Effect of sub lethal microwave radiation on bacterial growth, enzyme activity, and exopolysaccharide production

A dissertation thesis submitted to Nirma University

in partial fulfilment for the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY/ BIOTECHNOLOGY

Submitted by:

TOSHI MISHRA (11MMB020)

PREEMADA KUSHWAH (11MBT016)

Under the guidance of

Dr. Vijay Kothari

Acknowledgement

"Science is simply common sense at its best, that is, rigidly accurate in observation, and merciless to fallacy in logic." ― Thomas Henry Huxley

First and foremost, I would like to thank God for attaching the strings of my life with Science which I believe to carry on throughout my life. I thank you for all the good fortune you showered upon me during my dissertation.

I would like to express my gratitude to *Prof. Sarat Dalai*, Director in-charge, Institute of Science, Nirma University for all the support and inspiration. Also, I thank Nirma Education and Research Foundation (NERF)/ Nirma University for the infrastructure and facilities that helped me complete my dissertation work.

I would like to thank my mentor *Dr Vijay Kothari* for his guidance and all useful discussions we had during the development of this work. I thank him for introducing me to the wonders of scientific research and teaching me the value of time and discipline in science. He made me believe that the ability to stretch yourself gets you to the next level, and that is where the difference is made. Thanks a lot for putting so much effort, time and faith in me.

I gratefully acknowledge to my teachers *Dr. Sonal Bakshi, Dr. Mili Das, Dr. Amee Nair, Dr. Shalini Rajkumar, Dr. Nasreen Munshi* and *Dr. Sriram Seshadri* for providing every possible help. Also, a sincere thanks to *Sachin Prajapati,* and *Hasit Trivedi* for their co-operation.

I would like to thank my project partner *Preemada* for being so compatible throughout the dissertation work. I am thankful to my dissertation group- *Ina, Asha, Vaibhavi, Meera* and *Namrata* for helping me all the way and sharing their knowledge in every possible way.

I thank *Nishi* and *Neha* for standing up for me every time with immense love, care and support and for bearing with me even at times I was all irritable and cranky. Also I thank *Himanshu* for all the technical help and moral support. I thank all my friends in *Microbiology* batch for creating a positive atmosphere around me and being so sweet and helpful always.

Finally my parents who are the reason I thrive to be better in everything I do. Their pride in me has been the greatest motivation all through.

Toshi Mishra

Acknowledgement

It's my fortune to gratefully acknowledge the support and encouragement of some special individuals on completion of my MSc. dissertation. Foremost, I would like to express my sincere gratitude to my guide *Dr. Vijay Kothari*, Assistant Professor, Institute of science, Nirma University for picking me as his student and for his continuous motivation, understanding, support, patience, enthusiasm, constructive criticism, discipline, commitment, immense knowledge and constant moral support. His guidance supported me in all the time of research and thesis writing. I could not have imagined a better advisor and mentor for my dissertation. I sincerely thank Nirma Education and Research Foundation for providing so good facilities for studying. I am extremely indebted to *Prof. Sarat Dalai* for stimulating and fun-filled environment to work.

I gratefully acknowledge to my teachers *Dr. Mili Das, Dr. Shalini Rajkumar, Dr. Amee Nair, Dr. Nasreen Munshi, Dr. Sriram Seshadri, Dr. Sonal Bakshi* for providing helping hand. I thank *Mr. Sachin Prajapati* and *Mr. Hasit Trivedi* for providing me all the requirements required for the research on time.

I thank my dissertation partner *Toshi Mishra* for being so co-operative in the laboratory. I thank my fellow labmates *Vaibhavi Patel, Asha Thakkar* and *Namrata Srivastava* for all the support and fun we had. I convey my special gratitude to *Vaidehi Chemburkar, Rachna Sharma* and *Gursimran Kaur* whose selfless refreshing friendship, love, support, long lasting sense of humour, spirit of optimism mixed with pieces of advise which enabled me to bypass the most persistant hurdles and making more than a temporary place for study.

My sincere thanks to my family as I know I always had them to count on when times were rough. I am forever grateful to my grandparents and parents whose foresight and values paved the way to education and for their unconditional love and care. It is difficult to find words to express my gratitude to the great *Almighty* for health, mercy, power and wisdom that he gave me throughout my life.

Preemada Kushwah

List of abbreviations

EMF: Electromagnetic field

MW: Microwave

RADAR: Radio Detection and Ranging

EPS: Exopolysaccharide

PD: Power Density

GSM: Global System for Mobile communication

β-gal: β-galactosidase

GOS: Galacto-oligosaccharides

G6P: Glucose-6-phophatase

CPS: Capsular Polysaccharides

OD: Optical Density

DNSA: 3,5-dinitrosalicylic acid

CFS: Cell Free Supernatant

IU: International Unit

SD: Standard Deviation

NTG: Nitrosoguanidine

BSA: Bovine Serum Albumin

TCA: Trichloroacetic acid

ONPG: o-nitrophenol galactopyranoside

TY: Tryptone Yeast

Index

List of tables

List of figures

1. Prologue……..1-5
   1.1 Preamble……..2
   1.2 The Importance of the Study……...3
   1.3 Statement of the Problem……4
   1.4 Rationale of the Research Work……4
   1.5 Objectives……...5
2. Literature Review…….7-18
   2.1 Interactions of Microwave with Biological Materials……7
   2.2 Athermal (Non-thermal) Mechanisms of Interaction……7
   2.3 Thermal versus athermal effects……7
   2.4 Factors affecting Microwave effects……...9
   2.5 Biological effects of Microwave radiation……10
   2.6 Microwave mutagenesis……11
   2.7 Effect of Microwave on higher organisms……11
   2.8 Microwave and cellphones……12
   2.9 Effect of Microwave on growth and enzyme activity……....12
   2.10 Amylase……13
   2.11 Pectinase……15
   2.12 β-galactosidase……..16
   2.13 Glucose-6-phosphatase……17
   2.14 Xanthan gum……17
3. Effect of low power microwave on growth, enzyme activity (amylase and pectinase) and EPS production on different bacteria……..20-32
   3.1 Materials and Methods……..20
      3.1.1 Culture maintenance……..20
      3.1.2 Culture activation……...20
      3.1.3 Inoculum preparation……20
      3.1.4 Microwave oven and its maintenance……....20
      3.1.5 Microwave treatment to inoculums……21
      3.1.6 Growth measurement……..21
      3.1.7 Amylase estimation……..…22
      3.1.8 Pectinase estimation……23
      3.1.9 EPS quantification……...24
      3.1.10 Statistical Analysis……..24
   3.2 Results and Discussion…….25-32
4. Effect of low power microwave on growth, intra- and extracellular protein and intracellular enzymes (glucose-6-phosphatase and β-galactosidase)……34-40
   4.1 Materials and Methods…….……34

4.1.1 Test organisms……..34
4.1.2 Experimental outline…….34
4.1.3 Estimation of intra- and extracellular protein content……34
4.1.4 Estimation of G6P…….35
4.1.5 Estimation of β-galactosidase ……....36
4.2 Results and discussion……37-41
5. 44 To investigate mutagenic effect of MW on exopolysaccharide production in *X. campestris*.....42-49
5.1 Materials and Methods……..43
5.1.1 Test organisms……43
5.1.2 Microwave treatment to inoculums……43
5.1.3 Experimental outline……...43
5.2 Results and discussion…….45

6. Epilogue……..……50

7. Appendices……..51-60

Appendix I……52

Appendix II……..55

Appendix III…….57

Appendix IV…….58

Appendix V……...……59

Appendix VI…….….62

8. References……..63-70

List of Tables

Table 3.1 Experimental conditions……..22

Table 3.2 Effect of MW on growth and amylase activity in *B. subtilis* ……25

Table 3.3 Effect of MW on growth and amylase activity in *S. mutans* ……..……26

Table 3.4 Effect of MW radiation on growth and pectinase activity in *P. carotovora*…..27

Table 3.5 Effect of MW radiation on growth and pectinase activity in *B. subtilis*…….28

Table 3.6 Effect of MW on growth and EPS production in *X. campestris*…….……29

Table 3.7 Effect of MW on growth and EPS production in *S. mutans*……..30

Table 4.1 Effect of MW on growth, and protein synthesis in *B. subtilis*…….37

Table 4.2 Effect of MW radiation on glucose-6-phosphatase and β-galactosidase activity in *B. subtilis*…….……..37

Table 4.3 Effect of MW radiation on growth, and protein synthesis in *L. acidophilus*….38

Table 4.4 Effect of MW radiation on glucose-6-phosphatase and β-galactosidase activity in *L. acidophilus*……38

Table 4.5 Effect of MW radiation on growth, and protein synthesis in *E. coli*…….39

Table 4.6 Effect of MW radiation on G6P and β-galactosidase activity in *E.coli*…….39

Table 4.7 Comparison of effect of different MW exposure on growth, protein synthesis, and enzyme activity in the test organisms 40

Table 5.1 Effect of MW (90 W) on growth and xanthan produced by *X. campestris*…….45

Table 5.2 Growth and xanthan gum production by three different isolates selected randomly from plate corresponding to 6 min MW treatment……45

Table 5.3 Effect of MW (90 W) on growth and xanthan production by *X. campestris*……..46

Table 5.4 Effect of MW (450 W) on growth and xanthan produced in *X. campestris*……..47

Table 5.5 Growth and xanthan gum production by four different isolates selected randomly from plate corresponding to 3 min MW treatment……..47

Table 5.6 Effect of MW (450 W) on growth and xanthan production by *X. campestris*…….48

List of Figures

Figure 1.1 Position of microwave radiation in electromagnetic spectrum…….3

Figure 2.1 The orientation of dipoles in absence (a) and in presence (b) of electric field..9

Figure 2.2 The structures of amylose (A) and amylopectin (B)……..14

Figure 2.3 The action of β-gal on lactose converting it into galactose and glucose subunits……..16

Figure 2.4 The conversion of G-6-P to glucose……...……17

Figure 2.5 Structure of xanthan gum…….……18

Figure 3.1 Comparison of effect of different duration of MW exposure on growth and amylase activity in *B. subtilis*…….……..25

Figure 3.2 Comparison of different duration of MW on growth and amylase activity in *S. mutans*……..26

Figure 3.3 Comparison of different duration of MW on growth and pectinase activity in *P. carotovora*……....27

Figure 3.4 Comparison of different duration of MW on growth and pectinase activity in *B. subtilis*……...28

Figure 3.5 Comparison of effect of different duration of MW exposure on growth and EPS production in *X. campestris*…….30

Figure 3.6 Comparison of effect of different duration of MW exposure on growth and EPS production in *S. mutans*……..……31

Figure 4.1 Comparison of effect of different duration of MW exposure on growth, protein synthesis, glucose-6-phosphatase and β-galactosidase activity in *B. subtilis*……37

Figure 4.2 Comparison of effect of different duration of MW exposure on growth, protein synthesis, glucose-6-phosphatase and β-galactosidase activity in *L. acidophilus*……38

Figure 4.3 Comparison of effect of different duration of MW exposure on growth,

protein synthesis, glucose-6-phosphatase and β-galactosidase activity in *E. coli*……..39

Figure 5.1 Outline of the experiment to study the mutagenic effect of MW in *X. campestris*..……44

Prologue

1. Prologue

1.1. Preamble

Since many years, scientists are interested in studying the interaction of electromagnetic fields (EMFs) and various bio-system and its bioprocesses. All biological systems are electrochemical in nature so EMF may influence them. Attention has been focused on different frequency range waves, of which microwave (MW) is an important part. Microwaves (MW) are non-ionizing electromagnetic waves in 1mm to 1m wavelength range, with a wide frequency band between 300 MHz and 300 GHz [Banik et al., 2003]. They are a very important component of the electromagnetic spectrum as demonstrated by the increasing scope of applications. They have relatively short wavelengths and high frequencies compared to the extremely low frequency fields. They therefore have a greater energy which is sufficient to cause heating in conductive materials (Figure 1.1). Unlike the X-rays and gamma rays, which are ionizing, MW interaction with matter does not result in removal of orbital electrons. Rather, such interactions are known to cause effects like atomic excitations, increased atomic and molecular vibrations, rotations, and heat production [Davis and VanZandt, 1988]. Because of the nature of MW, its interaction mechanisms and the biological effects have previously not been associated with production of free radicals, oxidative modification of cell membranes, lipid peroxidation. These mechanisms are associated with ionizing radiations. Interest in the study of MW interaction with biological systems has been sustained for several decades. The first person to explore the bioeffects of MW fields was Antonin Gosset in 1924, when he and his co-workers used short waves to destroy tumors in plants with no damage to the plant itself [Bren, 1996]. During the 1930s, physicists, engineers, and biologists studied the effects of low frequency electromagnetic waves on biological materials. Studies of the effects of microwaves on bacteria, viruses and DNA were performed in the 1960s and included research on heating, biocidal effects, dielectric dispersion, mutagenic effects, etc. [Yaghmaee and Durance, 2005]. In recent year, the industrial MW applications have grown considerably, apart from the usage of domestic MW ovens. Some of the MW applications in industries are tampering of frozen product, thawing, blanching, baking, drying/dehydration/vacuum drying/freeze-drying, pasteurization, sterilization, cooking, etc. In modern world, MW radiations are used in FM radio, television, RADAR (Radio Detection and Ranging), and satellite-to-earth communications. Apart from its use in domestic ovens, many other applications of MW in different areas have been identified.

Applications of MW that are based primarily on its thermal effects include those in food processing industry [Decareau and Kenyon, 1970], disinfection and sterilization [Bhattacharjee et al., 2009; Kothari et al., 2011], moisture removal [Ozbek and Dadali, 2007], waste treatment [Beszedes et al., 2010], etc.

