800 V input gave a 4.93 log reduction of the spore of *A. niger*. Takeshita et al. (2003) proposed that pulsed UV-light can be used as an effective sterilizing method for the yeast *Saccharomyces cerevisiae* by investigating the mechanisms of damage of yeast cells treated by pulsed UV-light and continuous UV light.

Complete inactivation of *M. fructigena* conidia was obtained after a 40-s pulsed light treatment and 15 min heat at 41°C or after an 80-s light treatment and 10 min heat at 41°C. It is noted that microbial spores are more resistant than vegetative cells when they are exposed to UV treatment. As a result, more intense treatments are required for their inactivation (Farkas et al. 2007). Overall, more extensive research is needed to investigate microbial inactivation on food

surfaces by this technique. Therefore in order to further understand the potentials of pulsed UV light on *Penicillium roqueforti*, the responses of this microorganism to pulsed light treatment need to be understood through modeling. The inactivation of microorganisms via UV light has been shown to follow first-order kinetics in solution (EPA, 2003), but it has also been reported to display a sigmoidal shape with a shoulder and/or a tail (CFSAN-FDA, 2006). The previous chapters focused on the characterization and optimization of the pulsed light apparatus for treatments used in this study. These results were used as a guideline to determine the proper treatment procedure to construct inactivation model kinetics of *P. roqueforti*.

The objectives of this study were:

- 1) To evaluate the efficacy of pulsed UV-light treatment for inactivation of *Penicillium roqueforti* on agar surface;
- 2) To evaluate the pulsed UV-light destruction kinetics of *P. roqueforti* on agar surface.

6.3 Material and Methods

6.3.1 Preparation of Inoculum

Penicillium roqueforti (ATCC 10010) provided by Cedarlane Corporation (Burlington, Ontario, Canada) was used throughout this study. The strain was grown in Potato Dextrose Broth (PDB, Difco, spark, MD, USA) with shaking at 180 rpm at 24°C for 4 to 7 days. Cultures were maintained by spreading 0.1 ml of liquid culture on Potato Dextrose Agar (PDA, Difco, spark, MD, USA). After incubating the Petri plates at 24°C for 7 days, the spore crops were harvested by adding 5 ml of Tween 80 (Acros Organic, New Jersey, USA) onto the surface of PDA, then filtered by using 4 layers of cheese cloth. The resulting inoculum solution had 2.74×10^{10} colony forming units (CFU) mL⁻¹ and this cell suspension was serially diluted up to 10⁻², 10⁻³ and 10⁻⁵ before transferring to the test agar plate. A 0.1 ml of inoculum was transferred onto solid agar and spread on each PDA Petri plates using a sterile hockey stick.

6.3.2 Treatments

The Agar seeded plates were positioned on the tray at 5 cm from the UV lamp in the treatment chamber. At this distance, the effect of treatment time (1, 3, 5, 7 and 10 min) and input voltages of 500, 750 and 1,000 V were investigated. The initial cell concentrations at the various input voltages were set by 10^{-5} , 10^{-3} , 10^{-2} dilutions, respectively. Temperature of the treatment chamber was measured at 5 cm away from the light source using K type thermocouple (Omegaette HH306, Omega Engineering Inc., Stamford, CT, USA).

6.3.3 Inactivation of *P. roqueforti*

All Petri plates were incubated for 4 days at 24°C in the dark according to the supplier's protocol (ATTC); however enumeration was performed prior to 7 days (Tiwari et al., 2011), since the colonies have not grown to the level of overlapping each other and they can be distinguished easier. The impact of treatments on the inactivation of *P. roqueforti* was determined by enumerating the Colony Forming Units (CFUs) on each Petri plate. The log reduction was calculated by subtracting the log value of the control from that of treated sample.

6.3.4 Statistical analysis

An analysis of variance was performed using the general linear models procedure in SAS to compare the effects of different applied pulsed-light intensity (voltage input) and treatment time (number of pulses). All treatments were performed in triplicate, and all significant differences are reported at 95% ($P < 0.05$) confidence intervals.

6.3.5 Model development

6.3.5.1 Log-linear model

Traditionally, microbial inactivation resulting from application of both thermal and non-thermal processes have been described by the log-linear equation (Bialka, Demirci & Puri, 2008). This Model [Eq. (1)] follows the rules of first-order inactivation kinetics to describe microorganism destruction (Van Boekel, 2002).

$$\log \left(\frac{N}{N_0} \right) = -kt \quad \text{Eq. (1)}$$

Where N is the number of microorganisms at time t (CFU ml⁻¹), N_0 is the initial number of microorganisms (CFU ml⁻¹), t is the treatment time (sec or min), k is the first-order extinction coefficient (s⁻¹ or min⁻¹). From this equation, the classic “D-value” or the time necessary for a 1 log reduction can be calculated as the reciprocal of the first-order rate constant. The log-linear equation is simply appropriate for linear inactivation curves and research findings suggest that many inactivation curves are non-linear (Bialka et al., 2008).

6.3.5.2 Weibull model

The second model used in the study is the Weibull model [Eq. (2)] which is offered as a simple model for the description of microbial inactivation by thermal and non-thermal treatment methods (Van Boekel, 2002). In the case of UV-light treatment, inactivation curves are generally sigmoidal and exhibit concavity or convexity behaviors through downwards or upwards as a function of inactivation time or UV dose (Unluturk et al., 2010). The Weibull model usually describes this behavior accurately. This model is composed of two parameters (α and β) given in the following correlation (Van Boekel, 2002; Bialka et al., 2008):

$$\log \left(\frac{N}{N_0} \right) = -\frac{1}{2.303} \left(\frac{t}{\alpha} \right)^\beta \quad \text{Eq. (2)}$$

Where N is the number of microorganisms (CFU ml⁻¹), N_0 is the initial number of microorganisms (CFU ml⁻¹), t is the treatment time (sec or min), α is the characteristic time (s or min) and β is the shape parameter (unitless). Many survival curves exhibit concavity, either downwards or upwards, and the β parameter is used to describe this concavity. If $\beta < 1$, the curve displays upward concavity. This can be a sign of stress adaptation of surviving microorganism. If $\beta > 1$, the curve displays downward concavity that shows considerable damages of pulsed UV-light in the cells. In addition, by using α and β , reliable life (t_R) (indicating the time necessary for 90% reduction in the number of microorganism) can be calculated (Van Boekel, 2002). This parameter corresponds to the D-value for the first log reduction [Eq. (3)].

$$t_R = \alpha(2.303)^{\frac{1}{\beta}} \quad \text{Eq. (2)}$$

Where α characteristic time, β shape parameter.

6.4 Results and discussion

6.4.1 Pulsed UV-light and microbiological analysis

Increasing the treatment time significantly ($p < 0.05$) increased *P. roqueforti* inactivation. There were significant ($P < 0.05$) differences between all treatment times (1, 3, 5, 7 and 10 mins) when 500 V were applied. For the treatment sets using 750 V, the significant difference ($P < 0.05$) was demonstrated between 1 to 3 and 5 to 7 min. In the case of 1,000 V, treatment times of 1 to 3, 3 to 5 and 7 to 10 mins showed a significant difference ($P < 0.05$) on inactivation of *P. roqueforti* on agar surface. Therefore, it can be noted that at a specific energy, the reduction in population improved significantly with an increase in the treatment time. According to our results, there was also a significant ($P < 0.05$) increase in the reduction of population along with an increase in the voltage input for the same number of pulse. After 10 mins of pulsed light treatment, a log reduction of 3.74, 5.36 and 6.14 were obtained by 500, 750 and 1,000 V, respectively. Marquenie et al. (2003) also reported a maximal inactivation of 3 and 4 log units for conidia of *B. cinerea* and *M. fructigena* in vitro after pulse UV treatment of 120 s at the fluence of 0.10 J cm^{-2} . The treated fungal populations of *Aspergillus niger* and *Fusarium culmorum* were reduced by 3 to 4.5 log orders after 1,000 light pulses of the 3 J UV intensity light (Anderson et al., 2000). The maximal log reduction was close to 1 with a pulsed UV- light fluence of 1.2 Jcm^{-2} for *Aspergillus niger* inoculated in sugar syrup (Chaine and Levy et al., 2012); however, on agar, the log reduction for the same fungi was even lower than 1 for the same fluence.

Taking into account that solid agar used in the experiments was unnaturally contaminated, the initial number of mold spores (determined for the control samples) was higher than in normal conditions. Thus, it could be expected that, it is possible to achieve a complete decontamination by 6.14 log reduction (the highest mold reduction achieved in this experiment). Therefore, the results of this report shows that pulsed light can be considered as a promising techniques for fungi elimination or decontamination in the industry.

Over a 10 min treatment period, temperature in the treatment chamber increased from the initial 25°C to 27, 33 and 35°C when 500, 750 and 1,000 V, respectively were applied. Species of *Penicillium* have an optimum temperature in the range of 25 to 35°C (Food and Drug Administration, 2001). Therefore, the temperature as a result of pulsed UV treatment will not inactivate *Penicillium roqueforti* used in this study. Therefore, temperature in pulsed UV-light units was not of a major concern regarding the possible risk of detrimental effect on the quality of bread or pizza and hence would not compromise the integrity of this value-added product.

6.4.2 Inactivation kinetics model

Comparison of the predicted and experimental inactivation data is shown in Figure 6.1. From the inactivation curves, it can simply be observed that none of the inactivation curves (at different voltages) exhibit a linear trend. As indicated by the results, the Weibull model was found to more accurately estimate the microbial reductions obtained during pulsed UV light treatments. Bialka et al. (2008) also reported that first-order kinetics are not suitable for the estimation of microbial inactivation on berries treated with ozone or pulsed UV-light, but that the Weibull model can be successfully used to estimate the reductions of *E. coli* O157:H7 and *Salmonella enterica* on raspberries and strawberries. A modified Weibull model was chosen by Levy et al. (2012) to fit microbial reduction curves for *Bacillus subtilis*, *B. atrophaeus*, *B. cereus*, *Geobacillus stearothermophilus*, and *Aspergillus niger* treated by pulsed UV-light on solid agar. Koseki and Yamamoto (2007) showed that a linear model was not suitable to describe reduction of *E. coli* during high pressure processing. Their findings also demonstrate that the Weibull model as well as the modified Baranyi model could be used to accurately estimate this inactivation and were capable of fitting the tail of the survivor curve resulting from high pressure processing. The ability of this model to accurately estimate reductions of *P. roqueforti* can be seen in Figure 6.1.