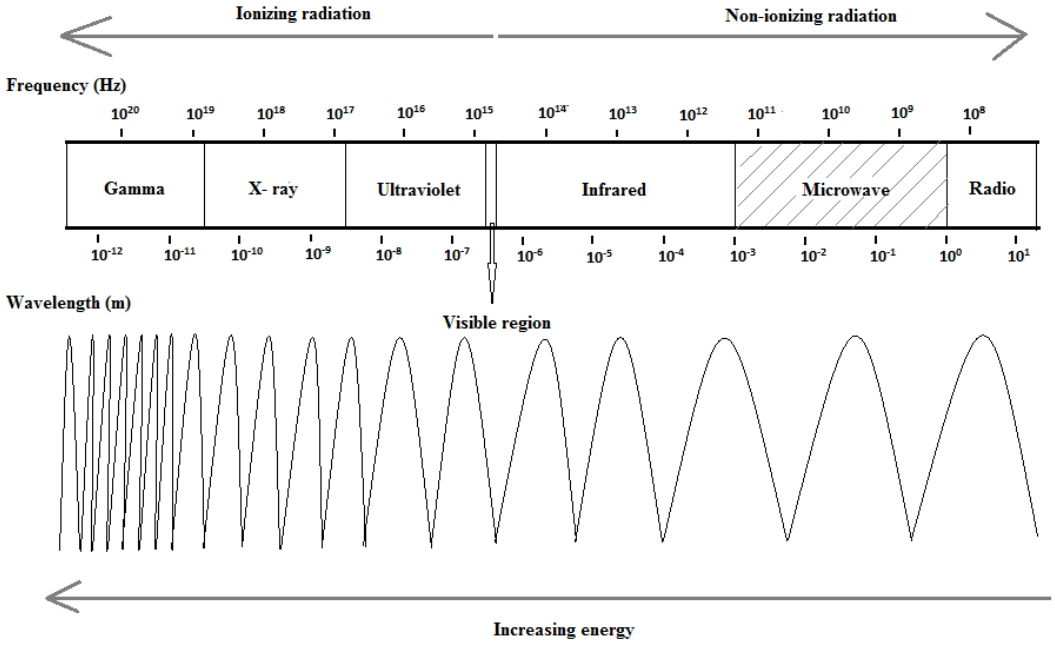


Figure 1.1 Position of microwave radiation in electromagnetic spectrum
(http://www.chem.ucalgary.ca/courses/350/Carey5th/Ch13/ch13-1.html)

MW irradiation is also used to disinfect household products such as sponges, kitchen utensils, syringes and medicinal devices where bacterial viability is reduced by MW treatment. Microwave assisted extraction (MAE) has emerged as a promising method for preparation of bioactive plant extracts. It is considered suitable for fast extraction of phenolic compounds, and also for extraction of heat-labile phytoconstituents [Gupta et al. 2012; Kothari et al., 2012; Mandal et al., 2007]. Research on non-thermal effects can open the door for new applications which may be based either solely on non-thermal effect, or both thermal and non-thermal effects. Either low power or high power (for very short duration, and with a provision of cooling water circulation to avoid heating) MW can be used for mutagenesis in plants [Jangid et al., 2010] and microorganisms [Lin et al., 2012; Li et al., 2009], protein unfolding [George et al., 2008], tumor detection based on electrical properties of tissues [Stuchly and Stuchly, 1983], rate enhancement of biochemical reactions [Bose et al., 2002; Sun et al., 2011], enzyme immobilization [Wang et al., 2011], and reduction of pathogenic population [Barnabas et al., 2010].

1.2. The Importance of the Study

Sub- lethal MW-microbes interaction: may change level of

- Growth rate [Rebrova et al., 1992; Golant et al., 1994; Shub et al., 1995]
- Metabolite activity [Dreyfuss et al., 1980]
- Plasmid amplification [Otludil et al., 2005]

- Rapid Strain identification [Spencer et al., 1985]
- Transformation efficiency [Fregel et al., 2008]
- Porosity of the cell membrane that allow uptake of drugs into MW-treated cell [Shamis et al., 2011]
- Secondary metabolites such as antibiotics [Himabindu et al., 2007]
- Lose of virulence of virulent strain [Moore et al., 1979; Tsuji and Yokoigawa 2011]
- Mutation [Lin et al., 2012; Li et al., 2009]

1.3. Statement of the Problem

Increasing applications of MW radiation has led to concerns globally due to the suspected bio effects associated with its exposure. Effect of MW, thermal and/or athermal, is inconclusive, complex, and controversial in literature. Thermal effect causes thermogenic effect while athermal effects are other than heat and such effects reported as somatic effect and/or genetic effect.

This study basically deals with the athermal effects and is aimed at investigating the hypothesis that the exposure of microbial cells to MW (low power) may cause athermal effect, which affect on growth of microbes, enzyme activity, and production of exopolysaccharides. Furthermore, we have also checked the effect of different intracellular enzymes on MW treated bacteria. Our study also gives information that MW athermal effects causes changes at genetic level and can be passed on to next generation.

1.4. Rationale of the Research Work

There are numerous and increasing applications of MW energy and technology in the industries, in homes, in medical, research institutions etc., and there is greater awareness and concern of the public over the suspected potential health hazards associated with such exposures [ICNIRP Guidelines, 1998]. There is therefore, a need for deeper understanding of the bio-effects of exposure to this radiation. Due to the ease of handing them in laboratory, microorganisms can be conveniently used to study the effect of MW on living systems. Besides, employing mutagenic frequencies of MW radiation for microbial strain improvement can be of considerable industrial significance.

1.5. Objectives:

1. To investigate the effect of low power MW on,
    a. Growth
    b. Extracellular enzyme (amylase and pectinase) activity in *Bacillus subtilis, Streptococcus mutans* and *Pectobacterium carotovora.*
    c. Exopolysaccharide (EPS) in *S. mutans* and *Xanthomonas campestris.*

2. To study the effect of low power MW on,

    a. Growth
    b. Protein synthesis
    c. Intracellular enzyme (Glucose-6-phosphatase and β- galactosidase) activity

3. To investigate mutagenic effect of MW on EPS production in *X. campestris*.

2. Literature Review

2.1. Interactions of MW with Biological Materials

The interactions can be considered at various levels of organization of a living organism: atomic, molecular, subcellular, cellular, entire organism. These interactions with biosystem arise because of three processes: (i) penetration by electromagnetic waves and their propagation into the living system, (ii) primary interaction of the waves with biosystem; and (iii) possible secondary effects arising from the primary interaction. One of the first fundamental steps in evaluating the effects of a certain exposure to radiation in a living organism is determination of the induced internal electromagnetic field and its spatial distribution. Further, various possible biophysical mechanisms of interaction can be applied. Any such interactions, which may be considered primary, elicit one or more secondary reactions in the living system. For instance, when MW energy absorption results in a temperature increase within cell (a primary interaction), the activation of the thermoregulatory, compensatory mechanism is a possible secondary interaction [Czerski, 1975; Czerski, 1975] While the primary interactions are becoming better understood, there is still insufficient attention devoted to the interaction mechanisms involving molecular level. Studies on the biological effects of MWs reveal several areas of established effects and mechanisms on the one hand and speculative effects on the other. There are known thermal and athermal interaction mechanisms of MWs with biological systems.

2.2. Athermal (Non-thermal) Mechanisms of Interaction

MW radiation seems to affect system in a manner, which cannot be explained by thermal effects alone [Spencer et al., 1985]. MW has ability to destroy bacterial cells at specific parameters without causing heating of the substrate [Barnabas et al., 2010]. MW plays role in dielectric saturation [Hyland, 1988], formation of oxidative stress [Sokolovic et al., 2008], protein unfolding [George et al., 2008], changing the structures by differentially partitioning the ions [Asadi et al., 2011], others chemical transformation of small molecules such as chemical bond cleavage [Oslen, 1966], vibrational resonance in DNA molecules [Edwards et al., 1985]. The oscillating EMF of MW couples energy into large biomolecules with several oscillations. When a large number of dipoles are present in one molecule (DNA, protein, RNA etc.) and kept under MW, enough energy can be transferred to the molecules, which would be able to break the bond.

2.3. Thermal versus athermal effects

Biological effects of MW radiation can be divided into two categories: thermal effects and non-thermal effects. Thermal effect is the one in which the MW energy is converted into heat energy in the living systems. These effects can be macroscopic where whole organisms or major portions of them participate in the heat transfer process, or microscopic where a cellular component like bound water is vaporized by the selective application of the MW

heating [Richmond, 1969]. The dielectric effect of MW on polar molecules has been known for more than a century [Debye, 1922]. Polar molecules are present in the cells in the form of water, DNA, and proteins and they respond to an electromagnetic field by rotating. This rotation creates an angular momentum which results in friction with neighboring molecules, thereby developing a linear momentum (vibrational energy) [Saifuddin et al., 2009]. In this way, radiation energy is converted into thermal energy. Effect generated from vibrational energy is thermal effect which occurs in a biosystem due to penetration of electromagnetic waves (MW) into biological materials and heating up the intra- and extra- cellular fluids by transfer of vibrational energy [Tahir et al., 2009]. However, MW thermal effect is different from conventional heating effect. Dipolar polarization and rotation of molecules in an attempt to align the dipoles with applied MW field (Fig. 2.1) produces effects which cannot be achieved by conventional heating [Zelentsova et al., 2004].

The non-thermal effect of MW is highly controversial and has been a matter of debate in scientific community. Non-thermal effects are postulated to result from a direct stabilizing interaction of electric field with specific (polar) molecules in reaction medium with no rise in temperature [Herrero et al., 2007]. Thermal effects solely cannot explain the manner in which the MW affects biological systems. Several studies have revealed that MW radiation can kill microbial cells; however it is still not clear if non-thermal effects of MW have any contribution to this. One of the mechanisms involved in killing of microorganisms by MW is by altering the permeability of the cell membrane. The alteration in the permeability of the membrane of the cells is reflected by cell shape changes observed under electron microscopy or by detecting leakage of intracellular protein or DNA using spectroscopy [Chen et al., 2007]. Due to the paucity of information regarding the exact mechanism involved in *'nonthermal microwave effects'* (also referred to as *athermal effects or MW specific effects*), their existence is highly controversial. Kozempel et al. (2000) attempted at detection of nonthermal effects of MW energy on microbes at low temperature in various test fluids. To separate thermal and nonthermal effects in a system, they developed a continuous experimental microwave process combining rapid energy input to the food system using microwave, with rapid removal of thermal energy utilising an efficient heat exchanger design. A continuous MW treatment (7 kW, 2450 MHz) was given to the test organisms in various test fluids like water, liquid egg, beer, apple juice, and tomato juice. They concluded that MW energy in the absence of other stresses did not kill microorganisms at low temperatures and there was no convincing evidence that MW energy could kill microorganisms without thermal energy. Dreyfuss and Chipley (1980) studied the effect of MW at sub-lethal temperature on *Staphylococcus aureus* and suggested the existence of a phenomenon different from thermal heating resulting in altered activity of various metabolic enzymes. There has been a plenty of reports favoring the existence of non-thermal effects [Dreyfuss and Chipley, 1980; Copty et al., 2006; Carta and Desogus, 2012], but the studies refusing the possibility of athermal effects [Vela and Wu, 1979; Kozempel et al., 2000; Shazman et al. 2007] can also not be neglected.

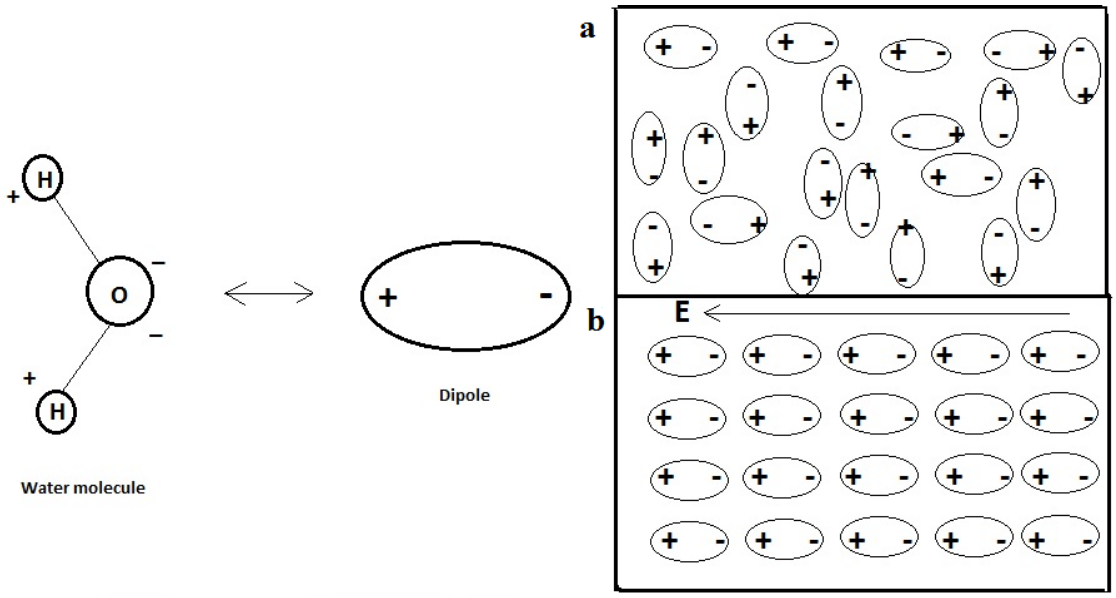


Figure 2.1 The orientation of dipoles in absence (a) and in presence (b) of electric field [Williams J.M., 2009]

2.4. Factors affecting MW effects

Interaction of MW with biological entities is influenced by multiple factors viz., MW power and frequency, far-field versus near-field location, duration of exposure, polarization, etc. Reports suggest that continuous and pulsed MW treatments have different effects on cells. Human diploid fibroblasts and rat granulosa cells were exposed to intermittent and continuous waves. With the help of alkaline and neutral comet assay, DNA strand breaks were determined. Single and double strand breaks occurred in both cell types, but the intermittent exposure showed a stronger effect in the comet assay than continuous exposure [Diem et al., 2005]. Normal human lymphocytes isolated from the peripheral blood were exposed for 5 days to 2450 MHz microwave radiation in both continuous and pulsed form. Spontaneous lymphoblastoid transformation was determined with an image analysis system and it was found that pulsed wave exposure enhanced transformation to a greater extent than continuous wave exposure [Czerska et al., 1992].

Duration of MW exposure seems to be a major determinant of MW effect on living cell. The time of exposure and power density are correlated in a way that decrease in power density (PD) could be compensated by increase in duration of exposure. Cells of *Escherichia coli* K12 AB1157 were irradiated with millimetre waves within the PD range of 10−20 to 10 4 W/cm^2 and it was found that decrease in PD could be compensated by increase in exposure time to achieve the same changes in chromatin conformation [Belyaev et al., 1992]. There have been reports suggesting that the length of post-treatment time following MW

exposure is also important in determining response of living cells to MW radiation. Sub-lethal MW radiation studies on *E. coli* revealed that cell-surface undergo modifications which are electrokinetic in nature, and cells revert back to original stat after 10 min of exposure [Shamis et al. 2011]. Low level MW radiation of 10 GHz frequency, 0.58 mW/cm^2 intensity, applied for 30-120 min caused loss of virulence in *Agrobacterium tumefaciens* strain B6, where 30-60% decrease in their ability to produce tumor and turnip disk in plant was observed. However, this loss in virulence was reversible within 12 h [Moore et al., 1979].

The medium/matrix in which the cells are embedded during MW exposure can also have its impact. The efficiency with which different solvents absorb microwaves and pass it on as heat to the surrounding molecules is indicated by dissipation factor (tan δ), expressed as:

$\tan \delta = \varepsilon'' / \varepsilon'$,

where, ε'' is the dielectric loss which indicates the efficiency of conversion of microwave energy into heat; ε' is the dielectric constant which is the measure of the ability of the material to absorb microwave energy. A reaction medium with a high tan δ at the particular operating frequency of a microwave synthesis reactor is required for good absorption [Herrero et al., 2007]. Different cells, organs and tissues of biological entities have varying dielectric properties, and thus are affected differently by MW radiation.