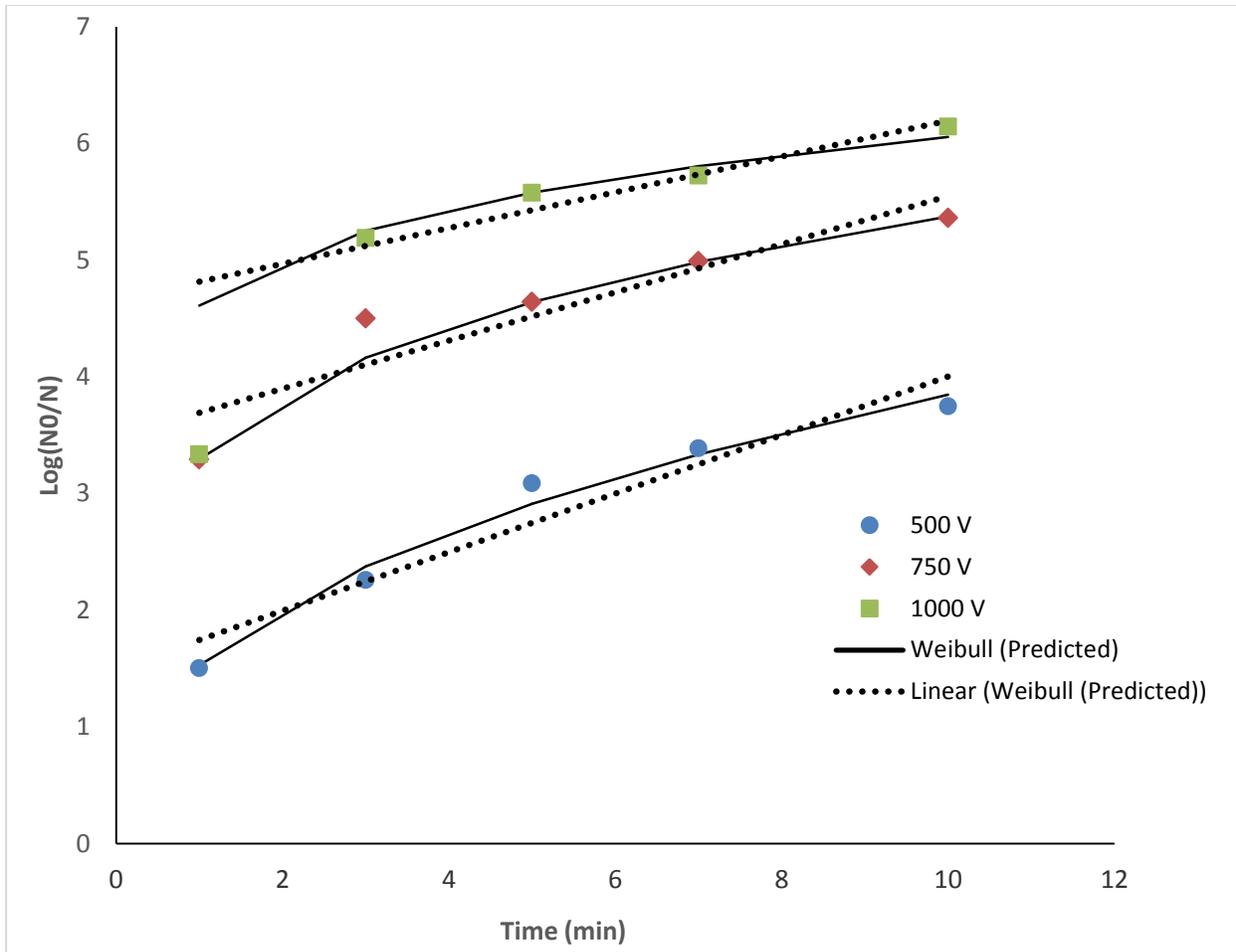


Figure 6.1 Inactivation of *Penicillium roqueforti* with pulsed UV light fitted with the first order and Weibull kinetics models. Inactivation was performed on solid agar at three different voltage inputs (500, 750 and 1,000 V) and five different treatment times (1, 3, 5, 7 and 10 mins). Data shown are mean and standard deviation values.

Goodness-of-fit parameters of log-linear and Weibull models for pulsed UV-light treatment are shown in Table 6.1 and 6.2. These parameters provide more insight into the shapes of the inactivation curves. Overall, the RMSE and R^2 values obtained for the Weibull model are less than and greater, respectively, than those obtained for the log-linear model with the exception of 750 V due to the existence of an outlier in the results. The scale parameter α is usually considered as a measure of the organism resistance (kinetic parameter) to treatment and decreases with exposure time. This value was found to be decreasing when voltage increased. For the three voltages, β values were less than 1, which accounts for curve's upward concavity, but also indicating that the remaining cells are less susceptible to pulsed UV-light, perhaps due to

lack of pulsed light penetrability. A trend can also be seen in the dose required for 1 log reduction (t_R). Values for t_R decrease when higher voltage is applied which indicates that the cells are inactivated quickly with higher voltages.

Table 6.1 Goodness-of-fit parameters of the first order kinetics models estimating reductions of *P. roqueforti* on solid agar after treatment with pulsed UV- light.

Voltage	RMSE	R ²	K
500 V	0.29317	0.9219	0.2497
750 V	0.7281	0.7296	0.2965
1,000 V	0.13248	0.9584	0.9584

Table 6.2 Goodness-of-fit parameters of Weibull models estimating reductions of *P. roqueforti* on solid agar after treatment with pulsed UV- light.

Voltage	α	β	t_R	RMSE	R ²
500 V	0.04326	0.40072	0.34687	0.1374	0.9828
750 V	0.0026	0.30359	0.04063	0.8163	0.6601
1,000 V	2.20E-09	0.11851	2.51E-06	0.0824	0.9839

6.5 Conclusion

In conclusion, by considering the impact of individual pulsed UV treatment's parameters and their interaction on inactivation of *P. roqueforti*, the most effective treatment conditions for inactivating this mold was 1,000 V for 10 min. In fact, pulsed UV light irradiation was very effective at inactivating the spores of *P. roqueforti*; however, the dose of energy per pulse and the position of xenon lamp will determine the lethality. Moreover, decontamination by pulsed UV-light has to account for possible interactions between the target surface and the microorganism. Therefore, further research on the influence of pulsed light on foods with more complex composition is needed to define the applicability of the technology in the decontamination processes. The experimental data also suggest that inactivation of *P. roqueforti*

by pulsed UV light does not appear to follow first-order kinetics. On the other hand, the inactivation kinetics of the tested microorganisms on solid agar was best described by Weibull model with the smallest RMSE and $R^2 \geq 0.92$.

6.6 REFERENCES

- Anderson, J. G., Rowan, N. J., Macgregor, S. J., Fouracre, R. A., Farish, O., & Member, S. (2000). Inactivation of Food-Borne Enteropathogenic Bacteria and Spoilage Fungi Using Pulsed-Light, 28(1), 83–88.
- Begum, M., Hocking, A. D., & Miskelly, D. (2009). Inactivation of food spoilage fungi by ultra violet (UVC) irradiation. *International journal of food microbiology*, 129(1), 74–7.
- Ben Saïd, N. E., Federighi, M., Bakhrouf, A., & Orange, N. (2010). Effectiveness of pulsed ultraviolet-light treatment for bacterial inactivation on agar surface and liquid medium. *Foodborne pathogens and disease*, 7(11), 1401–6.
- Bialka, K. L., Demirci, A., & Puri, V. M. (2008). Modeling the inactivation of *Escherichia coli* O157:H7 and *Salmonella enterica* on raspberries and strawberries resulting from exposure to ozone or pulsed UV-light. *Journal of Food Engineering*, 85(3), 444–449.
- Chaine, A., Levy, C., Lacour, B., Riedel, C., & Carlin, F. (2012). Decontamination of sugar syrup by pulsed light. *Journal of food protection*, 75(5), 913–7.
- CFSAN-FDA. 2000. Kinetics of microbial inactivation for alternative food processing technologies: Pulsed light technology. Atlanta, GA: Center for Food Safety and Applied Nutrition – Food and Drug Administration. Available at: <http://vm.cfsan.fda.gov/~comm/ift-puls.html>. Accessed 19 April 2006.
- EPA (Environmental Protection Agency). 2003. Appendix J. Pilot-scale and demonstration-scale testing. J-4, J-5. Ultraviolet Disinfection Guidance Manual. Washington, DC.: U.S. Environmental Protection Agency.
- Farkas, J. (2007). Physical methods of food preservation. *Food microbiology: fundamentals and frontiers*, 685-712.
- Hosseini, S., Azar-Daryany, M., Massudi, R., & Elikaei, a. (2011). Pulsed UV laser light on *Escherichia coli* and *Saccharomyces cerevisiae* suspended in non-alcoholic beer. *Iranian journal of microbiology*, 3(1), 31–5.
- Jun, S., Irudayaraj, J., Demirci, A., & Geiser, D. (2003). Pulsed UV-light treatment of corn meal for inactivation of *Aspergillus niger* spores, (2000), 883–888.

- Koseki, S., & Yamamoto, K. (2007). A novel approach to predicting microbial inactivation kinetics during high pressure processing. *International Journal of Food Microbiology*, 116, 275–282.
- Levy, C., Aubert, X., Lacour, B., & Carlin, F. (2012). Relevant factors affecting microbial surface decontamination by pulsed light. *International journal of food microbiology*, 152(3), 168–74.
- Marquenie, D., Michiels, C.W., Van Impe, J.F., Schrevens, E. & Nicolai, B.N. (2003). Pulsed white light in combination with UV-C & heat to reduce storage rot of strawberry. *ostharv Biology and Technology*, 28, 455– 461.
- Mcdonald, K. F., Curry, R. D., Clevenger, T. E., Unklesbay, K., Eisenstark, A., Golden, J., Morgan, R. D. (2000). A comparison of Pulsed and continuous ultraviolet light sources for the decontamination of surfaces, 28(5), 1581–1587.
- Takeshita, K. (2003). Damage of yeast cells induced by pulsed light irradiation. *International Journal of Food Microbiology*, 85(1-2), 151–158.
- Tiwari, K. L., Jadhav, S. K., & Kumar, A. (2011). Morphological and molecular study of different penicillium species, 7(2), 203–210.
- Turtoi, M., & Nicolau, a. (2007). Intense light pulse treatment as alternative method for mould spores destruction on paper–polyethylene packaging material. *Journal of Food Engineering*, 83(1), 47–53.
- Unluturk, S., Atilgan, M. R., Baysal, a H., & Unluturk, M. S. (2010). Modeling inactivation kinetics of liquid egg white exposed to UV-C irradiation. *International journal of food microbiology*, 142(3), 341–7.
- Van Boekel, M.A.J.S., 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology* 74, 139–159

CHAPTER 7

ANALYSIS OF FUNGAL DIVERSITY IN PRE-FORMED PIZZA BY SEQUENCING OF CLONED PCR-AMPLIFIED GENES ENCODING 18S rRNA

7.1 Abstract

Fungal contamination of food is a serious concern, both from a food safety and food trade standpoint. Thus, the identification and characterization of food spoilage but fungi and other microorganisms would be desirable in order to apply early intervention steps to limit the amounts of contaminated materials, particularly in cereal-based industries. In the present study, culture-dependent and independent methods were applied to study the ecology of preformed pizza produced in a pizzeria in Montreal. Classical microbiological analyses were performed to identify the main microbial groups present in the product. The average population of mesophilic aerobic bacteria (MAB), mesophilic anaerobic bacteria (MANB), lactic acid bacteria (LAB), molds and yeasts (M+Y) were 6.7 ± 0.5 , less than 2.3, 2.8 ± 0.6 and 5.4 ± 0.4 log CFU g⁻¹, respectively. Fungal diversity was analyzed by extracting DNA from naturally spoiled preformed pizza samples. Molecular methods including conventional PCR targeting the 18S rRNA gene of fungi, TA cloning of PCR-amplified fragments and sequencing followed by BLAST analysis were carried out to detect spoilage fungi in naturally spoiled pre-formed pizza. The cloning approach enabled the putative identification of *Saccharomyces cerevisiae*, *Saccharomyces sp. WW-W23*, *Penicillium expansum*, *Penicillium freii*, *Penicillium sp. HSL*, *Penicillium sp. ljg1*, *Rhodotorula mucilaginosa*, *Monascus fuliginosus*, *Hordeum jubatum*, *Galactomyces geotrichum* strains as well as uncultured fungus and uncultured eukaryote clones.

7.2 Introduction

Today, an important part of a balanced diet includes bakery products. This industry has been increasing and varying its production and pizza has become one of the most widely consumed fast food in the world. It has become popular because it can be sold ready to use, frozen, chilled or pre-cooked, is cheap and easy to prepare. Nevertheless, physical, chemical and microbial spoilage are of a concern in most cereal-based products including pizza. Spoilage

caused by fungi is considered as the dominant shelf life limiting factor and the major cause of economic losses in the bakery industry. In baked products such as pre-cooked pizza dough, the fungi may be inactivated during baking; however, they can contaminate the food again after processing (Pinho et al., 2000). Considering the above mentioned information, three types of fungi that are frequently found in pre-cooked pizza dough belong to the *Penicillium* and *Aspergillus* genera (Pinho et al., 2000). Numerous studies suggest that only a small fraction of microorganisms can be grown under typical laboratory conditions (Dorigo et al., 2005). Thus, culture-dependent methods do not necessarily provide reliable information about the targeted microbial communities (Palomba and Pepe, 2010). In recent years, a lot of attention has been paid to the use of molecular methods for the detection, quantification, identification and characterization of microorganisms in foods. These methods are currently considered as an indispensable tool that permits the accurate description of the microbial ecology of food (De Vuyst et al., 2007).

Polymerase Chain Reaction (PCR) using domain or taxon-specific probes in combination with separation techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and molecular cloning have revolutionized our understanding of the composition of food samples. The cloning and sequencing of rRNA genes have been used for the study of food microbiology for detection, identification and characterization of potential spoilage microorganisms. These methods are robust, reproducible and provide valuable information about microbial taxonomy (Diez et al., 2001). Although direct cloning has been used to characterize the microbial composition of several environments, few published studies have applied this approach to analysing food products. The aim of the present study was to analyze the diversity and composition of the fungal community in pre-formed pizza using complementary culture-dependent and culture-independent techniques.

7.3 Materials and methods

7.3.1 Sampling

The spoiled pre-cooked pizza samples were acquired from a pizza-manufacturing company (Les Aliments Rustica) in the city of Montreal QC, Canada. Collected samples had expiration dates of 21 days, according to the manufacturer. Sample collection was performed in the summer of 2013.

7.3.2 Preparation of pizza samples for microbiological analysis

All of the pizzas were aseptically removed from the package and weighed. A 25 g amount of the sample was then placed in a sterile stomacher bag containing 250 ml of buffered peptone water (in the proportion with pizza samples at 10:1). The mixture was then blended in a Stomacher 400 Lab Blender (Seward medical, London, UK) for 2 min or until homogenized. From the resulting mixture, serial decimal dilutions were conducted and the dilutions were spread-plated in triplicate onto different selective media (as mentioned in the next paragraphs). Results of all the microbial enumerations are expressed in CFU g⁻¹.

7.3.3 Enumeration of total bacteria

To determine the total population of mesophilic aerobic bacteria (MAB) and mesophilic anaerobic bacteria (MANB), Petri plates containing plate count agar (PCA, Oxoid, Hampshire, UK) were prepared. Plates were incubated at 35°C for 48 h either in aerobic condition or in anaerobic condition in a Forma anaerobic system, model 1025/1029 (Thermo Scientific, Marietta, Ohio, USA) (FDA, 1992). Colony Forming Units (CFU) was counted with a dark-field Quebec colony counter (Reichert technologies, Depew, NY, USA).

7.3.4 Enumeration of lactic acid bacteria

Anaerobic lactic acid bacteria (ANLAB) were enumerated by plating on de Man Rogosa Sharpe agar (MRS, Oxoid, Hampshire, UK), a selective medium acidified to pH 5.4 with tartaric acid, and incubating in a Forma anaerobic system at 37°C for 72 h (Terzaghi and Sandine, 1975). Aerobic lactic acid bacteria (ALAB) were enumerated by plating on M17 agar (Oxoid, Hampshire, UK) and incubating in aerobic conditions at 37°C for 48-72 h.

7.3.5 Identification of lactic acid bacteria

Fermentation of carbohydrates was determined using the API 50 CH strips and API 50 CHL medium (API system, Biomérieux, NC, USA). This is a standardized system consisting of 50 biochemical tests for the study of carbohydrate metabolism by microorganisms (Sana and

Hamdi, 2007). According to the manufacturer's instructions, API 50 CH along with API 50 CHL medium could be used for the identification of *Lactobacillus* and related genera (Ghanbari et al, 2009). To assure the purity of the isolates, well-isolated colonies were streaked onto MRS agar and incubated anaerobically for 24 h at 37°. Bacteria were transferred to API 50 CHL medium (5 ml) using a sterile swab in order to prepare heavy bacterial suspensions (S). Subsequently, a suspension with a turbidity equivalent to 2 McFarland was prepared by transferring a certain number of drops of suspension (S) into the ampoule provided by the manufacturer. The concentration was determined by spectrophotometer. 10 ml of pure water was dispensed into the incubation box with the strip placed in the incubation box. The wells of the API strips were filled to the required level with the bacterial suspension and topped off with mineral oil. The API strips were subsequently incubated at 37°C for 48 h.

7.3.6 Enumeration of molds and yeasts

To estimate the total number of molds and yeasts present, triplicate plates were poured with Potato Dextrose agar (PDA, Difco, spark, MD, USA). Tartaric acid was filtered by 0.22µ fisher band filter. The filter sterilized tartaric acid was added to the melted agar to reach the pH of 5.6. The plates were incubated at 24°C for 3-5 days, and then colonies were counted using a Quebec Colony Counter (Reichert technologies, Depew, NY, USA).

7.3.7 Extraction of nucleic acids

Bags containing spoiled samples of preformed pizza were aseptically opened and 1 gram of each sample (taken from a spoiled part of the sample) was weighed and placed into a sterile stomacher bag. Sterile 0.1% peptone water (1 ml) was added to each sample and the contents were homogenised in a Stomacher 400 Lab Blender (Seward Medical, London, UK) for 2 min. DNA was extracted using a Power Food Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA for the isolation of genomic DNA from food). According to manufacturer's instructions, in brief, approximately 1.8 ml of each homogenized food mixture was centrifuged at 13,000 rpm for 5 min at room temperature two times. Pellets were re-suspended in a lysing reagent that includes a detergent to break cell walls and remove non-DNA

organic and inorganic materials. Microbial cells were lysed using a combination of chemical and mechanical lysis conditions to increase yield for difficult cells. The process was followed by adding high concentrated salt solution to the mix, to allow binding of DNA to silica, but not non-DNA organic and inorganic materials that may still be present at low levels. The isolated DNA was finally diluted in an alcohol based wash solution used to further clean the DNA from residual salt and other contaminants. The eluted DNA, which did not contain EDTA, was stored at -20°C as recommended by manufacturer.

7.3.8 Quantification and purity of DNA

DNA concentration and purity of each extract were analyzed using a Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Yield of extraction were measured by following parameters; concentration (ng/ μ l), $A_{260/280}$ (absorbance at 260 nm / absorbance at 280 nm) and the $A_{260/230}$ ratio (absorbance at 260 nm / absorbance at 230 nm).

7.3.9 Verification of DNA quality

Extracted DNA was run on a 1% agarose gel for 2 h with voltage input of 55V, to verify its quality (shearing). The aliquot of 5 μ l of each extracted DNA were loaded on the gel and the electrophoresis was performed in 1X TAE buffer. Gels were stained with ethidium bromide for 20 min, destained in water for 5 min and viewed using a Red AlphaImager (Proteinsimple, Santa Clara, CA, USA). The size of the products was estimated using a λ DNA GeneRuler100-bp DNA ladder (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). The scale proposed by Lemarchand et al. (2005) was used to evaluate the degradation degree of the DNA in each extract; 1 = low (mean fragment size between 23 and 2 kb); 2 = medium (mean fragment size between 23 and 0.5 kb); 3 = high (mean fragment size between 23 and <0.5 kb) (Pakpour, 2012).

7.3.10 Polymerase chain reaction amplification

Fungal 18S rRNA genes were amplified from extracted genomic DNA using PCR primer sets A and B (Table 7.1). The intensity of the amplicons generated by each primer pair was compared by gel electrophoresis and staining with ethidium bromide, as described previously. Primer set C

was used for the sequencing of cloned fragments. All primers were obtained from Integrated DNA Technologies (Toronto, ON, Canada) and the PCR reactions were prepared in an Esco® PCR laminar flow cabinet (Esco Technologies Inc., PA, USA). PCR reaction mixtures and thermal cycling conditions were as described in Wu and Blomquist (2002) and the next paragraph. In order to determine the conditions giving the highest specificity, the annealing temperatures were optimized by temperature gradient PCR. Intensity and presence of only one amplicon were the criteria to determine the optimal temperature. *Rhizoctonia solani* as positive control and a negative control consisting of the reaction mixture without DNA, were used in each PCR run.