2.5. Biological effects of MW radiation

Research is being done worldwide on thermal and athermal effects of MW on different biological systems. *Phormidium* spp. Kutzing ISC31 (a cyanobacterium) grown in BG-11 medium was treated at a frequency of 2450 MHz using a microwave oven by combining five different intensities (180, 360, 540, 720 and 900 W/cm^2) and three pretreatments (10, 20 and 30 s). The content of chlorophyll *a* decreased with increase in intensity and exposure time. Synthesis of phycobiliproteins, phycocyanin, phycoerythrin, and allophycocyanin increased in all exposures except in 720 and 900 W/cm^2 (30 s). Photosynthetic rate compared to nitrogenase activity increased by all microwave exposures except at 180 W (10 s) and 720 W (10 s) as compared to control [Asadi et al., 2011]. Studies on *E. coli* and *S. aureus* suggested that physical damage caused by MW led to alterations in membrane permeability and consequently influx of extracellular Ca^{2+}. An increase in cell permeability of upto 89.8% and 19.7% was obtained after MW treatment in *S. aureus* and *E. coli* respectively [Chen et al. 2007]. Effect of MW on transformation efficiency was studied by Fregel et al. (2008), where calcium chloride competent cells of *E. coli* were given MW treatment at 180 W for 1 min, and the transformation efficiency was increased three-fold as compared to the classical method. Otludil et al., (2004) studied the effect of microwave (2450 MHz, 55 W) on the cellular differentiation of *Bacillus subtilis* YB 886 and its Rec derivatives YB 886 A4. It was found that organism's growth and amount of DNA decreased after MW exposure by 4% and 27% respectively, whereas the amount of RNA and plasmid was enhanced by 6.5% and 21% respectively. They noted an increase in the amount of specific protein synthesized during

DNA damage by SOS repair system, and its binding to din C promoter region, following MW exposure in rec+ bacteria.

2.6. Microwave Mutagenesis

A number of studies reveal that effects of MW can reach up to genetic level and can even result in stable mutation. Genetically stable mutant strains with higher nitrogenase activity and phosphate solubilizing capabilities of *Klebsiella pneumoniae* RSN19 were obtained by microwave (250W, 36 s) mutagenesis [Li et al., 2011]. Mutagenic potential of MW has also been demonstrated with respect to cellulase production in *Trichoderma viride*, wherein a compound mutagenesis by MW (700W, 15-195 s) and ultraviolet radiation was employed, and the mutants were found to be stable up to 9 generations [Li et al., 2009]. Lin et al. (2012) studied the effect of MW (400 W for 3 min) on *Lactobacillus rhamnosus* which induced >50% increase in L-lactic acid production than the parent strain, and the mutant generated was found to be stable up to 9 generations. Such studies together indicate the potential of MW as a substantial tool for strain improvement through mutagenesis. MW till now has not been exploited as widely UV has been as a mutagenic radiation. Identifying the frequencies, power range and exposure duration in MW region, which are most suitable for mutagenesis among microbes will certainly be of interest to fermentation industries.

2.7. Effect of MW on higher organisms

A study on effects of low power MW radiation on germination and growth rate in seeds of wheat (*Triticum aestivum*), bengal gram (*Cicer arietinum*), green gram (*Vigna radiata*), and moth bean (*Vigna aconitifolia*) showed that different treatments stimulated the germination and seedling vigour. Increase in power density resulted in reduced rate of germination [Ragha et al., 2011]. Effect of different MW power was studied on potato tuber biomass and it was found that for seed potatoes irradiated with microwaves (100 W) at frequency 2.45 GHz for 10 s, tuber weight was 7.9% higher compared to check sample. No significant change was observed at 38 GHz, 46 GHz, and 54 GHz [Jakubowski, 2010]. Treatment with low-level MW (35 GHz; surface power density 30 $\mu W/cm^2$) radiation for 10 s has been reported to induce chromatin condensation (increase in number of heterochromatin granules) in human cells and increased membrane permeability. However, the number of heterochromatin granules decreased to its initial level and membrane permeability was recovered after a few hours [Shckorbatov et al., 2011]. Low-intensity MW radiation effectively changed membrane functions in striated muscle and cardiac pacemaker cells in rats (Chernyakov et al., 1989). Exposure at 0.1-0.15 mW/cm^2 for 90 s or lesser time (frequencies between 54-78 GHz) either decelerated the natural loss of transmembrane potential in myocytes, or even increased it by 5-20 mV. Low intensity MW was also found to suppress and alter the T-peak on electrocardiography of in situ exposed myocardium, enhance respiration, alter membrane calcium binding, and reduce the contractibility of cardiomyocytes. Drop in blood pressure of rats caused by MW pulses persisted for several weeks indicating a stable effect. The

properties of blood plasma like dielectric permittivity and absorption coefficient could be altered by microwave radiation [Lu et al., 1999]. Hybridization profile of brain and testis DNA of mice exposed to low power MW (1 mW/cm^2; 2450 MHz) showed an additional band suggesting amplification of tandem sequences in particular region [Sarkar et al., 1994]. Elder (2003) concluded that exposure of rabbit eye to 2450 MHz MW at 150 W/kg for more than 30 min can induce cataracts. Induction of cataracts via thermal effects of high-power MW radiation is well established. Whether low-power MW are cataractogenic remains unclear. Yu and Yao (2010) reviewed non-thermal cellular effects of low-power microwave radiation on the lens and lens epithelial cells.

2.8. MW and cell phones

Within last decade, the world population has adopted the use of cell phones in a horrendous way. Increasing use of radiofrequency devices has become a trend as well as a need in a large section of society. Cellular phones transmit radiofrequency waves of very low intensity and there has been a lot of discussion on possible adverse effects of these radiations on health. Effect of electromagnetic radiation from communication towers on human and other forms of biodiversity has been a matter of concern. Panagopoulos and Margaritis (2002) exposed fruit flies to cell phone radiation (Global System for Mobile communication-GSM-900 MHz) at very low levels (3-7 mW/cm^2) for 6 min/day before hatching. The exposed groups in their adult life showed a loss in reproductive activity varying between 15-60%. Frey (1962) reported that exposure of heads of volunteers to low level of radar from a radar horn resulted in headaches, and that low level of radar can interact with nerve tissue to cause dysaesthesiae. Hocking and Westerman (2001) reported a neurological abnormality in a patient after accidental exposure of left side of the face to cellular phone radiation, which led to long-lasting loss of tactile sensitivity of the facial skin, unilateral left blurred vision, and pupil constriction. The worker took virtually 6 months to recover.

2.9. Effect of MW on growth and enzyme activity

MW effects on growth and other cellular activities are being studied for atleast more than 50 years. Growth rate of yeast *Saccharomycetes cerevisiae* either increased upto 15% or decreased up to 29%, when exposed to MW radiation having narrow frequency range of 41.8-42.0 GHz [Grundler et al., 1977]. When *S. aureus* culture was exposed in a controlled temperature experiment to microwave radiation (24 GHz) for 10, 20, 30, and 40 s, the activity of various enzymes like malate dehydrogenase, cytoplasmic adenosine triphosphatase, glucose-6-phosphate dehydrogenase, and cytochrome oxidase increased in microwave treated cells than microwave non treated cells; whereas membrane adenosine triphosphate, alkaline phosphatase and lactate dehydrogenase activity remained unaffected [Dreyfuss and Chipley, 1980]. The effect of MW radiation on 94 strains of Enterobacteriaceae was studied and it was found that microwave irradiation increased the enzyme activity of bacteria in suspension [Spencer et al., 1985]. Low power MW treatment (2450 MHz; 90 W; 2 min exposure) on *Aeromonas hydrophila* decreased its total protease activity by 33%. Urease activity and

aflatoxin production in *S. aureus* and *Aspergillus parasiticus* respectively was completely inhibited by MW exposure [Dholiya et al. 2012]. Low power (100 W, 60 s) microwave radiation reduced not only the cell number but also the acid resistance and verocytotoxin productivity in enterohemorrhagic *E. coli* [Tsuji and Yokoigawa, 2011]. Komarova et al. (2008) investigated influence of MW on soil bacteria. They observed both the suppression and the stimulation of the growth for different bacterial species under the impact of microwaves. Spore suspensions responded to microwave radiation upon a shorter time of exposure than suspensions of vegetative bacterial cells. The influence of microwave radiation on the biomass accumulation and the intensity of other physiological processes in streptomycetes species led to changes in the number and activity of these microorganisms in the soil microbial complex.

2.10. Amylase

Amylase was the first enzyme to be discovered and isolated in 1833 by Anselme Payen as diastase. The first amylase was produced in 1894 from a fungal source which was used as a pharmaceutical aid for digestive disorders. Biodin and Effront were the first to use *B. subtilis* and *B. mesentericus* for the production of α- amylase on commercial scale using large fermentors. Amylases are starch-degrading enzymes that are widely distributed in plants, animals and microbial kingdom. Amylase forms complex with iodine to form intense blue colour and this forms the basis of a method for quantitative determination of amylase [Singh et al., 2011].

Starch is a major reserve carbohydrate component of all higher plants and in some cases it accounts for as high as 70% of the undried plant material. It occurs in the form of water insoluble granules but when they are heated in water the hydrogen bonds that hold the granules together begin to weaken and this permits them to swell and gelatinize.
Starch is a heterogeneous polysaccharide composed of two high molecular weight entities called amylose and amylopectin (Fig 2.2). These two polymers have different structures and physical properties. Amylose is composed of linear chains of α-1,4 linked D-glucose residues and therefore extensively degraded by α-amylase. Because of the shape and molecular structure of amylase, it is not stable in aqueous solution and thus precipitates spontaneously.
Amylopectin may account for 75- 85% of most starches. It has molecular weight in excess on $10^7 – 10^8$ and has a branched structure composed of chains of about 20 – 25 α-1, 4 linked D-glucose residues. In aqueous solutions, amylopectins are relatively stable due to branched molecules and are not able to form compact aggregates. The hydrolysis of starch results in formation of short-chain polymers called dextrins, then the disaccharide maltose, and ultimately glucose [Souza, 2010].

α- Amylases:

α- Amylases (1,4-α-glucan-glucanohydrolases) are extracellular enzymes which hydrolyze α-1,4-glycosidic bonds. Their action is not affected by α-1,6-glycosidic bonds although they do not split.

Bacteria that can produce α-amylases are: *B. subtilis, B. amyloliquefaciens, B. cereus, Lactobacillus, Pseudomonas, Arthrobacter, Escherichia, Proteus, Serratia, etc.*
Fungi from the genera *Aspergillus, Penicillium, Cephalosporium, Mucor, Candida, Neurospora* and *Rhizopus* can also produce α- amylases.
α- amylases are produced industrially from *Bacillus* and *Aspergillus* species. For industrial production, α- amylases are produced either in batch or in fed-batch fermentation. The rate of formation of enzyme is low in exponential growth, but as the growth rate decreases and spore formation begins, amylase production increases.

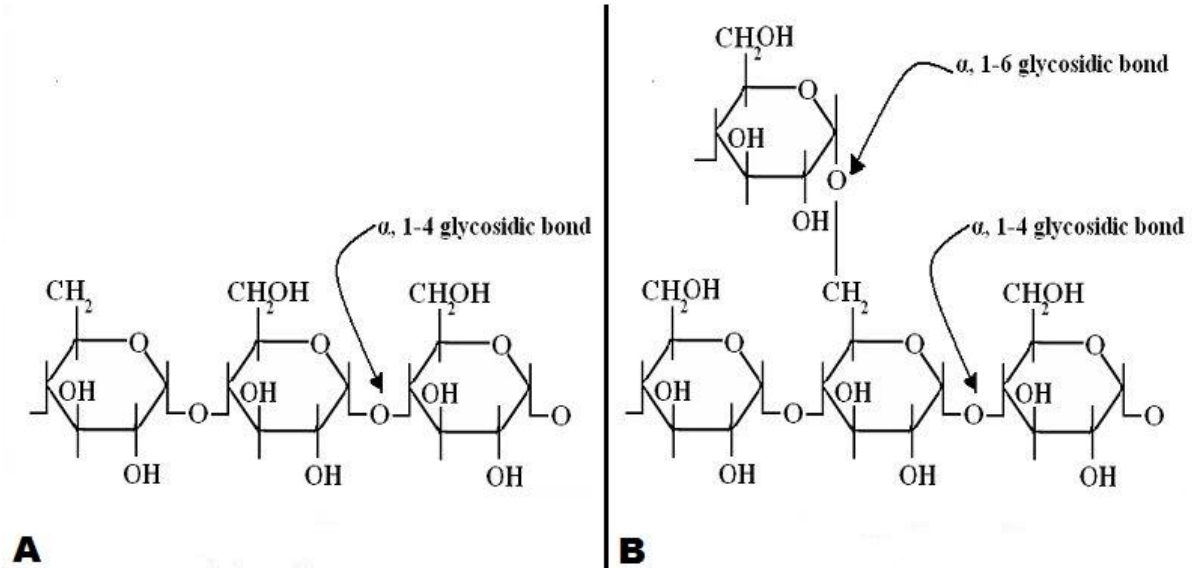


Figure 2.2 The structures of amylose (A) and amylopectin (B)
http://www.rsc.org/Education/EiC/issues/2006Sept/MakingMostStarch.asp

β- Amylases:

β- Amylases (α- 1,4-glucan-maltohydrolases) are produced by *Bacillus polymyxa, B. cereus,* B. *megaterium, Streptomyces* sp., *Pseudomonas* sp., and *Rhizopus japanicus* but usually these are of plant origin. Bacterial β- amylases have a much greater heat resistance (>70%) than plant β- amylases.

Applications [Crueger and Crueger, 1984; Aiyer, 2005]

Removal of starch sizer from textile (desizing)
In textile industry, sizing agent like starch is used in fabric production for fast and secure weaving process. It also prevents the loss of string by friction, cutting and generation of static

electricity on the string by giving softness to the surface of string due to laid down warp. From the woven fabric, starch is removed by amylase ensuring that the warp-thread remains intact.

Fermentation of starch to ethanol

For the production of ethanol, starch is a widely used substrate due to its low price and easy availability. The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using α-amylase or any amylolytic microorganism followed by fermentation where sugar is converted into ethanol.

Detergent industry:

The use of amylase in detergent industry enhances the rate of stain removal and makes the detergent environmentally safe. Amylases have the capacity to degrade the residues of starchy foods such as potato, custard etc.

Food industry:

α- amylases are widely used in baking industry where they are added to the dough of bread to degrade the starch into dextrins. This enzyme helps in enhancing the rate of fermentation and produces additional sugar in the dough that improves the taste of bread. Amylases are also extensively used in processed-food industry such as production of cakes, fruit juices and starch syrups.

Paper industy:

These enzymes are used in pulp and paper industry where the starch of coated paper is modified in such a way that it improves the writing quality of the paper. It also enhances the stiffness, strength and erasing ability of the paper.

2.11. Pectinase

Pectinases are a group of enzymes that break down pectic polysaccharides of plant tissues into simpler molecules like galactouronic acids. Pectinases are the most widely distributed enzymes in bacteria and fungi. Pectinases account for 10% of the overall manufacturing of enzyme preparations. Pectinolytic enzymes are widely used in the food industry for juice and wine production. Acid pectinases are widely used in extraction, clarification, and removal of pectin in fruit juices, in maceration of vegetables to produce pastes and purees, and in wine making are often produced by fungi, especially *Aspergillus niger*. In case of fruit juice extraction using pectinases, the yield is increased by more than 90% compared to conventional mechanical juicing, besides improving organoleptic and nutritional properties. The commercially available pectinases used in food processing industry are associations of polygalactouronases, pectic lyases and pectin methyl esterases [Semenova et al., 2006]. Alkaline pectinases are generally produced by bacteria, particularly species of *Bacillus*, but are also made by some filamentous fungi and yeasts [Kashyap et al.,2000 ; Hoondal et al.,

2002; Kapoor et al., 2001]. They are used in the processing of textile fibers such as flax, jute and hemp, coffee and tea fermentation, vegetable-oil extraction and the treatment of paper pulp [Kashyap et al.,2001; Hoondal et al., 2002; Zhang et al., 2000; Kapoor et al., 2001]. Pectinases are also used in bioscouring of cotton where noncellulosic impurities from raw cotton are removed by pectinases [Li and Hardin, 1998].