Table 7.1 Overview of the oligonucleotide sequences used in the study.

Primer set *	Primer name	Sequence (5' → 3')	Specificity	References
A	NS1f	GTAGTCATATGCTTGTCTC	Universal for fungi	(Wu et al., 2002)
	NS4r	CTTCCGTCAATTCCTTTAAG	Universal for fungi	(Wu et al., 2002)
B	NS1f	GTAGTCATATGCTTGTCTC	Universal for fungi	(Wu et al., 2002)
	NS6r	GCATCACAGACCTGTTATTGCCTC	Universal for fungi	(Wu et al., 2002)
C	M13 f	GTAAAACGACGGCCAG	Plasmid linker region	(Messing et al., 1982)
	M13 r	CAGGAAACAGCTATGAC	Plasmid linker region	(Messing et al., 1982)

*: Primer sets A and B were used for the generation of PCR products for TA cloning. Primer set C was used for the sequencing of PCR products cloned with the Easy T vector system.

7.3.11 Temperature gradient PCR assay for primer pairs A and B

The extracted genomic DNA served as a template for amplification of 1,100 and 1,400 bp fragments of the 18S rRNA gene by using NS1f-NS4r and NS1f-NS6r (universal fungal primers for 18S), respectively. In brief, all PCR amplifications were performed in a total volume of 50 ul containing 1-5 ng of template DNA extracted from the spoiled samples, 1 X buffer (Denville Scientific, Saint-Laurent, QC, Canada), 500 µM of each primer (Integrated DNA Technologies, Toronto, ON, Canada), 1.5 U of Hot Start *Taq* DNA polymerase (Denville Scientific, Saint-

Laurent, QC, Canada), 200 mM of each dNTP (Bioshop Canada Inc., Burlington, ON, Canada) and molecular grade water (Sigma-Aldrich, Oakville, ON, Canada) to reach a total volume of 50 μ l (Wu et al., 2002). PCR amplifications were conducted using a Veriti Thermocycler (Applied Biosystems, Foster City, CA, USA) with the following temperature cycling profile: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 50, 52, 54, 56, 58, 60°C for 50 sec, and extension at 72°C for 90 sec, followed by 5 min at 72°C for the final extension. Aliquots (13 μ l) of the amplification products were examined by electrophoresis on 1.5% agarose gels and ethidium bromide staining. A GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific Inc.) was used to estimate the size of the products.

7.3.12 PCR on spoiled samples

PCR on spoiled samples were carried on by using primer set A and B with the optimized annealing temperature of 50°C and 52°C respectively. The PCR reaction mixture and cycling profile were identical to those of temperature gradient PCR.

7.3.13 Purification of PCR products

The PCR products were purified using a SpinSmart PCR purification kit (Denville Scientific Inc., Metuchen, NJ, USA). The samples were processed according to the protocol supplied by the manufacturer. The purification is based on the following principle: DNA was first absorbed to the silica-membrane of the spin column in the presence of high salt concentration, whereas the contaminants passed through during centrifugation. Salts were resolved and then washed away with an ethanol-containing buffer. Afterwards, DNA was eluted with 15 to 50 μ l of elution buffer. The concentration and quality of the purified PCR products were determined with a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific), as described hereinabove.

7.3.13 Cloning

Cloning was performed using a TA cloning kit pGEM-T Easy vector system A1380 (Promega, Madison WI, USA) as described by the manufacturer. In brief, 1 to 3 μ l of PCR products were ligated as specified in the kit's protocol (Table 7.2). PCR amplicons were cloned

into a pGEM[®]-T Easy vector (3,015 bp) provided with protruding thymidines at the cloning site, taking advantage of the adenosinic overhangs left by the *Taq* DNA polymerase at the 3' termini of the DNA. The vector molar ratio is shown in Table 7.2. The ligation reaction was carried out at room temperature for two hours. The ligation mixture was subsequently transformed into high efficiency competent cells of *Escherichia coli* (JM109). After 20 mins of incubation on ice, cells were heat shocked for 60 sec at 42°C and then incubated for an additional 150 mins in S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose (Fisher Scientific, NY, USA) at 37°C under shaking condition (150 rpm). After incubation, small volumes (100 to 200 µl) were spread on pre-warmed LB agar plates (Luria-Bertani, Difco, spark, MD, USA) 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 15% agar, pH 7.0, ampicillin with a final concentration of 100 µg per ml, IPTG 0.5 nM, X-Gal 80 µg per ml (Fisher Scientific, NY, USA) and cells were allowed to grow overnight at 37°C. Four to six white colonies (positive recombinants) for each sample were selected from each transformation and screened by PCR for the presence of an insert, using primers M13 f and M13 r.

Table 7.2 Cloning conditions

Reagents	Samples							
	1	2	3	5	8	9	11	12
2X Rapid ligation buffer	5ul	5ul	5ul	5ul	5ul	5ul	5ul	5ul
pGEM-T easy Vector (50ng)	1ul	1ul	1ul	1ul	1ul	1ul	1ul	1ul
PCR product	3ul	1ul	1ul	2ul	1ul	3ul	1ul	3ul
T4 DNA legase (3weiss units/ul)	1ul	1ul	1ul	1ul	1ul	1ul	1ul	1ul
Nuclease-free water	0	2ul	2ul	1ul	2 ul	0ul	2ul	0ul
Approximate Vector molar ratio	1-Jan	1-Feb	1-Feb	1-Feb	1-Jan	1-Feb	1-Feb	1-Feb
Sample concentration in cloning reaction	27ng	36ng	37ng	46 ng	39.5ng	18.1ng	53ng	24 ng

Colony PCR was then performed using the vector-specific primers M13f and M13r and a single colony as template. Four to six colonies per sample were used as a template for the PCR. Each PCR reaction contained 1X PCR buffer, 200 nM of each primer (Integrated DNA Technologies, Toronto, ON, Canada), 80 mM dNTPs (Bioshop Canada Inc., Burlington, ON, Canada), 1 U Hot-Start *Taq* DNA polymerase (Denville Scientific, Saint-Laurent, QC, Canada), and molecular grade water (Sigma-Aldrich, Oakville, ON, Canada) in a total volume of 50 μ l. PCR amplifications were conducted in a Veriti Thermocycler (applied Biosystems, CA, USA). The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min, and then 10 min at 72°C for the final extension. Aliquots (13 μ l) of the PCR products were verified for the correct insert length on 1.5% agarose gels and ethidium bromide staining. The size of the products was estimated using a GeneRuler100-bp DNA ladder (Thermo Fisher Scientific Inc.).

7.3.14 Sequencing

Cloned PCR products that contained the desired insert were selected for sequencing. In total, 28 clones were sequenced. Sequencing was performed at the McGill University and Genome Québec Innovation Centre (Montreal, QC, Canada) using a 3730 × 1 DNA Analyzer system (Applied Biosystems, Carlsbad, CA, USA). Sequences were checked for putative chimeras using DECIPHER (Wright et al. 2012) and chimeras were removed before comparing sequences with those in the Ribosomal Database project (RDP; <http://rdp.cme.msu.edu>) and BLASTn (<http://blast.ncbi.nlm.nih.gov>). Sequences were aligned with related sequences in the database using ClustalX version 2.1 (Larkin et al. 2007). Phylogenetic analysis was performed using programs found in the PHYLIP software package version 3.695 (Felsenstein et al., 1995). The aligned sequences were bootstrapped 1,000 times using SEQBOOT and a DNA distance matrix calculated with DNADIST using the Kimura 2-parameter model. Phylogenetic trees were then created using the Fitch-Margoliash method in FITCH, and a consensus tree was constructed using CONSENSE. Trees were displayed using TREEVIEW version 1.6.6.

7.4 Results and discussion

7.4.1 Enumeration of microbial population

In this work, the microbial diversity of 10 spoiled pizza samples was studied. The samples were examined for the presence of mesophilic aerobic bacteria (MAB), mesophilic anaerobic bacteria (MANB), lactic acid bacteria (LAB), molds and yeasts (M+Y) (Table 7.3). MAB varied from 6.5×10^5 to 2.2×10^7 CFU g⁻¹ with 40% of the samples having counts of about 10^6 CFUg⁻¹. MANB counts ranged from less than 10^1 to 2.7×10^2 . These numbers are considerably lower than MANB counts 1.6×10^4 reported by Rodríguez et al. (2003) in study of the shelf life of pre-baked pizza dough. In all preformed pizza samples, counts of LAB ranged between 1.2×10^2 and 1.6×10^4 CFU g⁻¹. This was in agreement with 1.0×10^4 CFU g⁻¹ which was reported as enumerated LAB in the previous study by Rodríguez et al. (2003). However, another study (Ricciardi et al. 2005) showed that LAB counts ranged from 10^7 and 10^8 CFU g⁻¹ in fermented bread. Since LAB is used to produce this fermented product, it is expected to find more LAB counts on the product.

The fungal counts in the current study are higher, ranging from 3.95×10^4 to 9.17×10^5 CFU g⁻¹ (Table 7.3). These counts are comparable with those reported by Rodríguez et al. (2000) which indicated yeasts and molds counts in the range of 3.78 to 6.09 log CFU g⁻¹ in sliced wheat flour bread (with preservative) after 18 days of storage at room temperature. Yeast numbers depend especially on the inoculation ratio of the dough. It was shown by Hammes et al. (2005) that regardless of the inoculum's origin, yeasts in dough may originate from the flour or the other ingredients of the dough and from the bakery environment.

Table 7.3 Microbial populations in spoiled pre-formed pizza. Data are the results of the microbiological analysis of 10 samples.

Samples	Microbial populations (log CFU g ⁻¹)			
	MAB	MANB	LAB	Y+M
1	6.8	1.6	2.5	5.3
2	7.1	<1	3.0	5.2
3	6.1	<1	2.1	4.9
4	6.0	1.8	2.1	4.6
5	7.4	2.4	2.9	5.2
1	5.9	<1	2.1	5.4
2	6.8	<1	4.2	5.6
3	5.8	<1	2.9	6.0
4	6.5	1.8	3.0	5.9
5	7.2	1.2	2.71	5.8
Average	6.6±0.5		2.8±0.6	5.4±0.4

Mean values of the CFU g⁻¹ for each sample, analyzed in triplicate, are reported. Refer to materials and methods for media specifications and incubation conditions.

7.4.2 Identification of LAB isolates

Isolates of LAB from spoiled pizza samples were identified based on the fermentation of carbohydrates. Identification tables were prepared as (+/-) according to colour change in evaluation of results of API strips reactions. Numerical profiles of strains were identified by adding positive values in indicative table. The biochemical profile obtained for each isolate was determined using the APIWEB identification software and database Smith et al. (1994) reported that LAB, particularly *Leuconostoc mesenteroides*, were responsible for the spoilage of crumpets

packed in CO₂:N₂ (3:2). In the present study, *Lactobacillus plantarum* and *Pediococcus acidilactici* were found as the dominant LAB strains in 10 samples (Table 7.4). (Robert, Gabriel, & Fontagné-Faucher, 2009) also found these two species together with other LAB when they studied the biodiversity of lactic acid bacteria in French wheat sourdough. (V. Rodríguez et al., 2003) identified the following genera in prebaked pizza dough: *Leuconostoc* (*L. mesenteroides*), *Pediococcus* and *Lactobacillus*. Indeed, several factors including the degree of dough hydration, the type of cereal used and the leavening temperature result in the vast variability in the numbers and types of species found in bakery products (De Vincenzi et al., 1994). In addition, factors resulting from substrates present in the cereal fraction and from endogenous and microbial enzymes add to this unpredictability (De Vuyst and Neysens, 2005).

Table 7.4 Identification of LAB by biomedical analysis. Biomedical profile was compared with the closest relatives in the APIWEB database. One replicate per sample were used.

Sample NO.	Closest match	Confidence level (%)
1	<i>Lactobacillus plantarum</i>	98
2	<i>Lactobacillus plantarum</i>	94
3	<i>Pediococcus acidilactici</i>	96
4	<i>Lactobacillus plantarum</i>	94
5	<i>Pediococcus acidilactici</i>	96
6	<i>Pediococcus acidilactici</i>	90
7	<i>Pediococcus acidilactici</i>	94
8	<i>Pediococcus acidilactici</i>	90
9	<i>Pediococcus acidilactici</i>	88
10	<i>Pediococcus acidilactici</i>	88

7.4.3 Concentration and purity of the isolated DNA after Extraction

The DNA concentration and purity of each extract DNA from spoiled samples are reported in Table 7.4. The A_{260/280} ratio (absorbance at 260 nm / absorbance at 280 nm) and the A_{260/230} ratio (absorbance at 260 nm / absorbance at 230 nm) are used to evaluate the purity of DNA extracts. An A_{260/280} ratio >1.8 and A_{260/230} ratio between 2.0 and 2.2 is recognized as an indication of the purity of DNA solutions and extracts. When the A_{260/280} ratio is lower than 1.8 and the A_{260/230} ratio is lower than 2, proteins, carbohydrates, phenol and other contaminants that absorb at or near 280 or 230 nm may be present (Pakpour, 2012). According to this information,

purity of sample number 3, 5, 6, 7, 9, 10, 11, 12 are acceptable, however sample number 1, 2, 4, 8 might have some impurities and contaminants. Overall, concentration of the samples is low and needed to be increased through purification step.

Table 7.5 Concentration and purity of extracted DNA from spoiled samples

Sample number	Concentration (ng/μl)	A_{260/280} ratio	A_{260/230} ratio
1	11.3	1.68	0.31
2	10.4	1.58	0.06
3	10.3	1.92	0.65
4	25.5	1.49	0.48
5	14.7	2.04	0.50
6	4.7	2.08	0.36
7	6.2	2.03	0.06
8	7.3	1.56	0.39
9	9.4	2.54	0.76
10	9.1	2.23	0.05
11	4.6	2.15	1.49
12	2.9	2.6	0.2

Extracted DNA was subsequently verified for their quality and shearing by running on agarose gel electrophoresis. The result is not shown as the gel only contained dimers; it could have been because of low concentrations of DNA as it is shown in Table 7.5.

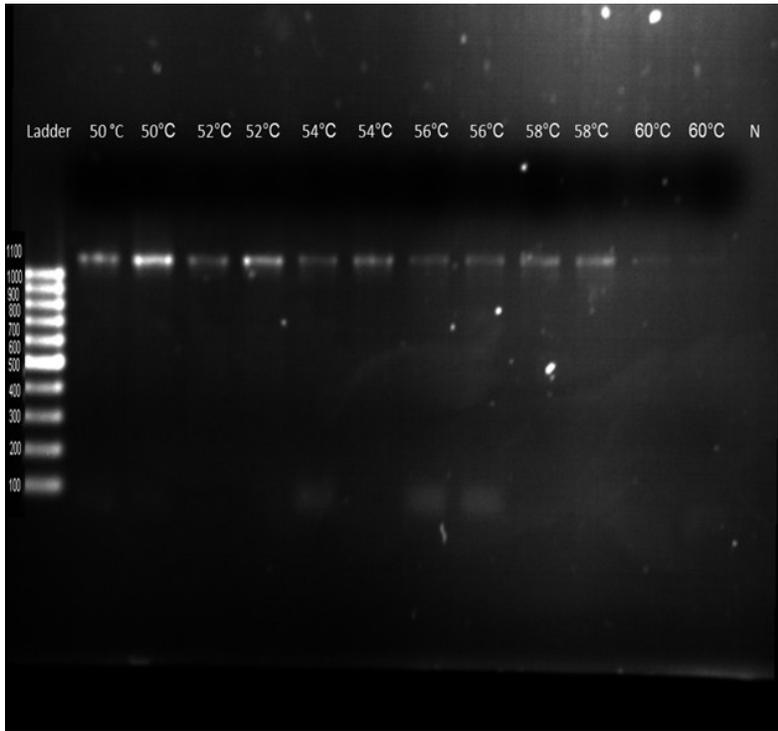
7.4.4 Temperature gradient PCR

Temperature gradient PCR assays were performed to determine the proper annealing temperature for primer sets A and B. Results illustrated that the optimal annealing temperatures are 50 and 58°C for primer set A and B, respectively, based on the intensity of the DNA bands and presence of only one amplicon obtained in these temperatures (Figure 7.1).

7.4.5 Conventional PCR on spoiled samples

Fungal 18S rDNA was amplified from extracted genomic DNA (from spoiled pizza samples) using PCR primer sets A and B. Since primer set B generated a more intense band when stained with ethidium bromide, it was selected for the rest of the PCR reactions in the present experiment (Figure 7.1). Due to the fact that no product was obtained for the samples number 4, 6, 7 and 10, these samples were excluded from the remaining part of the experiment (Figure 7.2).

A



B

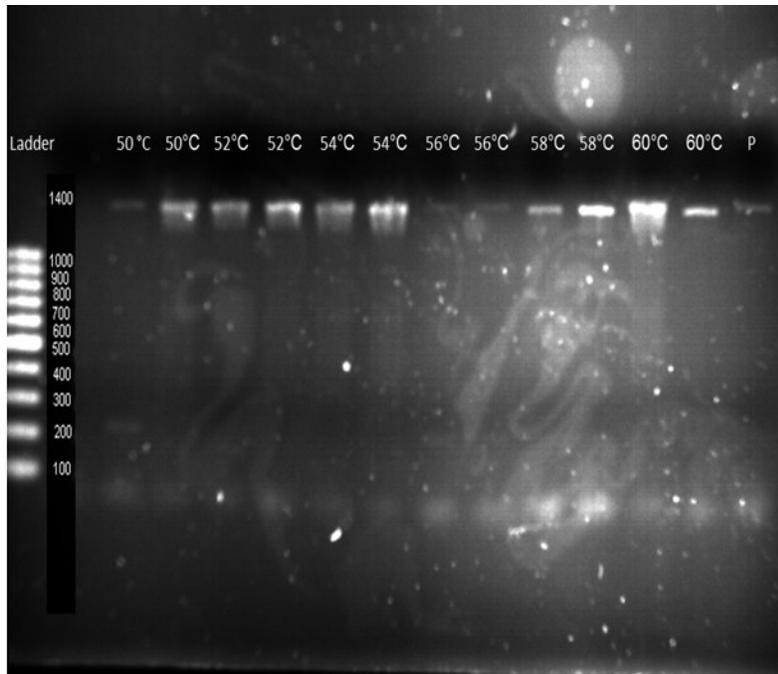


Figure 7.1 Temperature Gradient PCR for by perimer set A (A) and primer set B (B). Two replicates per temperature are used. Ladder shows 100 to 1,000 bp.

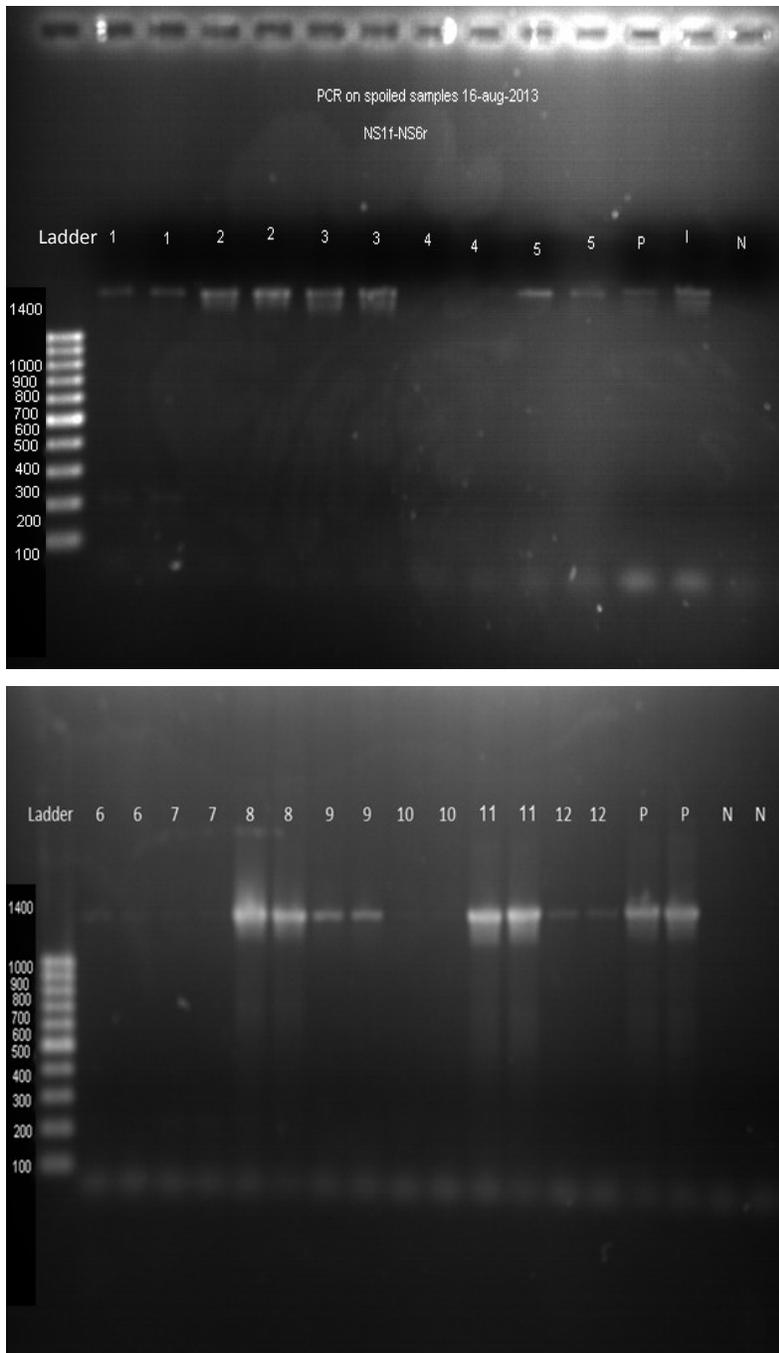


Figure 7.2 PCR performed on spoiled samples using primer set B with 58°C annealing temperature. Two replicates per temperature are used. Ladder shows 100 to 1,000 bp.