2.12. β-galactosidase (β-gal)

Taking the most common example of *Escherichia coli*, β-gal is a tetramer of four identical 1023-amino acid chains. All of these chains contain five domains out of which the third one comprises much of the active site [Matthews, 2005]. β-gal can be obtained from microorganisms, plants, animals and from recombinant sources. Microbial β-gal is of great interest due to its thermostability, thermoresistant and thermoacidophilic properties [Asraf and Gunasekaran, 2010]. To increase the stability and reusability, β-gal are immobilized on the surface of both organic and inorganic materials by adsorption, covalent attachment, chemical aggregation, entrapment and microencapsulation [Hussain, 2010]. β-gal is important due to its lactose hydrolysing properties (Fig 2.3) which has various industrial applications such as development of lactose hydrolysed products for lactose intolerant people.

The formation of galacto-oligosaccharides (GOS) during lactose hydrolysis favours the growth of intestinal bacterial microflora. GOS possess bifidogenic properties i.e. they favour the growth of colonic bifidobacteria reducing the number of potential pathogenic bacteria. These bifidobacteria provides anticarcinogenic effects, reduction in serum cholesterol, improved liver function, reduction of the colon cancer risk and improved intestinal health. These properties of GOS find application in prebiotic food ingredients. GOS are also used as low calorie sweeteners, food ingredients, and cosmetic additives. They are included in a wide variety of foods such as soft drinks, cookies, cereals, chewing gums, candies, ice cream, yoghurts, powdered milk, clabbered milk, etc.[Rosenberg, 2006].

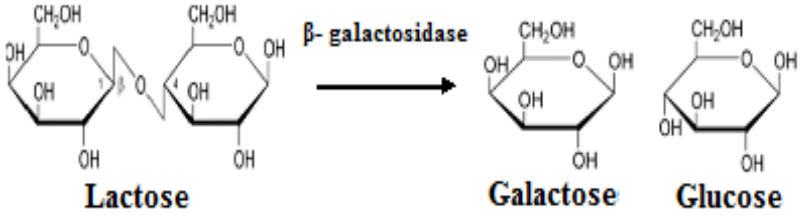


Figure 2.3 The action of β-gal on lactose converting it into galactose and glucose subunits

Another important industrial role of β-gal lies in its ability of whey utilization. Whey has enormous therapeutic applications but the problem was that only an insignificant part of whey is used for production of protein concentrates or permeates. The major part of whey remained unutilised and was disposed off causing severe water pollution. So, the bioconversion of whey into commercially important products like ethanol and β-gal was introduced since the market demand of β-gal has increased due to increase in number of lactose intolerant people [Asraf and Gunasekaran, 2010].

2.13. Glucose-6-phosphatase

Glucose-6-phosphatase (G6P) is an enzyme that carries out the hydrolysis of glucose-6-phosphate to form free glucose and phosphate group (Fig 2.4). This reaction is the final step of gluconeogenesis and plays an important role in maintaining the blood glucose levels.

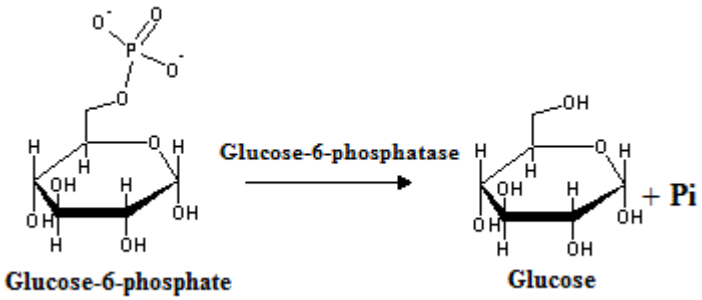


Figure 2.4 The conversion of G-6-P to glucose

Deficiency of G6P causes a metabolic disease called glycogen storage disorder and it is characterized by poor tolerance to fasting, retardation in growth and hepatomegaly due to accumulation of fat and glycogen in the liver [Froissart et al., 2011].

2.14. Xanthan gum [Kumar et al., 2011; Morris and Harding, 2009; Sanford et al., 1984]

Polysaccharides made by microbes are secreted from the cell to form a layer over the surface of the organism. Their functions are mainly protective as they act as physical barrier by binding and neutralizing bacteriophage. They also prevent phagocytosis by other organisms or the cells of the immune system. They also have roles in adhesion and penetration of the host. In appropriate environment they may also prevent dehydration. Xanthan or commonly called xanthan gum is a highly electronegative or polyanionic extremely large molecule with an average molecular weight ~2.5×10^6 g mol^{-1} secreted by *Xanthomonas campestris* which is responsible for cabbage blight. The production of xanthan gum is around 10000 tonnes a

year and it costs around US $14 per kg.The original commercial producer of xanthan was Kelco Ltd (now CPKelco). Xanthan has a β(1—>4)-linked glucan main chain with alternating residues substituted on the 3-position with a trisaccharide chain containing two mannose and one glucoronic acid residue (Fig 2.5). Some of the mannose residues may also carry acetyl groups.

Xanthan was approved as food grade by the US Food and Drug Administration nearly 30 years ago. Because of its property of solubility in hot and cold water and its very high thickening and suspending potential it is used as viscosifier in food industry. Xanthan suspensions have high acid stability and hence it is popular in sauces, syrups, toppings and salad dressings. The high freeze-thaw stability of xanthan suspensions makes it particularly attractive for the frozen food industry. Xanthan has a high suspending and stabilizing properties that are used by the animal feed industry for transporting liquid feeds with added vitamins and other supplements, which would otherwise sediment out during transport or with storage time. It is used as an additive to fruit drinks to reduce tooth decay. It is also used in cosmetic industry, shampoos, suspending adhesive agents for wallpapers, for producing sharp prints with dyes in textile industry.

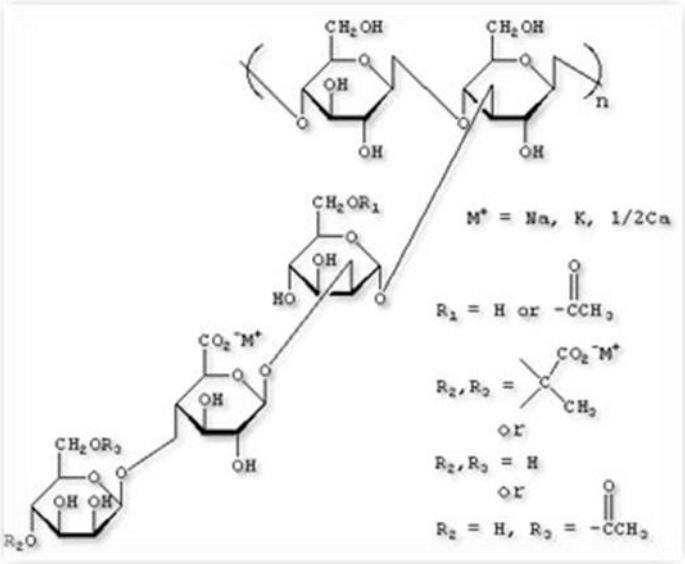


Figure 2.5 Structure of xanthan gum
(http://www.biokimkimya.com/content.asp?id=14&v=c&d=p&pid=1061&l=EN)

The capsular polysaccharides (CPSs) are often highly immunogenic, and may have evolved their unusual diversity as a way of avoiding antibody responses, advantage of this feature can be taken in the development of vaccines.

3. Effect of low power microwave on growth, enzyme activity (amylase and pectinase) and EPS production

3.1. Materials and Methods

3.1.1. Culture maintenance

Bacteria were maintained on nutrient agar (HiMedia, Mumbai). All the cultures were maintained at 4-8°C. Paraffin slants were also prepared for the same by overlaying light paraffin oil (light; density, 0.830-0.860 g/L) on the slants such that it covers the slant fully preventing air exposure. Glycerol stocks (glycerol at 10% v/v concentration; Merck) were also prepared for the same broths and stored at -20°C in cryovials. Subculturing was done for all the test organisms once in a month.

3.1.2. Culture activation

Culture activation was done in such a way that we had exponential growth phase while performing the experiment. Bacteria was activated just one day before experiment by picking colonies from working plate and streaking it on a new sterile plate/slant containing respective media using sterile wire-loop.

3.1.3. Inoculum preparation

A loopful of culture was transferred to nutrient agar slant/plate and incubated for 24 hours (72 hours for *Xanthomonas campestris* and 48 hours for *Streptococcus mutans*). The growth temperature for all the test organisms was maintained as given in the Table 3.1. Few isolated colonies were suspended in sterile normal saline (0.85% NaCl), standardized to 0.5 McFarland turbidity standard.

3.1.4. MW oven and its maintenance

For MW treatment, MW kitchen oven (Electrolux® EM30EC90SS) was used. This MW oven generates 2.45 GHz MW at different power (90-900 W). Baseplate (30 cm diameter) of the oven rotated at a speed of 5 rpm. 70% alcohol was used to clean MW oven before starting the experiment.

MW treatment

- Frequency: 2450 MHz
- Power: 90W
- Treatment: Continuous MW
- Physiology of cells: Overnight grown bacterial cell culture, 72 hours old culture for *X. campestris* and 48 hours old culture for *S. mutans*
- Inoculum volume: 5 ml in normal saline
- Duration of exposure: 2, 4, 6 min

Increase in power of MW, can result in heat generation in the system. But as we are interested in athermal effect of MW so generation of heat should be avoided. Therefore, low power MW (90 W) was chosen.

3.1.5 MW treatment to inoculum

Inoculum was prepared (0.5 McFarland equivalent) by suspending colonies in 20 mL N-saline so as to avoid the problem of heterogeneity. 5 mL inoculum from it was added to sterile 15 mL screw cap flat bottom glass vial (Borosil®). Glass vial that contained inoculum was kept along with control (glass vial containing inoculum but not to be treated with MW) in ice for 5 min so as to bring the initial temperature to the same level. To avoid the thermal effect, the glass vial was placed into ice containing beaker (100 mL; Borosil®) in such a way that ice remains between base of beaker and base of vial, and the vial was also covered with ice up to its neck. Hence, the ice did not allow the temperature to increase. This system (vial+beaker) was placed in the centre of the base plate and the MW oven was operated at low power (90 W) for different duration of time. The non-MW treated inoculum was used as control. After the treatment was over, MW treated inoculum was inoculated as soon as possible (within 5 min) in media. The inoculum size was kept 5% (v/v). The internal temperature of MW oven was checked and maintained at room temperature before MW treatment.

3.1.6 Growth measurement:

Sterility control (uninoculated autoclaved medium) was used as blank for optical density measurements. Vortexing was done before taking OD (Optical Density) to disperse the microbial cells. OD for all the tubes was measured at 625 nm (Spectronic 20D+, Thermo scientific). In case of *S. mutans*, the growth obtained in the medium was in particulate form. To solve this problem, the culture medium containing growth was first centrifuged at 7500 rpm (nüve®, high speed cooling centrifuge NF 800R) for 15 min and the pellet obtained was resuspended in 5 ml of distilled water and was vortexed (Cyclo mixer CM 101; Remi). This suspension was used for growth measurements and distilled water was used as blank. All the experiments were performed in triplicate and mean value was recorded along with standard deviation.

3.1.7. Amylase estimation [Nigam and Ayyagari, 2008]:

Reagents:

1. DNSA reagent: 30.0 g sodium-potassium tartrate (*HPLC* Mumbai, AR) was dissolved in 50 mL distilled water. 20 mL of 2 M NaOH was added to it. After mixing, 1.0 g of DNSA (3,5-dinitrosalicylic acid; *HPLC* Mumbai, LR) powder was added and final volume was adjusted to 100 mL.

2. Buffer: 0.1 M citrate buffer of pH 6.0 (Appendix II).
3. Substrate: 1% starch solution was prepared by adding 1 g of starch in 100 mL of citrate buffer.

Table 3.1 Experimental conditions

Organism	Medium for inoculum growth	Inoculum age (h)	Medium	System (mL)	Incubation temperature (° C)	Incubation time (h)	Incubation condition
For amylase							
B. subtlis (MTCC 619)	1% starch containing nutrient agar	24	1% Starch containing minimal media	5	30	24	Static
S. mutans (MTCC 497)		48	BHI (3.7g/L) containing 1% starch		35	20	
For pectinase							
P. carotovora (MTCC 1428)	1% pectin containing nutrient agar	24	1% pectin containing minimal media	5	30	92	Static
B. subtilis (MTCC 619)		24	1% pectin containing minimal media		30	90	
For EPS							
X. campestris (MTCC 2286)	Nutrient agar	72	Tryptone Yeast (TY)	30	28	72	Shaking (120 rpm)
S. mutans (MTCC 497)		48	BHI containing 2% sucrose		35	72	Shaking (100 rpm)

Procedure:

1. Culture broth after growth measurement was centrifuged at 7500 rpm for 10 min and cell free supernatant (CFS) was used as a live enzyme source.
2. 0.5 mL buffer, 0.5 mL CFS (replaced with distilled water in the case of blank), and 1.0 mL substrate was pipetted out in a test tube, and incubated at 50^{o} C for 30 min.
3. Following incubation 2 mL DNSA reagent was added to each test tube and the tubes were kept in boiling water bath for 5 min. Absorbance was read at 540 nm and the amount of sugar released was estimated by plotting the values of OD on standard curve of maltose (Appendix IV).
   Using the amount of sugar produced, enzyme activity was calculated.

$$\text{Enzyme activity (IU)} = \frac{\text{Net amount of sugar produced (µg)}}{\text{MW X T}}$$

MW = Molecular weight of maltose (342 g/mol)

T = Reaction time (30 min)

3.1.8. Pectinase estimation [Patil et al., 2012]

Reagents:

Substrate: 0.5% pectin (HiMedia) in 0.025 M acetate buffer (pH 6.0).
DNSA reagent.

Procedure:

1. After growth measurement, culture broth was centrifuged at 7500 rpm for 10 min and CFS was used as live enzyme source.
2. 0.5 mL of CFS (acetate buffer in case of blank), and 0.5 mL of substrate solution was added and incubated at 50^{o} C for 10 min.
3. 1.0 mL of DNSA reagent was added and the tubes were put in boiling water bath for 5 min.
4. To remove the unutilized pectin, each sample was centrifuged at 7500 rpm for 15 min.
5. Absorbance of the supernatant was measured at 575 nm and the amount of sugar produced was estimated by plotting the values of OD on standard curve of monogalacturonic acid (Appendix IV). Reading of sterility control was subtracted from each to avoid interference of sugar present in any form in the inoculation medium.
6. Enzyme activity was calculated using the equation given below:

$$\text{Enzyme activity (IU)} = \frac{\text{Net amount of sugar produced (µg)}}{\text{MW X T}}$$

MW = Molecular weight of monogalacturonic acid (Sigma-Aldrich; 212.12 g/mol)

T = Reaction time (10 min)

3.1.9. EPS quantification [Li et al., 2012]:

Procedure:

1. 20 ml of CFS was collected after centrifugation at 7500 rpm for 10 min. This supernatant was precipitated with two volumes of chilled acetone (Merck; AR) i.e. 40 mL in one run.