7.4.6 Concentration and purity of PCR products

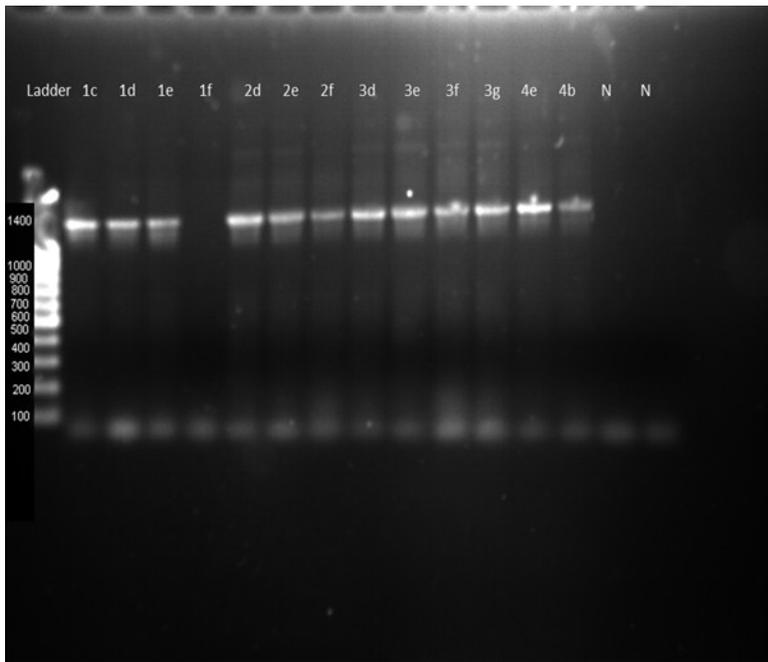
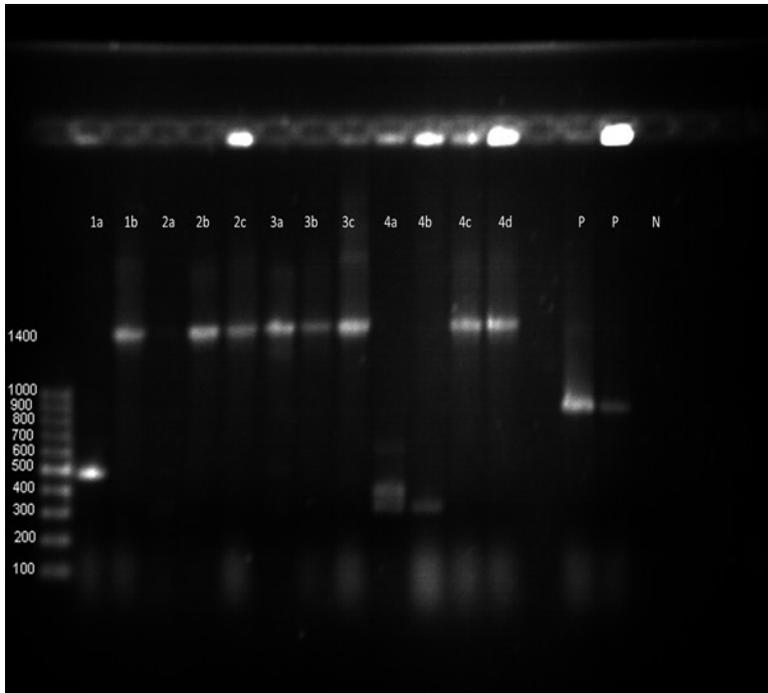
The PCR products were purified and the concentration of the isolated DNA after purification is shown in Table 7.6. As it was mentioned before, the concentration (ng/μl), $A_{260/280}$ ratio and the $A_{260/230}$ ratio are used to evaluate the purity of PCR products. An $A_{260/280}$ ratio >1.8 and $A_{260/230}$ ratio between 2.0 and 2.2 is recognized as an indication of the purity of DNA solutions and extracts. When the $A_{260/280}$ ratio is lower than 1.8 and the $A_{260/230}$ ratio is lower than 2, proteins, carbohydrates, phenol and other contaminants that absorb at or near 280 or 230 nm may be present (Pakpour, 2012). According to this information, almost all of the samples in Table 7.6 shows an acceptable purity based on $A_{260/280}$ ratio.

Table 7.6 Concentration and purity of the purified PCR products spoiled samples.

Samples number	Concentration (ng/μl)	$A_{260/280}$ ratio	$A_{260/230}$ ratio
1	9	2.47	0.02
2	36	1.30	0.01
3	37	1.72	0.07
5	23	1.86	0.04
8	39.5	1.93	0.05
9	6.6	1.83	0.07
11	53	1.85	0.45
12	8.1	1.81	0.05

7.4.7 18S rDNA clone libraries

Amplicons from primer set B were cloned into the pGEM-T Easy vector and screened on LB agar with X-gal and IPTG. Four to six clones per sample were screened for the presence of the insert prior to sequencing using the M13 primers. A total of 28 amplified PCR products from these clones were sent for sequencing based on their size (primer set C, Table 7.1).



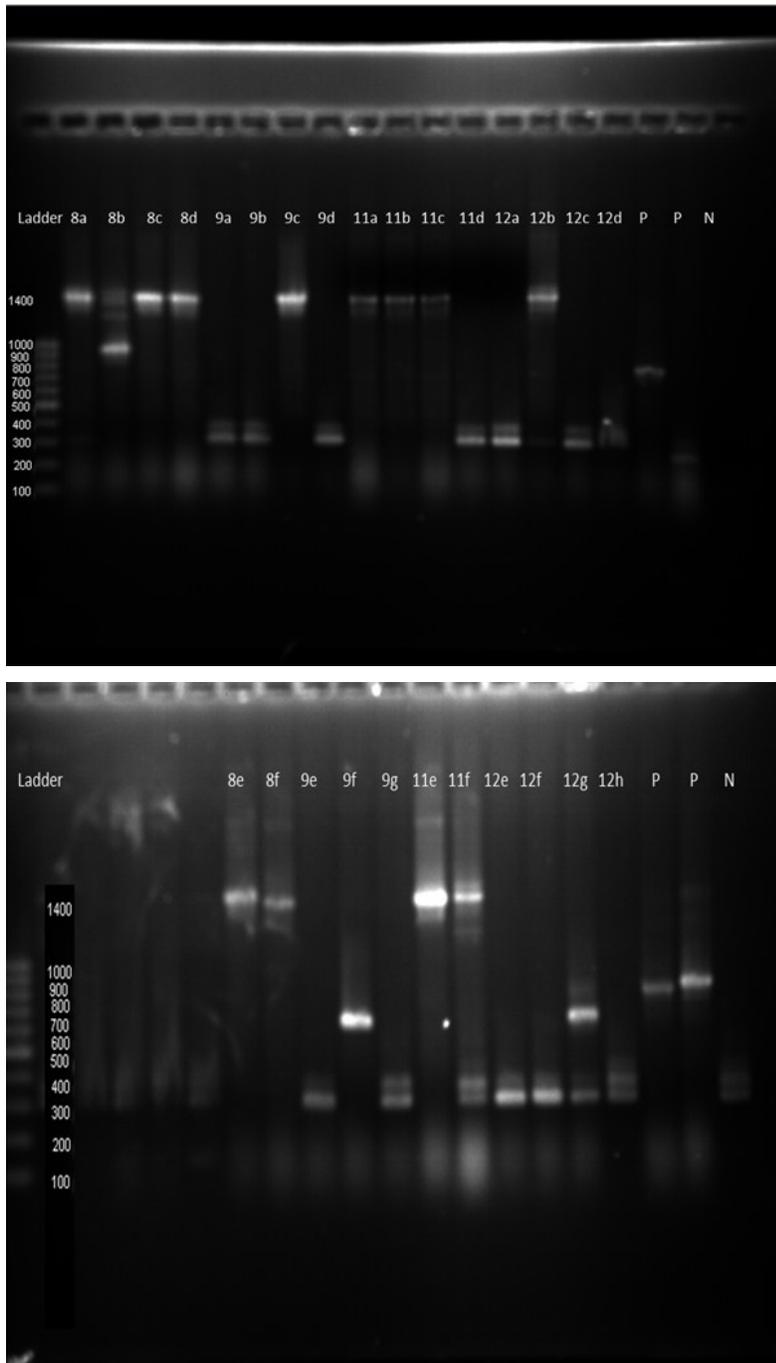


Figure 7.3 Four images show PCR performed after cloning using primer set C. The amplicons with insert of the expected size are equal to 1,400 bp. Bands showing less than 1,000 bp do not have the desired inset. Number of clones are showed as 1, 2, 3... and number of replicates for each clone are showed as a, b, c, d, P = Positive control N = Negative control.

7.4.8 Diversity of fungal biota

To our knowledge, this is the first study to investigate the fungal microbiota in preformed pizza using molecular microbiological analysis. Overall, 28 clones (from samples number 1, 2, 3, 4, 8, 9, 11, 12) with the desired insert were selected and sequenced. Sequencing of these clones revealed that 9 different species of fungi were present, including 12 different strains (Table 7.6). The dominant species in the preformed pizza shared 99–100% similarity with *Penicillium sp.* and *Saccharomyces sp.* Of these 28 clones, 7 were classified as belonging to the *Penicillium* species (sample 2, 8, 9, 11) including *Penicillium expansum*, *Penicillium sp.* HSL, *Penicillium sp.* Ljg. A total of six clones (from samples 2, 8, 11, 12) were identified as either *Saccharomyces cerevisiae* or *Saccharomyces sp.* WW-W23. *Penicillium* is a genus of ascomycetous fungi that can be found on foodstuffs, leather, and fabrics. They are of economic importance in the production of antibiotics (e.g. penicillin), organic acids, and cheeses (Tiwari and Kumar, 2011). However, the common occurrence of *Penicillium* species in food also causes spoilage issues. Some species produce toxins and may render food inedible or even dangerous. (Seiler et al., (1976) reported *Penicillium* spp. such as *P. notatum*, *P. expansum*, and *P. Viridicatum*, were the predominant spoilage molds in bakery products with a high water activity, i.e., >86%. Karin et al. (2005) also indicated that *Penicillium roqueforti* is the major contaminant of rye bread. Rodríguez et al. (2000) investigated the effect of modified atmosphere packaging on the shelf life of sliced wheat flour bread and identified *Penicillium* spp. as the most important spoilage agent in this product.

S. cerevisiae, also known as baker's yeast, has a long history of use in the area of food processing. It has been used for centuries as a leavening agent for bread and as a fermenter of alcoholic beverages. With a prolonged history of industrial applications, this yeast has also been the model for various studies in the principles of microbiology (Anderson, 1992). The presence of *S. cerevisiae* in the product might be due to contamination of the bakery environment with commercial baker's yeast. The investigation of the microbiota of sourdough bread by culture-dependent and culture-independent methods has revealed that the majority of all yeasts isolated from this product is *S. cerevisiae* (Iacumin et al., 2009). *S. cerevisiae* has also been found to be dominant among yeasts in studies of microbial characterization of sourdough for sweet baked products (Palomba et al., 2010).

Rhodotorula mucilaginosa was the third most frequently fungus identified in this study, with 5 clones (samples 1, 2, 11) sharing high identity (100%) with this species (Table 7.6). This microorganism is an anamorphic genus of heterobasidiomycetous yeasts, existing normally in air and soil. *Rhodotorula* species can also be isolated from human skin, stool, food and fruits and produce pink, orange and red pigments (Kreger-Van Rij, 1984). Red yeasts are the predominant yeasts recognized in many studies of food products and are primarily members of the genera *Rhodotorula*, *Rhodosporidium* and *Sporobolomyces* (Hagler et al., 1987). Although most species of the genus *Rhodotorula* are nonpathogenic, some of them are infectious (Mokhtari et al., 2011). *Rhodotorula* is characterized by: no ballistoconidia, no fermentation ability, no starch-like compounds, and no xylose in whole-cell hydrolyzates (Nagahama et al., 2001).

Three colonies from sample 3 shared a similarity of 99-100% with yeast *Monascus fuliginosus*. This microorganism is a species of fungus belonging to the genus *Monascus van Tieghem*. Species of this genus are commercially important in the production of various Asian fermented foods, e.g. red-rice and food-colouring pigments (Steinkraus, 1984). *Monascus fuliginosus van Tieghem* is a cosmopolitan fungus found in soil, cooked potatoes, soya, sorghum, tobacco, rice, oat and silage (Moharram et al., 2011).

A single clone from sample number 1 shared 99% identity with *Hordeum jubatum* (foxtail barley). This plant is a short-lived perennial plant species indigenous to western North America that has become naturalized in eastern North America. It is most prevalent in soils with a high water table and high salinity (Kemuel et al., 1988).

Clone 27 from sample 4 had a high level of identity (99%) with *Galactomyces geotrichum*. *Galactomyces* is an arthroconidial ascomycetous genus (Kurtzman and Robnett, 1998; Ueda-Nishimura et al., 2000). *G. geotrichum* is considered as a plant pathogen and is a common post-harvest fungus disease of citrus known as sour rot (Pimenta et al., 2005).

Table 7.7 Tentative identification of clones by sequencing and BLAST analysis. Sequences were aligned with the closest relatives in the GenBank database with BLAST.

Clone	Sample	Phylum	Closest match	Identity (%)	Accession Number	Sequence Size of the clones
Clone 1	1	Tracheophyta	<i>Hordeum jubatum</i>	99	AF168852	781
Clone 2	2	Ascomycota	<i>Saccharomyces cerevisiae</i>	99	AY99853.1	800
Clone 3	2	Ascomycota	<i>Saccharomyces sp. WW-W23</i>	99	DQ345280.1	563
Clone 4	3	Ascomycota	<i>Monascus fuliginosus</i>	99	HM188430.1	800
Clone 5	3	Ascomycota	<i>Monascus fuliginosus</i>	100	HM188430.1	734
Clone 6	3	Ascomycota	<i>Monascus fuliginosus</i>	100	HM188430.1	561
Clone 7	4		<i>Uncultured fungus clone</i>	99	JN054689.1	561
Clone 8	4		<i>Uncultured fungus clone</i>	99	JN054689.1	849
Clone 9	8	Ascomycota	<i>Penicillium expansum strain</i>	99	JX470345.1	759
Clone 10	8	Ascomycota	<i>Penicillium expansum strain</i>	100	JX470345.1	734
Clone 11	8	Ascomycota	<i>Penicillium freii</i>	99	JX470344.1	816
Clone 12	9	Ascomycota	<i>Penicillium freii</i>	99	JX470344.1	809
Clone 13	11	Ascomycota	<i>Penicillium sp. HSL</i>	99	JX910356.1	745
Clone 14	11	Ascomycota	<i>Penicillium sp. HSL</i>	99	JX910356.1	798
Clone 15	11	Ascomycota	<i>Saccharomyces cerevisiae</i>	100	KF447113.1	808
Clone 16	12	Ascomycota	<i>Saccharomyces cerevisiae</i>	99	KF447113.1	828
Clone 17	8	Ascomycota	<i>Saccharomyces cerevisiae</i>	100	KF447113.1	766
Clone 18	8	Ascomycota	<i>Saccharomyces cerevisiae</i>	99	KF447113.1	797
Clone 19	9		<i>Uncultured fungus clone</i>	99	KC337083	814
Clone 20	11		<i>Uncultured fungus clone</i>	99	KC337083	826
Clone 21	11	Basidiomycota	<i>Rhodotorula mucilaginosa</i>	100	KC186124	803
Clone 22	1	Basidiomycota	<i>Rhodotorula mucilaginosa</i>	100	KC186125	803
Clone 23	1	Basidiomycota	<i>Rhodotorula mucilaginosa</i>	100	KC186126	802
Clone 24	1	Basidiomycota	<i>Rhodotorula mucilaginosa</i>	100	KC186127	803
Clone 25	2	Basidiomycota	<i>Rhodotorula mucilaginosa</i>	100	KC186128	766
Clone 26	2	Ascomycota	<i>Penicillium sp. ljl1 Galactomyces</i>	99	KC833482	691
Clone 27	4	Ascomycota	<i>geotrichum</i>	99	JQ668740	529
Clone 28	4		<i>Uncultured eukaryote</i>	99	JX394808	728

7.5 Conclusion

Based on culture dependant analysis, a scale of the number of colony forming units of observed microorganisms was determined. In decreasing order of size, the scale is as follows: mesophilic aerobic bacteria (MAB), molds and yeasts (M+Y), mesophilic anaerobic bacteria (MANB), and finally lactic acid bacteria (LAB). Based on biochemical analysis, *Lactobacillus plantarum*, *Pediococcus acidilactici* were identified as LAB isolate in the product. Analysis of the clone libraries using cultivation-independent techniques revealed low diverse fungi communities in preformed pizza samples. The microbiota, as revealed by PCR-cloning, was composed of mold affiliated to *Penicillium* genus and yeast affiliated to the *Saccharomyce* and *Rhodotorula* genus. Preformed pizza is a popular food for all categories of people, including children, the elderly and it is considered unsafe for such people when the product with a high count of bacteria is consumed. Beside safety issues, pizza spoilage contributes to the annual loss of bakery product as a staple food around the world. This emphasizes the importance of quality control procedures aimed at reducing spoilage microorganism (particularly mold and yeast) counts in bread. Classical microbiology as well as modern genetic methods, such as PCR-cloning and sequencing can be useful in tracking the spoiling agents within and after the baking process and in understanding the role of different microorganisms in spoilage of the pizza.

7.6 REFERENCES

- Anderson, A. 1992. Yeast genome project: 300,000 and counting.
- Badger, S., & Ungar, I. A. (1989). The effects of salinity and temperature on the germination of the inland halophyte *Hordeum jubatum*, (Ungar 1984).
- De Vincenzi S, Gobbetti M, Corsetti A, Rossi J, La Rosa F (1994). Identification and clustering of LAB and yeasts from wheat sourdoughs of central Italy. *Ital. J. Food Sci.*, 6: 85-94.
- Dorigo, U., Volatier, L., Humbert, J.-F., 2005. Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Research* 39 (11), 2207- 2218.
- De Vuyst L, Vancanneyt M (2007) Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiol* 24:120–127
- Diez B, Pedros-Alio C, Massana R (2001) Study of Genetic Diversity of Eukaryotic Picoplankton in Different Oceanic Regions by Small-Subunit rRNA Gene Cloning and Sequencing. *Applied and Environmental Microbiology* 67:2932- 2941