For *X. campestris*:

2. After 30 min, the precipitation was visibly seen and it was filtered by Whatman™ # 1 filter paper (125 mm Ø; Whatman International Ltd., England). Before filtering, the pre-weight of each filter paper was noted.
3. The filter papers were dried at 60^{o} C for 24 h and the dried sample was then weighed (post-weight) to calculate the total EPS produced.
4. Pre-weight was subtracted from the post-weight of each filter paper to obtain the weight of EPS produced.

For *S. mutans*:

5. After 30 min, a thin layer of EPS was formed at the base of conical flasks. The liquid was decanted and the EPS containing flask was dried at 60^{o} C for 24 h.
6. The pre-weight and the post-weight of conical flasks were noted, and by subtracting pre-weight from the post-weight, total EPS produced was obtained.

EPS produced (g/L:): $\frac{Weight\ obtained\ (g)\ X\ 1000}{20}$

3.1.10. Statistical analysis:

All the experimental data obtained was subjected to statistical analysis using two-tailed t-test in Microsoft® Excel (Appendix V). At $p<0.05$, data was considered statistically significant (95% confidence level) and at $p<0.01$, data was considered highly significant (99% confidence level).

Null hypothesis (H_o) is MW irradiated sample = Control

At $p<0.05$, null hypothesis was rejected whereas in case where $p>0.05$, null hypothesis was accepted.

3.2. Results and Discussion

3.2.1. Effect on growth and amylase activity

Effect of MW on growth of microbial cells and its amylase activity was investigated in two different microorganisms (*B. subtilis* and *S. mutans*). The results of microbial growth from MW treated and MW non-treated inoculums were expressed in terms of OD and the results of amylase activity were expressed in IU (International Unit).
While calculating % change of growth in MW treated cells in terms of OD and amylase activity in terms of IU, the value for non-treated cells were taken as 100%.

Table 3.2 Effect of MW on growth and amylase activity in *B. subtilis*

Duration of MW treatment (min)	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Amylase activity (IU/mL) (Mean ± SD) ($X\ 10^{-3}$)	% change compared to control
0	0.093 ± 0.005	0.00	44.28 ± 1.60	0.00
2	0.086 ± 0.005	-7.52	23.34 ± 0.00	-47.28**
4	0.086 ± 0.005	-7.52	34.50 ± 0.44	-22.08
6	0.100 ± 0.000	7.52	14.42 ± 0.00	-67.43**

** $p < 0.01$; minus sign indicates a decrease over control

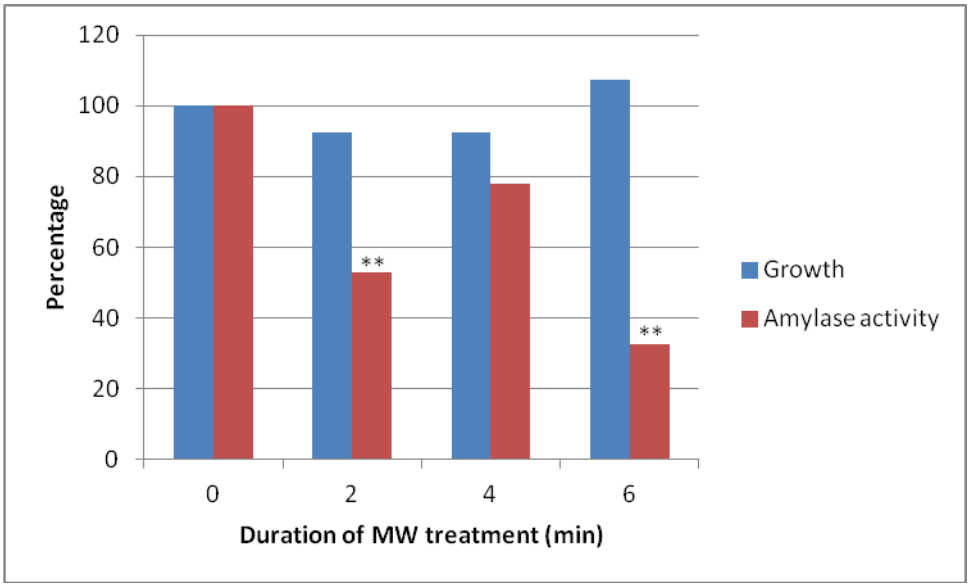


Figure 3.1 Comparison of effect of different duration of MW exposure on growth and amylase activity in *B. subtili*

Table 3.3 Effect of MW on growth and amylase activity in *S. mutans*

Duration of MW treatment (min)	Growth (OD_{625}) [Mean ± SD]	% change compared to control	Amylase activity (IU/mL) [Mean ± SD] X ($X10^{-3}$)	% change compared to control
0	0.077 ± 0.000	0.00	26.06 ± 0.00	0.00
2	0.079 ± 0.000	2.59*	27.98 ± 0.00	7.36
4	0.077 ± 0.001	0.00	28.38 ± 0.00	8.90*
6	0.074 ± 0.000	-3.89*	22.56 ± 0.00	-13.43*

*p<0.05; minus sign indicates a decrease over control

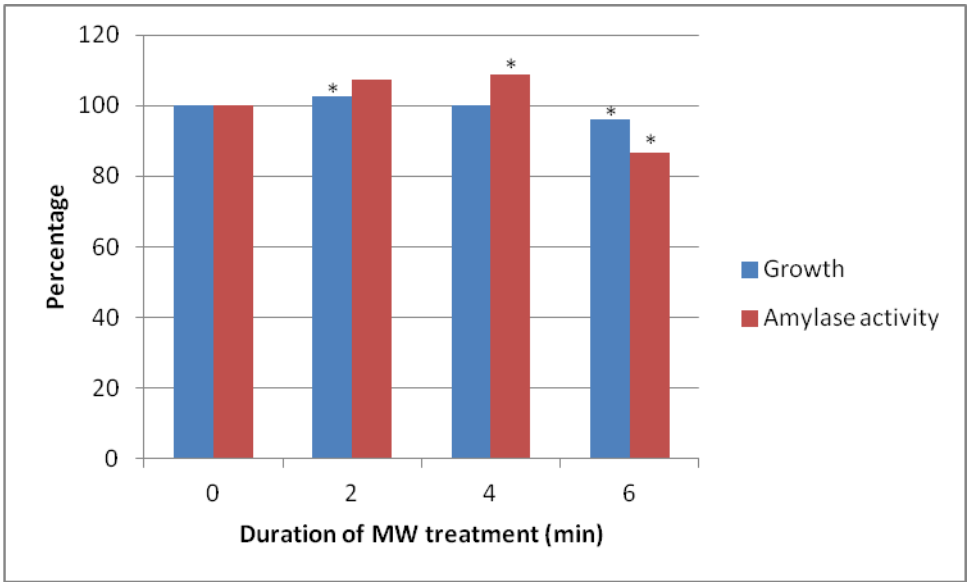


Figure 3.2 Comparison of different duration of MW on growth and amylase activity in *S. mutans*

Growth of *B. subtilis* remained unaffected by MW exposure for all durations (Table 3.2). However amylase activity suffered a significant decrease after 2 min and 6 min exposure. *S. mutans* responded differently to MW treatment than *B. subtilis*. Growth of the former experienced a little enhancement after 2 min and 6 min treatment, whereas the amylase activity was influenced by 4 min and 6 min duration of MW exposure. Albeit small, but significant increase in amylase activity of S. mutans was observed after 4 min MW treatment. Amylase has been among the most important microbial enzymes with wide-ranging applications in baking, brewing, and textiles [Aiyer, 2005]. In case of both the organisms

growth and amylase activity were affected by MW exposure independently, despite starch being the major carbon source in the medium.

3.2.2. Effect on growth and pectinase activity:

Effect of MW on growth of microbial cells and its pectinase activity was performed on two different microorganisms (*P. carotovora* and *B. subtilis*). The results of microbial growth from MW treated and MW non-treated inoculums were expressed in terms of OD and the results of amylase activity were expressed in IU.

While calculating % change of growth in MW treated cells in terms of OD and amylase activity in terms of IU, the value for non-treated cells were taken as 100%.

Table 3.4 Effect of MW radiation on growth and pectinase activity in *P. carotovora*

Duration of MW treatment (min)	OD_{625} [Mean ± SD]	%change compared to control	Pectinase activity (IU/mL) [Mean ± SD] ($X\ 10^{-2}$)	%change compared to control
0	0.483 ± 0.007	0.00	55.08 ± 8.44	0.00
2	0.523 ± 0.021	8.28	173.56 ± 5.82	215.10**
4	0.367 ± 0.036	-24.01*	30.64 ± 4.30	-44.37*
6	0.488 ± 0.007	1.03	26.88 ± 0.00	-51.19*

*$p<0.05$, **$p<0.01$; minus sign indicates a dcrease over control

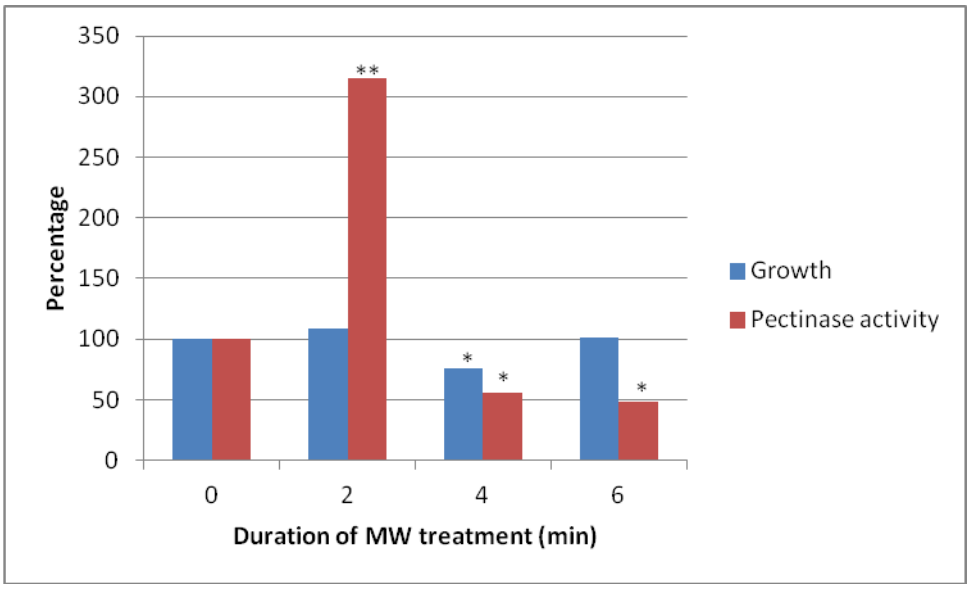


Figure 3.3 Comparison of different duration of MW on growth and pectinase activity in *P. carotovora*

Table 3.5 Effect of MW radiation on growth and pectinase activity in *B. subtilis*

Duration of MW treatment (min)	OD_{625} [Mean± SD]	%change compared to control	Pectinase activity (IU/mL) [Mean± SD] ($X\ 10^{-2}$)	%change compared to control
0	0.416 ± 0.017	0.00	1.30 ± 0.00	0.00
2	0.417 ± 0.001	0.24	0.00 ± 0.00	-100*
4	0.421 ± 0.001	1.20	220.90 ± 2.06	16892**
6	0.416 ± 0.008	0.00	84.56 ± 2.06	6404*

*$p<0.05$, **$p<0.01$; minus sign indicates a decrease over control

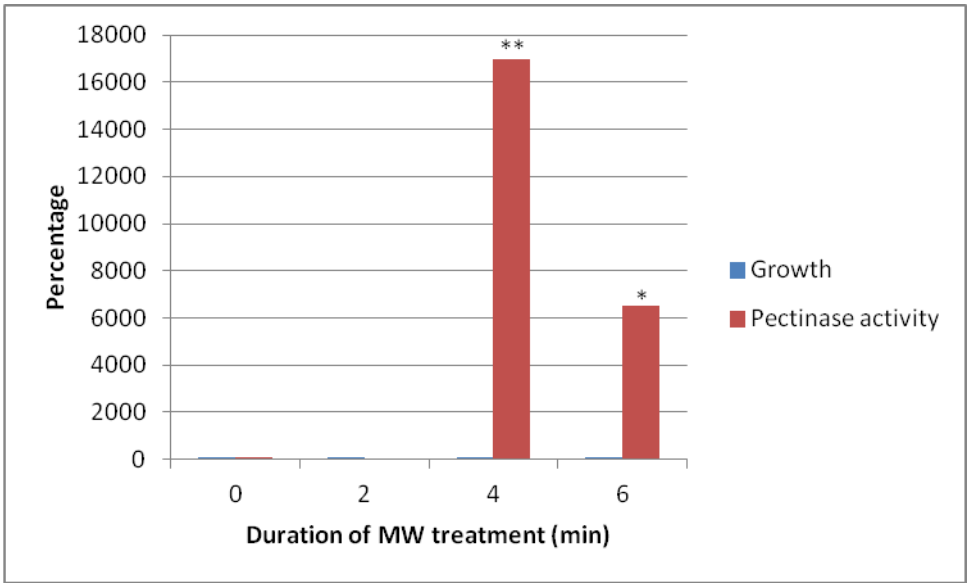


Figure 3.4 Comparison of different duration of MW on growth and pectinase activity in *B. subtilis*

Pectinase activity was influenced significantly by all durations of MW exposure in both the test organisms, *P. carotovora* and *B. subtilis* (Table 3.4 and 3.5). MW exposure of 2 min resulted in a significant increase in the pectinase activity in *P. carotovora*, whereas 4 min and 6 min MW exposure almost halved the pectinase activity. Pectinase has been implicated as an

important virulence factor for plant pathogens like *P. carotovora* [Lei et al., 1985]. If a reliable protocol can be developed for attenuation of pectinase activity in such phytopathogens, it can be interesting with respect to control of crop damage by these organisms. In such cases, where MW radiation reduces expression of particular virulence factor, it may be used for attenuation of pathogenic strains. The potential use of MW irradiation to improve vaccine preparation productivity and efficacy against *Fusobacterium necrophorum* was indicated by Craciun et al. (2009).

Though the pectinase activity in *B. subtilis* was heavily influenced by MW treatment, its growth remained unaffected. Inability of low power MW radiation to cause any change in growth of *B. subtilis* was observed while growing this organism in both pectin containing medium (Table 3.5), as well as in starch containing medium (Table 3.2). Previously also, while investigating influence of low power MW on multiple prokaryotic and eukaryotic microorganisms, we found MW radiation unable to influence growth of *B. subtilis* [Dholiya et al., 2012]. Tahir et al. (2009) also reported that viability of *B. subtilis* remained unaffected by short MW exposures in controlled temperature experiments. Neither colony morphology nor cell shape was altered due to MW exposure for 60-180 s.

It is interesting to note that MW treatment for the same duration had quite opposite impact on the pectinase activity of *B. subtilis* and *P. carotovora*. The former completely lost its pectinase activity after 2 min MW exposure, the later experienced a heavy increase (215.10%) in the same (Table 3.4 and 3.5). MW exposure of 4 and 6 min caused a significant decrease in the pectinase activity of *P. carotovora*, whereas same duration MW exposures caused a significant increase in the pectinase activity of *B. subtilis*. Pectinases are enzymes of commercial importance, particularly for depectinization in fruit juice industry [Prathyusha and Suneetha, 2011].

3.2.3. Effect on growth and EPS production:

Effect of MW on growth of microbial cells and its amylase activity was performed on two different microorganisms (*X. campestris* and *S. mutans*). The results of growth are expressed in terms of OD and that of EPS production in terms of g/L.

Table 3.6 Effect of MW on growth and EPS production in *X. campestris*

Duration of MW treatment (min)	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Amount of EPS produced (g/L) (Mean ± SD)	% change compared to control
0	0.735 ± 0.007	0.00	0.52 ± 0.04	0.00
2	0.775 ± 0.007	5.44*	0.56 ± 0.00	7.69

4	0.805 ± 0.007	9.52*	0.76 ± 0.00	46.15**
6	0.815 ± 0.007	10.88**	0.80 ± 0.04	53.84*

*$p<0.05$; **$p<0.01$

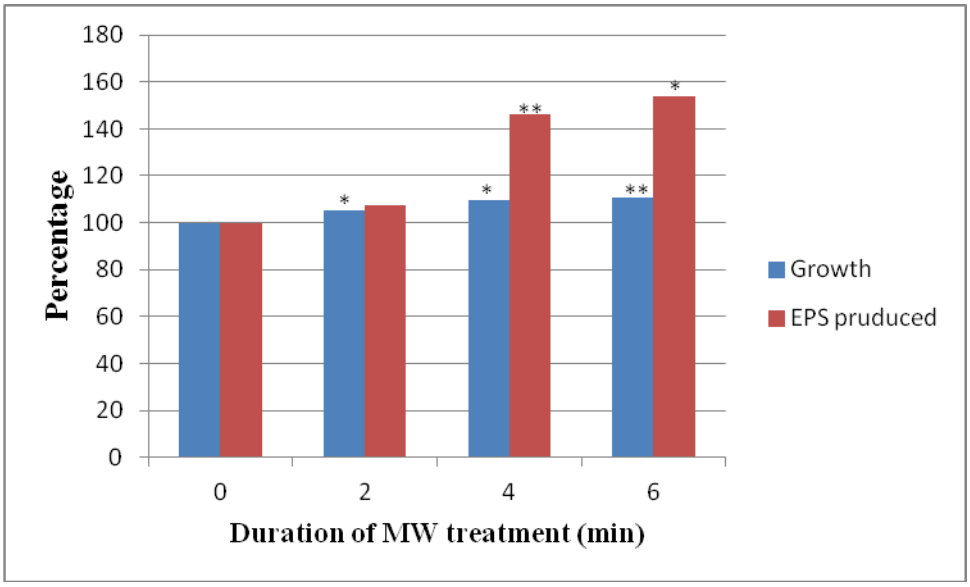


Figure 3.5 Comparison of effect of different duration of MW exposure on growth and EPS production in *X. campestris*

Table 3.7 Effect of MW on growth and EPS production in *S. mutans*

Duration of MW treatment (min)	Growth (OD_{625}) [Mean ± SD]	% change compared to control	Amount of EPS produced (g/L) [Mean ± SD]	% change compared to control
0	0.730 ± 0.024	0.00	6.60 ± 0.20	0.00
2	0.870 ± 0.005	19.17**	5.05 ± 0.10	-23.48*
4	1.790 ± 0.014	145.20**	7.85 ± 0.70	18.93
6	0.830 ± 0.007	13.69*	6.35 ± 1.25	-3.78

*$p<0.05$, **$p<0.01$; minus sign indicates a decrease over control

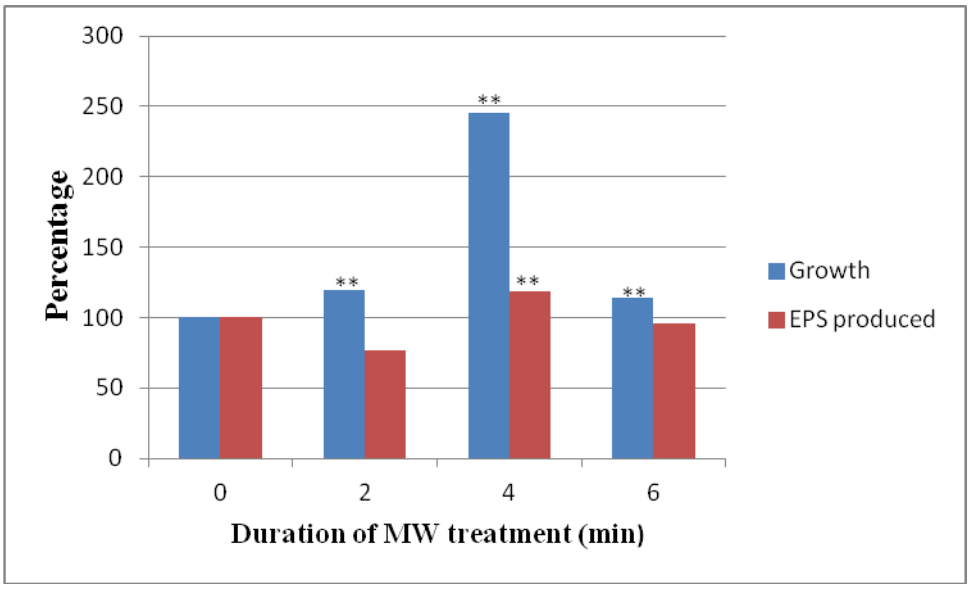


Figure 3.6 Comparison of effect of different duration of MW exposure on growth and EPS production in *S. mutans*

MW treatment for all the durations was able to cause a significant increase in growth of both the EPS producers, *X. campestris* and *S. mutans* (Table 3.6 and 3.7). A 2 min MW exposure to *S. mutans* resulted in a significant decrease (23.48%) in EPS production by *S. mutans*, whereas the same duration of MW treatment had no effect on EPS production by *X. campestris*. Similarly, MW treatments of 4 and 6 min duration significantly enhanced EPS production by *X. campestris*, but had no effect on EPS production by *S. mutans*. This variation in response to MW radiation of different microbial species is likely to be due to the fact that different species have varying susceptibilities to MW radiation [Najdovski et al. 1991]. There seemed to be no correlation between effect of MW on EPS production, and that on growth i. e. growth and EPS production were affected by MW treatment independent of each other. Enhancement of microbial polysaccharide production by MW treatment or any other method can be of considerable industrial interest, as they have found multiple applications in fields including food, pharmaceutical, medical (e.g. development of vaccines), cosmetics, etc. [Sandford et al., 1984]. EPS produced by *X. campestris* (xanthan gum) has been a product of commercial importance since long. It is useful as a packaging material, in tooth-pastes, in oil well drilling, and has a market value of approximately US$ 14 per kg [Morris and Harding, 2009].

Response of *S. mutans* to MW treatment was not the same, when grown in two different media. MW exposure of 4 min was not able to induce any change in growth of *S. mutans* in starch containing medium (Table 3.3), whereas this exposure of 4 min heavily induced its growth in sucrose containing BHI (Table 3.7). Medium composition may not necessarily have a role in this, because effect of MW radiation on microbial cells can be random, as can happen with ultraviolet radiation or any other part of electromagnetic spectrum. The possibility of non- uniform distribution of MW radiation in different layers of irradiated microbial suspension can be the major reason for random effect of MW (which

may be difficult to reproduce) on given test organism even when experiments on non-thermal effects are performed under identical conditions [Rojavin and Ziskin, 1995].

Increase in enzyme activity following MW treatment may be due to direct effect of MW radiation on genetic or cellular machinery of the test organism, and this effect may or may not be heritable. However, increased enzyme activity, particularly in case of extracellular enzymes may not necessarily be due to genetic effect of MW radiation. It may be due to increased secretion of extracellular enzymes, as MW are known to alter membrane permeability in both gram-positive and gram-negative bacteria [Chen et al., 2007]. Reports dealing with the biological effects of low-level MW radiation, which did not produce significant thermal induction, suggest that molecular or membrane interaction of MW with living systems can lead to alteration of function [Buckle, 1985]. While comparing effects of sub-lethal MW radiation and conventional heating on the metabolic activity of *Staphylococcus aureus*, Dreyfuss and Chipley, (1980) reported increase in specific activities of many key enzyme systems due to MW radiation, which could not be explained solely by thermal effects. Ability of MW irradiation to increase the enzymatic activity of bacterial suspensions has also been demonstrated in members of the family Enterobacteriaceae [Spencer et al., 1985].

Results of present study suggest in favor of existence of MW specific athermal effects on microbial systems. However, such studies (either in favour of non-thermal MW effects at present can only add fuel to the controversy over non-thermal effects of MW radiation rather than settling it. For this controversy to end, it is required to have data about impact of microwaves of varying frequencies and power for different time durations on both prokaryotic and eukaryotic biological systems. Such data can be of help in deciphering the mechanism of MW specific effects on cells and their biomolecules. Due to the ease of handing them in laboratory, microorganisms can be conveniently used to study the effect of MW on living systems, and further probing into the underlying mode of their action. Additionally, employing mutagenic frequencies of MW radiation for microbial strain improvement can be of considerable industrial significance. Whether the MW effects are heritable, also remains an interesting problem to investigate.

4. Effect of low power microwave on growth, protein synthesis and intracellular enzymes (glucose-6-phosphatase and β-galactosidase)

4.1 Materials and Methods

4.1.1 Test organisms

Bacillus subtilis (MTCC No. 619)

Lactobacillus acidophilus (MTCC No. 447)

Escherichia coli **(**MTCC No. 1687**)**

4.1.2 Experimental outline

1. Inoculum was prepared in normal saline and was standardised to the turbidity of 0.5 McFarland standard and MW treatment was given to the inoculum as described in chapter 1 (Page no.) for 3 and 6 min
2. Inoculum (radiated/irradiated) was inoculated in enzyme (β-galactosidase) production medium (Appendix I)
3. All inoculated media were incubated at 35° C temperature until visible growth was observed.
4. After incubation, growth was estimated through turbidity measurement (OD_{625}).
5. Cells were removed from media to obtain CFS by centrifugation and CFS was used for the estimation of extracellular protein
6. Cell lysate was obtained for the estimation of intracellular protein, glucose-6-phosphatase and β-galactosidase activity.
7. Cell lysis was performed with little modification in the protocol as described in Harley and Prescott, (2002). Cell lysis was done by suspending the pellet obtained after centrifugation in 0.1 M phosphate buffer (pH 6.2). To this 0.05 mL of lysozyme (HiMedia) solution (2 mg/mL) was added with gentle agitation and kept for 1 h at room temperature. The tubes were cooled to 5° C in chilled water and were placed in refrigerator for 1 h. After 1 h 5 drops of chloroform (Merck; AR) were added and the tubes were agitated manually to mix the chloroform. This reaction mixture was kept for 2 h at room temperature for *E. coli*, and for overnight for the lysis of *L. acidophilus* and *B. subtilis*. The tubes were centrifuged at 7500 rpm for 10 min to obtain intracellular content of the cells.

4.1.3 Estimation of intra- and extracellular protein content [Nigam and Ayyagari, 2008]

Reagents:

1. Biuret reagent: It was prepared by dissolving 0.3 g $CuSO_4$ and 0.9 g Na-K-tartrate in 50 mL of 0.2 N NaOH solution. After mixing, 0.5 g of KI was added and final volume was made up to 100 ml by using 0.2 N NaOH.
2. Stock solution of bovine serum albumin (BSA; HiMedia) of concentration 5 mg/mL in distilled water was used for preparing standard.

Procedure:

1. CFS was used a source of extracellular protein.
2. 1.0 mL of CFS (distilled water in case of blank) was mixed with 1.5 mL of Biuret reagent and incubated at 37º C for 10 min.
3. The formation of purple colour indicated the presence of protein in the sample. Absorbance was measured at 520 nm. For estimation of intracellular protein, cell lysate in equal volume was used instead of CFS.
4. The protein concentration was calculated by plotting the values of optical density on standard curve for BSA.
5. Total protein was calculated by adding intra- and extracellular protein.

4.1.4 Glucose-6-phosphatase estimation [Nigam and Ayyagari, 2008]:

Reagents:

1. Buffer: 0.1 M citrate buffer of pH 6.0 containing 1.0 mM EDTA.
2. Substrate: 1 mM Glucose-6-phosphate in same buffer.
3. Molybdate reagent: 6% ammonium molybdate in distilled water.
4. 10% Trichloroacetic acid (TCA; Central Drug House, LR)
5. Acidified ferrous sulfate solution: 2 g $FeSO_4$. $7H_2O$ was mixed in 20 mL distilled water which was then acidified with 0.5 mL of 0.9 N H_2SO_4. Final volume was made upto 25 mL.
6. 9 N H_2SO_4: 6 mL H_2SO_4 was added in 19 mL of distilled water.

Procedure:

1. Cell lysate was used as a live enzyme source.
2. 0.5 ml of cell lysate (distilled water in case of blank), 0.5 ml of substrate and 1 ml of buffer was taken in test tubes. It was incubated at 50º C for 30 min.
3. 10 ml of 10% TCA was added to terminate the reaction.
4. 1 ml from each tube was taken into fresh test tubes to which 1.5 ml distilled water and 0.5 ml of 9 N H_2SO_4 was added.
5. The mixture was allowed to stand for 15 min and then 0.5 ml of molybdate reagent was added.

6. The mixture was vortexed and was incubated in dark for 15 min. The test tubes be capped with aluminium foil for incubation.
7. 0.5 ml of ferrous sulphate reagent was added to each tube and it was again incubated in dark for 15 min.
8. Absorbance was measured at 660 nm for all tubes and estimation of amount of phosphate released was calculated by plotting the values of optical density on standard plot for inorganic phosphate (Appendix 1V).