- De Vuyst L, Vandamme P & Huys G (2007) Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. *Appl Environ Microb* 73: 6262–6269.
- Felsenstein J (1995) Confidence limit on phylogenies: an approach using the bootstrap. University of Washington, Seattle . *Evolution* 39: 783–791
- Ghanbari M, Rezaei M, Jami M, Nazari RM (2009). Isolation and characterization of *Lactobacillus* species from intestinal contents of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*). *Iran. J. Vet. Res.*, 10: 152-157.
- Hammes WP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH & Vogelmann SA (2005) Microbial ecology of cereal fermentations. *Trends Food Sci Tech* 16: 4–11.
- Hagler, A.N. and D.G. Ahearn (1987) Ecology of aquatic yeasts. In: Rose AH and Harrison JS (Eds) *The yeasts*, second edition, vol. 1 (pp. 181–205). Academic Press, London, United Kingdom.
- Iacumin, L., Cecchini, F., Manzano, M., Osualdini, M., Boscolo, D., Orlic, S., & Comi, G. (2009). Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food microbiology*, 26(2), 128–35.
- KARIN I. SUHR. (2005). *Food Microbiology and Safety Inhibition of Fungal Growth on Wheat and Rye Bread by Modified Atmosphere Packaging and Active Packaging Using*, 70(1).
- Kreger-van Rij, N. J. W. (1984). Systems of classification of the yeasts. In: Kreger-van Rij, N. J. W. (ed.). *The Yeasts: A Taxonomic Study Third revised and enlarged edition* Elsevier. Amsterdam
- Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* 73, 331–371.
- Larkin M, Blackshields G, Brown N, Chenna R, McGettigan PA, McWilliam H, et al. *Clustal W and Clustal X version 2.0*. *Bioinformatics* 2007;23:2947-8.
- Lemarchand, K., Berthiaume, F., Maynard, C., Harel, J., Payment, P., Bayardelle, P., Masson, L. and Brousseau, R. (2005) Optimization of microbial DNA extraction and purification from raw wastewater samples for down-stream pathogen detection by microarrays. *Journal of Microbiological Methods*, 63, 115-126
- Messing, J., Vieira, J., Biochemistry, D., Paul, S., & Mn, U. S. A. (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest strategy) DNA phage inserts with controlled orientation restriction fragments multiple cloning sites sequencing The strategy of shotgun cloning with M13 is based on obtaini, 19, 269–276.
- Moharram, A. M., M, E. M., & Ismail, M. A. (2011). Chemical Profile of *Monascus ruber* Strains. *Food Technology and Biotechnology*, Vol.50 No.4 .490- 499

- Mokhtari, M., Etebarian, H. R., Mirhendi, S. H., & Razavi, M. (2011). Identification and phylogeny of some species of the genera *Sporidiobolus* and *Rhodotorula* using analysis of the 5.8s rDNA gene and two ribosomal internal transcribed spacers. *Archives of Biological Sciences*, 63(1), 79–88.
- Nagahama, T., Hamamoto, M., Nakase, T., Takami, H., & Horikoshi, K. (2001). Distribution and identification of red yeasts in deep-sea environments around the northwest Pacific Ocean. *Antonie van Leeuwenhoek*, 80(2), 101–10.
- Pakpour, S. (2012). A multi-criteria decision-making approach for comparing sample preservation and DNA extraction methods from swine feces. *American Journal of Molecular Biology*, 02(02), 159–169.
- Palomba, S., Blaiotta, G., Ventrino, V., Saccone, A., & Pepe, O. (2010). Microbial characterization of sourdough for sweet baked products in the Campania region (southern Italy) by a polyphasic approach. *Annals of Microbiology*, 61(2), 307–314.
- Pimenta, R. S., Alves, P. D. D., Corrêa, A., Lachance, M.-A., Prasad, G. S., Rajaram, ... Rosa, C. a. (2005). *Geotrichum silvicola* sp. nov., a novel asexual arthroconidial yeast species related to the genus *Galactomyces*. *International journal of systematic and evolutionary microbiology*, 55(Pt 1), 497–501.
- Pinho, B. H., & Furlong, E. B. (2000). The occurrence of molds, yeasts and mycotoxins in pre-cooked pizza dough sold in Southern Rio Grande do Sul. *Brazilian Journal of Microbiology*, 31(2), 99–102.
- Ricciardi A, Parente E, Piraino P, Paraggio M, Romano P (2005). Phenotypic characterisation of LAB from sourdoughs for Altamura bread production in Apulia (Southern Italy). *Int. J. Food Microbiol.*, 98: 63-72.
- Robert, H., Gabriel, V., & Fontagné-Faucher, C. (2009). Biodiversity of lactic acid bacteria in French wheat sourdough as determined by molecular characterization using species-specific PCR. *International journal of food microbiology*, 135(1), 53–9.
- Rodríguez, M., Medina, L. M., & Jordano, R. (2000). Effect of modified atmosphere packaging on the shelf life of sliced wheat flour bread. *Die Nahrung*, 44(4), 247–52.
- Rodríguez, V., Medina, L., & Jordano, R. (2003). Influence of modified atmosphere packaging on the shelf life of prebaked pizza dough with and without preservative added. *Die Nahrung*, 47(2), 122–5.
- Sana, M., Mejri, M., & Hamdi, M. (2007). Microflora distribution and species ratio of Tunisian fermented doughs for bakery industry, 6(September), 2122–2129.
- Steinkraus, K.H. (ed.) 1984 *Handbook of Indigenous Fermented Foods*. New York & Basel: Marcel Dekker.
- Tiwari, K. L., Jadhav, S. K., & Kumar, A. (2011). Morphological and Molecular Study of Different *Penicillium* Species, 7(2), 203–210.
- Terzaghi B.E., Sandine W.E. 1975. *App. Microbiol.* 29: 807–813

Ueda-Nishimura, K. & Mikata, K. (2000). Two distinct 18S rDNA secondary structures in *Dipodascus* (Hemiascomycetes). *Microbiology* 146, 1045–1051.

Wu, Z., Wang, X.-R., & Blomquist, G. (2002). Evaluation of PCR primers and PCR conditions for specific detection of common airborne fungi. *Journal of Environmental Monitoring*, 4(3), 377–382.

CHAPTER 8

GENERAL SUMMARY & CONCLUSION

Preformed pizza is a popular food for all categories of people, including children and the elderly. It is considered unsafe for people when the product with a high count of mold is consumed. Beside safety issues, pizza spoilage contributes to the annual loss of bakery product as a staple food around the world. This emphasizes the importance of quality control procedures aimed at reducing spoilage microorganisms (particularly mold and yeast) counts in bakery products. The industry is constantly looking for new preservation technologies to ensure the safety of ready-to-eat bread and pizza products, while maintaining their organoleptic properties. One such approach is pulsed UV-light treatment.

Preliminary studies showed that voltage, treatment time (number of pulses), distance from the pulsed UV-light source, and position in the pulsed UV-light source treatment chamber play a significant role in the destruction of *Penicilium roqueforti* as one of the predominant spoiling molds in baked products. Maximum destruction was achieved at high voltage and minimum distance from the pulsed UV-light source. However, due to the increase in treatment area as the distance from the pulsed UV-light source increases, sample surface area must be considered before choosing the appropriate combination of pulsed UV-light factors.

Different inoculation methods including the random spot and spread methods were investigated. The results showed that depending on the feasibility of each method, both random spot and spread methods can be selected for inoculating mold onto the sample surface. Parameters which need to be considered in choosing the best inoculation method are detectability of mold grouping and homogeneity of the colonies' distribution on the surface of the substrate.

Three sets of pulsed UV light treatments were applied on pizza samples, namely 500 V, 1 min (minimum treatment); 750 V, 5 min (intermediate treatment) and 1,000 V, 10 min (maximum treatment). The results showed that 8, 32, and 40% of pizza samples were mold-free after the 40 days storage with minimal, intermediate and maximal pulsed UV-light treatment, respectively. The bread samples were inoculated with *Penicilium roqueforti*, and treated with different pulsed UV input voltage (500 and 1,000 V), treatment time (2 and 7 min) and distance from the light source (5 and 15 cm). Samples treated with the more intense voltage (1,000 V) showed steady surviving population less than 10^2 CFU g^{-1} over the storage time of 21 days.

However, 500 V treated breads and controls showed growth on every analysis day (more than 10^3 CFU g^{-1}). Yet, many irregularity and unpredictability in the results had been seen especially when treatments with lower level of voltage input were performed. This inconsistency might be because of the non-homogeneous nature of the vegetative cell inoculum and of the irregularity of the surface of pizza samples. Therefore, a more homogenous source of inoculum such as spore suspension has to be applied in future experiments in order to observe more consistent and reliable inactivation results corresponding to the level of pulsed light intensity, even at lower levels.

The effectiveness of a pulsed UV light was evaluated for the decontamination of *P. roqueforti* on the surface of solid agar using the process parameters of treatment time (1, 3, 5, 7 and 10 min) and voltage input (500, 750 and 1,000 V). As a result, the most effective treatment condition for inactivating this mold was 1,000 V for 10 min. The population of *P. roqueforti* was reduced after 10 min of exposure to pulsed light by 3.74, 5.36 and 6.14 log CFU ml^{-1} , respectively at 500, 750 and 1,000 V. The experimental data also suggest that inactivation of *Penicillium roqueforti* by pulsed UV light does not appear to follow first-order kinetics. On the other hand, the inactivation kinetics of the tested microorganisms on solid agar was best described by the Weibull model with the smallest RMSE (Root Mean Squared Error) and $R^2 \geq 0.92$. RMSE of Weibull model for 500, 750 and 1,000 V were 0.1374, 0.8163 and 0.824, respectively.

Culture-dependent and independent methods were applied to study the ecology of preformed pizza. Based on culture-dependent analysis, the average population of mesophilic aerobic bacteria (MAB), mesophilic anaerobic bacteria (MANB), lactic acid bacteria (LAB), and molds and yeasts (M+Y) were 6.5 ± 0.5 , less than 2.43, 2.7 ± 0.6 and 5.3 ± 0.4 log CFU g^{-1} , respectively. Based on biochemical analysis, *Lactobacillus plantarum* and *Pediococcus acidilactici* were identified as LAB isolates in the product. Analysis of the clone libraries using cultivation-independent techniques revealed low diverse fungi communities in preformed pizza samples. The microbiota, as revealed by PCR-cloning, was composed of mold affiliated to *Saccharomyces cerevisiae*, *Saccharomyces* sp. WW-W23, *Penicillium expansum*, *Penicillium freii*, *Penicillium* sp. HSL, *Penicillium* sp. ljl1, *Rhodotorula mucilaginosa*, *Monascus fuliginosus*, *Hordeum jubatum*, and *Galactomyces geotrichum* strains.

Pulsed UV light irradiation was very effective at inactivating the spores of *Penicillium roqueforti*; however, the dose of energy per pulse and the position of xenon lamp will determine the lethality. Moreover, decontamination by pulsed UV-light has to account for possible interactions between the target surface and the microorganism. Therefore, further research on the influence of pulsed light on foods with more complex composition is needed to define the applicability of the technology in decontamination processes.

Future Recommendations

The voltages used in this study did not eliminate the population of *P. roqueforti* on the surface of pre-formed pizza; therefore, in order to improve the microbial load reduction by pulsed UV-light treatment, further studies utilizing higher voltages (1,000-3,500 V) seem to be necessary. In addition, the advantage of using higher voltages is a decrease in the treatment time, which is desirable for commercial purposes. The present studies have focused on the pulsed UV-light destruction of *P. roqueforti* as it is one of the main concerns in shelf stable bakery products. However, it would be advantageous to obtain pulsed UV-light destruction kinetics for other mold and yeast strains that cause spoilage in these types of product.