4.1.5 Estimation of β-galactosidase activity: [Asay et al., 2008]

Reagents:

1. Buffer: TM buffer (50 mM Tris-Cl + 10 mM $MgSO_4$)
2. Substrate: 5 mM ONPG (HiMedia, LR) solution in TM buffer.
3. 0.6 M Na_2CO_3

Procedure:

1. Cell lysate was used as a live enzyme source.
2. 1.1 mL of cell lysate was added to tubes containing 0.2 ml ONPG (o-nitrophenyl β-D-galactopyranoside) solution and 0.2 mL of TM buffer (Appendix II).
3. The tubes were vortexed and kept at 30° C until the yellowish colour appears. In case of *E. coli*, the colour developed in 2 h while in case of *L. acidophilus* and *B. subtilis*, yellow colour developed after an overnight incubation.
4. The reaction was stopped by adding 2 mL of 0.6 M Na_2CO_3.
5. The absorbance was measured at 410 nm.
6. The β-galactosidase activity was calculated using the expression

$$A = \varepsilon dc$$

Where, ε = molar extinction coefficient of ONP which was taken 4500 M^{-1} cm^{-1} [Becerra et al.,1998]

d= thickness of the sample which was taken as 1 cm

c = molar concentration

A = absorbance at 410 nm

4.2 Results and Discussion

Table 4.1 Effect of MW on growth and protein synthesis in *B. subtilis*

Duration of MW treatment (min)	OD_{625} (Mean ± SD)	% change compared to control	Extracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Intracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Total protein (mg/ml) (Mean ± SD)	% change compared to control
0	0.077 ± 0.001	0.00	1.127 ± 0.128	0.00	0.309 ± 0.139	0.00	1.425 ± 0.031	0.00
3	0.086 ± 0.009	11.68	1.116 ± 0.149	-0.97	0.298 ± 0.000	-3.55	1.419 ± 0.037	-0.42
6	0.082 ± 0.003	6.49	1.053 ± 0.128	-6.56*	0.298 ± 0.128	-3.55	1.350 ± 0.000	-5.26

*$p < 0.05$; minus sign indicates a decrease over control

Table 4.2 Effect of MW radiation on G6P and β-galactosidase activity in *B. subtilis*

Duration of MW treatment (min)	Amount of inorganic phosphate released (µg/ml) (Mean ± SD)	% change compared to control	Amount of ONP released (M) (Mean ± SD) ($X\ 10^{-4}$)	% change compared to control
0	82.19 ± 0.00	0.00	1.44 ± 0.02	0.00
3	103.93 ± 0.00	26.45	1.08 ± 0.02	-25.00**
6	104.58 ± 0.00	27.24*	1.54 ± 0.01	6.94

*$p < 0.05$, **$p < 0.01$; minus sign indicates a decrease over control

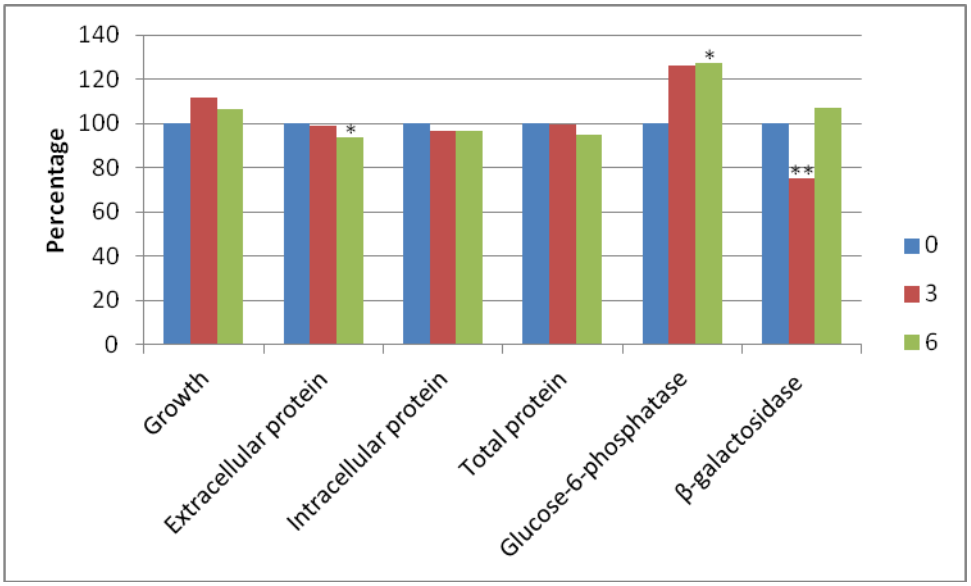


Figure 4.1 Comparison of different duration of MW radiation on growth, protein synthesis, glucose-6-phosphatase and β-galactosidase activity in *B. subtilis*

Table 4.3 Effect of MW on growth and protein synthesis in *L. acidophilus*

Duration of MW treatment (min)	OD_{625} (Mean ± SD)	% change compared to control	Extracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Intracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Total protein (mg/ml) (Mean ± SD)	% change compared to control
0	0.217 ± 0.002	0.00	0.927 ± 0.449	0.00	0.209 ± 0.127	0.00	1.136 ± 0.096	0.00
3	0.220 ± 0.003	1.38	0.768 ± 0.000	-17.15	0.309 ± 0.139	47.84**	1.043 ± 0.016	-8.18
6	0.208 ± 0.002	4.14	0.800 ± 0.417	-13.70	0.303 ± 0.139	44.97**	1.069 ± 0.067	-5.89

** $p < 0.01$; minus sign indicates a decrease over control

Table 4.4 Effect of MW radiation on G6P and β-galactosidase activity in *L. acidophilus*

Duration of MW treatment (min)	Amount of inorganic phosphate released (µg/mL) (Mean± SD)	% change compared to control	Amount of ONP released (M) (Mean ± SD) (X 10^{-4})	% change compared to control
0	86.10 ± 0.00	0.00	0.76 ± 0.06	0.00
3	89.58 ± 0.00	4.04	0.54 ± 0.04	-28.94*
6	109.36 ± 0.00	27.01**	0.56 ± 0.05	-26.31*

* $p<0.05$, ** $p<0.01$; minus sign indicates a decrease over control

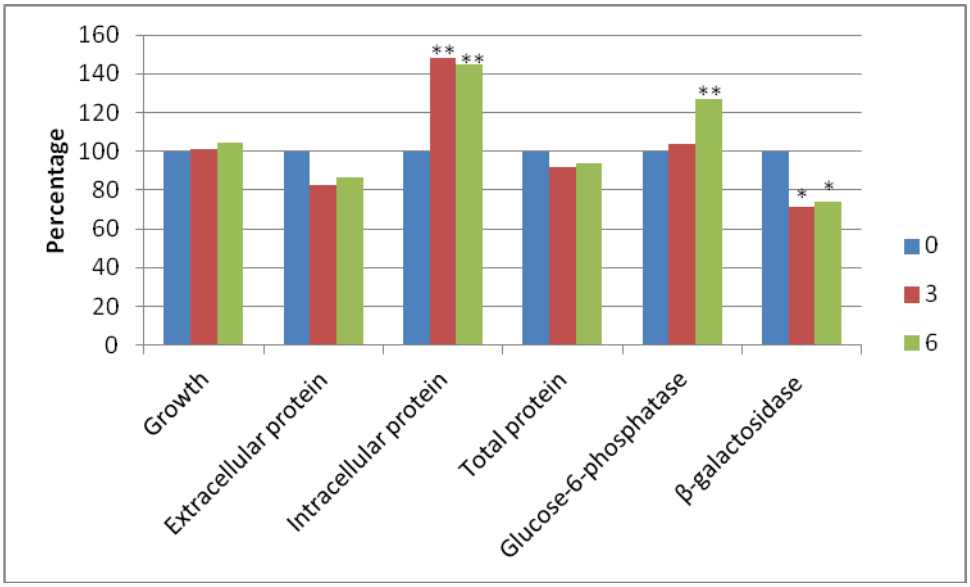


Figure 4.2 Comparison of different duration of MW radiation on growth, protein synthesis, glucose-6-phosphatase and β-galactosidase activity in *L. acidophilus*

Table 4.5 Effect of MW radiation on growth and protein synthesis in *E. coli*

Duration of MW treatment (min)	OD_{625} (Mean ± SD)	% change compared to control	Extracellular protein (mg/mL) (Mean ± SD)	% change compared to control	Intracellular protein (mg/mL) (Mean ± SD)	% change compared to control (Mean ± SD)	Total protein (mg/mL) (Mean ± SD)	% change compared to control
0	0.206 ± 0.000	0.00	0.819 ± 0.128	0.00	0.245 ± 0.000	0.00	1.074 ± 0.030	0.00
3	0.213 ± 0.004	3.39	0.798 ± 0.128	-2.56	0.234 ± 0.128	-4.48	1.014 ± 0.031	-5.58
6	0.221 ± 0.009	7.28	0.787 ± 0.149	-3.90	0.171 ± 0.000	-30.20**	0.958 ± 0.045	-10.80

**$p<0.01$; minus sign indicates a decrease over control

Table 4.6 Effect of MW radiation on G6P and β-galactosidase activity in *E. coli*

Duration of MW treatment (min)	Amount of inorganic phosphate released (µg/mL) (Mean± SD)	% change compared to control	Amount of ONP released (M) (Mean ± SD) (X 10^{-4})	% change compared to control
0	235.69 ± 0.00	0.00	2.67 ± 0.07	0.00
3	283.52 ± 0.00	20.29*	2.02 ± 0.06	-24.34**
6	280.91 ± 0.00	19.18*	2.41 ± 0.01	-9.73*

*$p<0.05$, **$p<0.01$; minus sign indicates a decrease over control

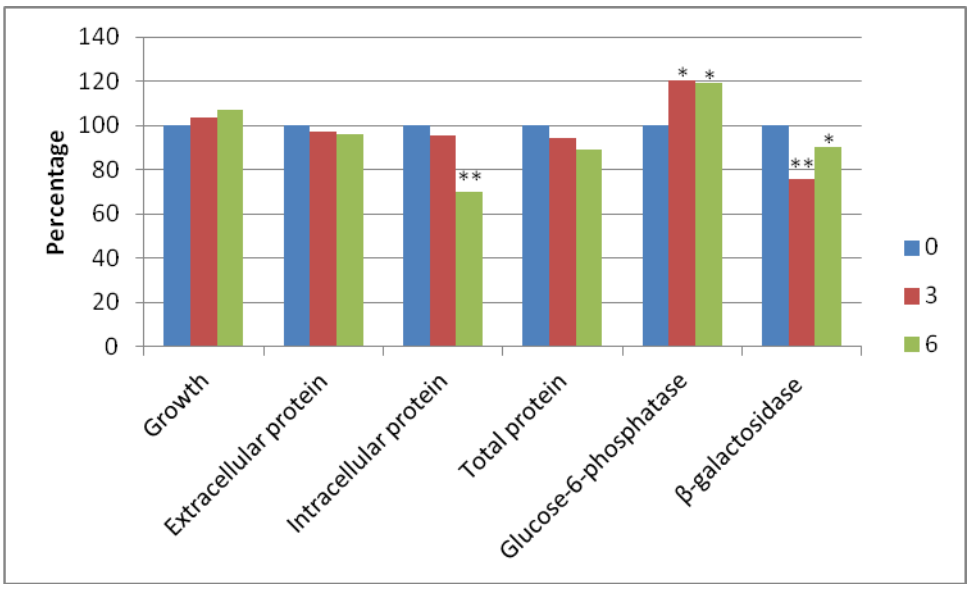


Figure 4.3 Comparison of different duration of MW radiation on growth, protein synthesis, glucose-6-phosphatase and β-galactosidase activity in *E. coli*

Table 4.7. Comparison of effect of different MW exposure on growth, protein synthesis, and enzyme activity in the test organisms

	B. subtilis		*E. coli*		*L. acidophilus*	
	3 min	**6 min**	**3 min**	**6 min**	**3 min**	**6 min**
Growth	-	-	-	-	-	-
Extracellular protein	-	↓*	-	-	-	-
Intracellular protein	-	-	-	↓*	↑**	↑**
Total protein	-	-	-	-	-	-
G6P	-	↑*	↑*	↑*	-	↑**
β- gal	↓**	-	↓*	↓*	↓*	↓*

*$p<0.05$, **$p<0.01$; ↑and ↓ indicates an increase and decrease over control respectively

Effect of MW on growth, protein synthesis, and enzyme activity in *B. subtilis* are presented in Table 4.1-4.2. MW treatment had impact neither on growth of *B. subtilis*, nor on the amount of total protein synthesized. Though intracellular protein synthesis remained unaffected by MW, a decrease in the amount of extracellular protein was noted after MW treatment of 6 min duration. Change in the estimated amount of extracellular protein may be due to effect of MW on protein synthesis *per se* or on its secretion through modification of membrane permeability. Alteration of membrane permeability upon MW treatment in *E. coli* and *Staphylococcus aureus* has been reported by Chen et al., 2007. However, Woo et al. (2000) found no effect of MW radiation (till 40º C) on amount of protein released in *B. subtilis*. Although, no significant change was found following MW treatment in the total intracellular protein content of *B subtilis* (Table 4.1), significant alterations in the activity of intracellular enzymes G6P and β-gal were noted after MW treatment of 6 min and 3 min duration respectively (Table 4.2).

Effect of MW on growth, protein synthesis, and enzyme activity in *L. acidophilus* are presented in Table 4.3-4.4. MW treatment did not cause any change in the capacity of *L. acidophilus* for growth, and extracellular protein secretion. Total protein content remained unaffected following MW treatment; however a significant increase (> 40%) was observed in the intracellular protein content for both durations of MW treatment tested. G6P activity experienced a significant increase (27.01%) following a 6 min MW treatment, whereas almost identical decrease in the β-gal activity was observed following both durations of MW treatment (Table 4.4).

Effect of MW on growth, protein synthesis, and enzyme activity in *E. coli* are presented in Table 4.5-4.6. As with other two test organisms, growth and total protein estimated in *E. coli* also remained unaffected by both durations of MW treatment. Activity of

both the intracellular enzymes was affected by MW treatment for 3 min as well as 6 min. G6P activity experienced a significant increase, whereas β-gal activity underwent significant decrease following MW treatment. This is in contrast to results reported by Asay et al. (2008), they did not find non-thermal effects to alter β-gal activity in *E. coli* C29.

Our results (Table 4.1-4.6) indicate that MW treatment (2.45 GHz; 90 W) for 3 min and 6 min duration had no effect on growth and total protein synthesis of any of the three test organisms. 6 min MW exposure was able to cause a significant decrease in extracellular and intracellular protein content of *B. subtilis* (Table 4.1) and *E. coli* (Table 4.5) respectively. Reduction in protein synthesis in microorganisms subjected to MW irradiation has also been reported earlier [Belyaev, 1992; Otludil et al., 2004]. G6P activity in all the test organisms experienced a significant increase following MW treatment for either one or both exposure times. β-gal activity in all the three test organisms experienced a significant decrease following MW treatment for either one or both exposure times. MW exposure of 3 min was able to significantly reduce β-gal activity in all three test organisms. MW exposure of 6 min was able to significantly induce G6P activity in all the three test organisms. Increase in the catalytic activity of intracellular enzymes (lactate dehydrogenase and cytochrome c oxidase) in *E. coli,* following MW (18 GHz) treatment was reported by Shamis et al. (2012). Involvement of non thermal effects of MW in altering activity of many intracellular enzymes in *Staphylococcus aureus* was suggested by Dreyfuss and Chipley (1980). Effect of MW treatment on extracellular and intracellular protein content varied from organism to organism. The observed alterations in protein content or enzyme activity may be due to mutagenic effects of MW, or it might have resulted from changes in the membrane permeability of the test organisms induced by MW radiation. Alterations in the permeability of cell walls and cell membranes of both gram-positive and gram-negative microbes, induced by MW irradiation has been reported by Bollet et al. (1991). Excitation of cell macromolecules by MW has been suggested to change enzyme activity and nucleic acid synthesis [Chipley, 1980; Frohlich, 1978]. Marka et al. (1971), and Bollet et al. (1981) did report ability of MW radiation to induce alterations in enzyme activity and protein synthesis.

In the present study, estimations of protein content and enzyme activity were made on the cell population originated from MW treated inoculum, and not directly on the MW treated cells. Therefore, the alterations in protein content or enzyme activity might have been transferred from the originally MW treated cells to their daughter cells (who did not receive direct MW exposure). The cells receiving direct MW exposure may get their biomolecules such as DNA altered in some manner. Maximum absorption of MW by DNA has been indicated at 2.45 GHz, and the dielectric properties of DNA are reported to reduce at this frequency (Edwards et al., 1984). However, the effect exerted by MW on DNA can be repairable [Rojavin and Ziskin, 1995]. As in this study, thermal effect of MW was avoided by putting the inoculum in ice during MW treatment, whatever alterations have been observed are most likely a result of *specific MW effects* (athermal effects).

.

5. To investigate mutagenic effect of MW on EPS production in *X. campestris*

5.1. Materials and Methods

5.1.1. Test organism:

Xanthomonas campestris (MTCC No. 2286)

5.1.2. MW treatment to inoculum:

- Frequency: 2.45 GHz
- Power: 90W
- Treatment: Continuous MW
- Physiology of Cells: 72 hours old culture for *X. campestris*.
- Inoculum volume: 5 ml (0.5 McFarland equivalent)
- Duration of exposure: 2, 4, 6 min
- Microwave treatment was given to the inoculum as described in Chapter 1.

Note: In case of MW exposure of 450 W, the duration was 3 min. Also the treatment was given in a 250 ml of Borosil® beaker with ice in a 30 ml screw-cap glass vial (Borosil®).

5.1.3. Experimental outline:

1. Activated culture of *X. campestris* was obtained on nutrient agar plate.
2. Colonies were suspended in 20 ml of normal saline and was set to 0.5 McFarland turbidity standard.
3. Equal amount of inoculum (5 ml) was distributed in 15 ml screw cap sterile vials for MW treatment.
4. After MW treatment, the inoculum from each treatment (untreated in case of control) was inoculated in TY broth (Appendix I) for EPS production keeping the inoculum size 5% v/v (EPS slot 1).
5. At the same time, nutrient agar plates were streaked from each of the treated and untreated inoculums.
6. From the results of EPS slot 1 (as described in the Figure 5.1), the time of exposure showing highest increase in EPS was selected (6 min in our case).
7. From the streaked plate of 6 min, 3 isolated colonies were picked at random and were again streaked on 3 different agar plates 6A, 6B and 6C respectively.
8. The plates contained daughter cells of a single colony and so were assumed to have same genetic makeup (named as master plates).
9. Now from each plate, 6 colonies were suspended in vials containing 5 ml of normal saline.
10. Again, it was inoculated in TY broth for EPS production keeping the inoculum size 5% v/v (EPS slot 2). From all 3 vials containing the inoculum (6A, 6B and 6C), new nutrient agar plates were also streaked.

11. The results of EPS slot 2 were compared with the control and overproducing colony was identified (6A in our case). This was the 0^{th} generation of colony 6A.
12. Again from the plate 6A, 6 colonies were selected and inoculated in broth for EPS production (EPS slot 3), and new plate was streaked.
13. The results of EPS slot 3 gives the 1^{st} generation result of colony 6A and from the new plate streaked, the same process was carried out up to 5 generations.

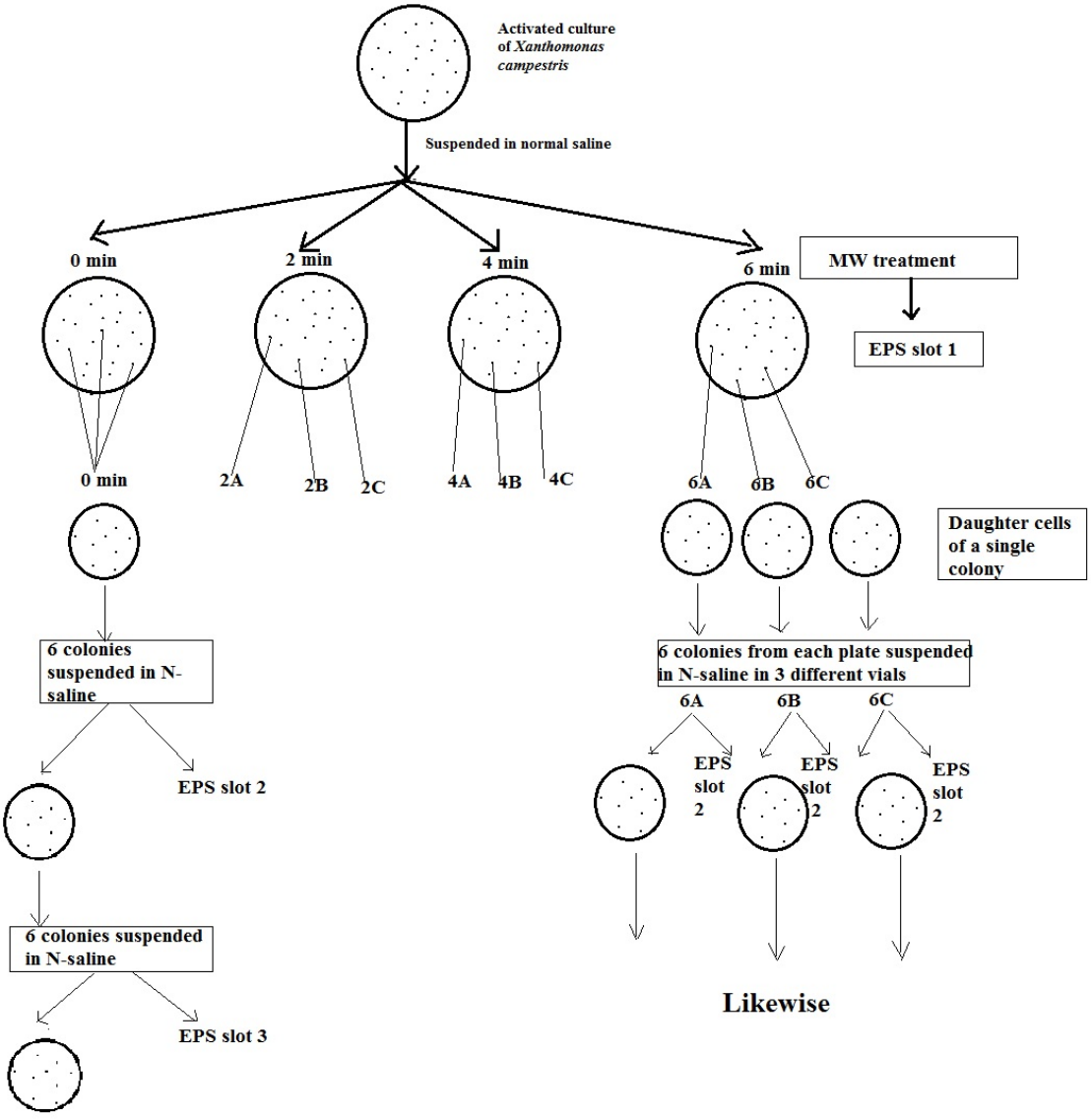


Figure 5.1 Outline of the experiment to study the mutagenic effect of MW in *X. campestris*

5.2. Results and Discussion

This experiment was carried out at two different power i.e 90 W and 450 W. For calculating percentage change, OD of non-treated cells was taken as 100%.

Table 5.1 Effect of MW (90 W) on growth and xanthan produced in *X. campestris*

Duration of MW treatment (min)	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced (g/L) (Mean ± SD)	% change compared to control
0 (control)	1.280 ± 0.014	0.00	0.720 ± 0.28	0.00
2	1.295 ± 0.021	1.17	0.800 ± 0.28	11.11
4	1.280 ± 0.014	0.00	0.880 ± 0.80	22.22
6	1.260 ± 0.000	-1.56	1.040 ± 0.40	44.44*

*$p<0.05$; minus sign indicates a decrease over control

The results of MW treatment on growth and xanthan production in 2, 4 and 6 min exposure are shown in Table 5.1. As the xanthan production significantly increased by 44.44% in 6 min MW treatment, we selected three isolated colonies (6A, 6B and 6C) randomly for further experiment.

Table 5.2 Growth and xanthan gum production by three different isolates selected randomly from plate corresponding to 6 min MW treatment (90 W)

Duration of MW treatment (min)		Growth (OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced (g/L) (Mean ± SD)	% change compared to control
0		0.886 ± 0.010	0.00	0.640 ± 0.00	0.00
6	6A	1.042 ± 0.003	17.60**	0.920 ± 0.00	43.75**
	6B	0.962 ± 0.208	8.57	0.600 ± 0.00	-6.25
	6C	1.040 ± 0.042	17.38**	0.680 ± 0.08	6.25

**$p<0.01$; minus sign indicates a decrease over control

The results of growth and xanthan production of colonies 6A, 6B and 6C as compared to control are given in Table 5.2. In colony 6A, xanthan production increased significantly by 43.75% as compared to control, so daughter cells of this single colony were selected for generation studies.

Table 5.3 Effect of MW (90 W) on growth and xanthan production in *X. campestris*

Duration of MW exposure (min)	Generation number									
	0		1		2		3		4	
	Growth (OD_{625})	Xanthan production (g/L)	Growth (OD_{625})	Xanthan production (g/L)	Growth (OD_{625})	Xanthan production (g/L)	Growth (OD_{625})	Xanthan production (g/L)	Growth (OD_{625})	Xanthan production (g/L)
0	0.892 ± 0.003	0.80 ± 0.00	0.872 ± 0.003	1.05 ± 0.00	1.370 ± 0.00	0.80 ± 0.00	1.032 ± 0.010	1.10 ± 0.00	1.030 ± 0.007	1.65 ± 0.00
6	1.010 ± 0.003	1.15 ± 0.00	0.890 ± 0.007	1.47 ± 0.00	1.410 ± 0.014	0.85 ± 0.00	1.050 ± 0.014	1.10 ± 0.00	1.062 ± 0.010	1.7 ± 0.00
% change compared to control	13.22*	43.75*	2.06	40.47*	2.91*	6.25	1.74	0.00	3.10	3.03

* $p<0.05$

The data which we got upto 4 generations has been compiled in Table 5.3. Interestingly, xanthan production in 0^{th} generation i.e 43.7% was approximately equal to the one which we got in Table 5.1 i.e 44.4%. In 1^{st} generation this pattern was still unchanged and a significant increase of 40.0% was obtained. These data suggests that the MW must have induced some mutation which was passed on to the next generation. From 2^{nd} generation onwards, there was no change in the xanthan production in 6 min MW treated inoculum as compared to control, which indicated that the mutation generated by MW treatment might have been reverted back.

Table 5.4 Effect of MW (450 W) on growth and xanthan produced in *X. campestris*

Duration of MW treatment (min)	**Growth (OD_{625}) (Mean ± SD)**	**% change compared to control**	**Amount of xanthan produced (g/L) (Mean ± SD)**	**% change compared to control**
0 (control)	1.130 ± 0.014	0.00	1.065 ± 0.02	0.00
3	0.945 ± 0.028	16.37*	1.300 ± 0.05	22.06*

*$p<0.05$; minus sign indicates a decrease over control

Table 5.5 Growth and xanthan gum production by four different isolates selected randomly from plate corresponding to 3 min MW treatment (450 W)

Duration of MW treatment (min)		**Growth (OD_{625}) (Mean ± SD)**	**% change compared to control**	**Amount of xanthan produced (g/L) (Mean ± SD)**	**% change compared to control**
0		1.400 ± 0.000	0.00	0.90 ± 0.00	0.00
3	3A	1.320 ± 0.084	-5.71	0.97 ± 0.03	-7.77
	3B	1.320 ± 0.042	-5.71	1.15 ± 0.07	27.77
	3C	1.075 ± 0.007	-23.21**	1.35 ± 0.07	50.00**
	3D	1.390 ± 0.014	-0.71	0.85 ± 0.10	-5.55

**$p<0.01$; minus sign indicates a decrease over control

Table 5.6 Effect of MW (450 W) on growth and xanthan production in *X. campestris*

Duration of MW exposure (min)	Generation number							
	0		1		2		3	
	Growth (OD_{625})	Xanthan production (g/L)	Growth (OD_{625})	Xanthan production (g/L)	Growth (OD_{625})	Xanthan production (g/L)	Growth (OD_{625})	Xanthan production (g/L)
0	1.400 ± 0.000	0.90 ± 0.00	0.755 ± 0.021	1.20 ± 0.00	1.140 ± 0.000	2.50 ± 0.05	1.355 ± 0.021	1.70 ± 0.03
3	1.075 ± 0.007	1.35 ± 0.07	0.830 ± 0.014	1.45 ± 0.02	1.125 ± 0.021	2.20 ± 0.50	1.200 ± 0.000	1.70 ± 0.05
% change compared to control	-23.21**	50.0**	9.93	20.83**	-1.31	-12.0	11.43**	0.00

** $p<0.01$; minus sign indicates a decrease over control

MW treatment at 450 W also showed a similar kind of result where in 0^{th} and 1^{st} generation, the 3 min treated cells showed a significant increase in xanthan production. However, in this case the % increase in both 0^{th} and 1^{st} generation are quite different i.e. 50.0% and 20.83% respectively (Figure 5.5). In subsequent generations, no increase in xanthan was found and the results were same as that of control. In our experiment on 450 W, we also estimated the amount of intracellular protein in 3 min treated cells as compared to control. In both 0^{th} and 1^{st} generation, the amount of intracellular protein decreased by 43.24% and 14.16% respectively.

Pasiuga et al., 2007 studied the long-term effects of low-level (frequency 35 GHz, surface power density 30 mW/cm^2, exposure time 10 s) MW radiation on mutation frequency of *Drosohila melanogaster*. It was reported that in first three generations, the efficiency of repair system was reduced due to MW treatment and after 4-5 generation, this efficiency got increased. Therefore, in our experiment, one possible explanation in reversion of mutation can be the role of repair system.

The mutagenic effects of MW have not been established yet, but there have been a few reports wherein MW was able to produce stable mutants. Lin et al., (2012) mutated the strains of *Lactobacillus rhamnosus* for increased lactic acid production and they have reported their mutant to be stable up to nine generations. Jangid et al., (2009) studied the effect of a compound mutation of MW and ultraviolet on *Trichoderma viride* for increased cellulose production. The mutant generated was also found to be stable up to 9 generations. The biosynthetic pathway of xanthan comprises of various stages starting from conversion of simple sugars to nucleotidyl derivative precursors, assembly of pentasaccharide subunits and finally secretion of polymer. Proteins related to the stages of xanthan biosynthesis have been proposed to be encoded by the *xpsI* or *gum* region. In an experiment carried out by Li et al., (2012), *X. campestris* XG30-18 was radiated by MW and then treated with nitrosoguanidine (NTG) and a mutant strain named as MW-42-NTG-6 was obtained. The strain had the ability to produce high-viscosity and acid resistant xanthan gum, but the information about yield was not available. Katzen et al., (1998) studied the effects on xanthan biosynthesis and plant virulence of *X. campestris gum* mutans. They reported that although xanthan gum is not essential for plant virulence, specific changes in the last stages of xanthan biosynthesis point to a reduction of the aggressiveness of *X. campestris* against the host.

Epilogue

Results of the present study suggest that exposure of MW radiation has some athermal effects on the tested bacterial system. In this work, MW produced significant effects in the irradiated microorganisms compared to the control, and the effect on EPS production in *X. campestris* (though not so stable) was also shown to be inheritable, i.e. genetic stability of the MW mutation was not found to be long-lasting.

It will certainly be interesting and useful to continue investigating effect of microwaves of varying frequencies and power on living systems (both eukaryotic and prokaryotic), with the objective of deciphering the mechanism of MW athermal effects on cells and their biomolecules, and determining the type of effect i.e. reversible vs. heritable; morphological, physiological or genetic.

Particularly identification of the mutagenic frequencies can be of use in industrial strain improvement programmes.

At present a large part of general population is exposed chronically to non-thermal MWs from different types of mobile communication. The current safety standards set by ICNIRP (International Commission for Non-Ionizing Radiation Protection) which are based largely on thermal effects of MW does not seem to be totally safe which needs to be re-evaluated.

Due to the ease of handing them in laboratory, microorganisms can be conveniently used to study the effect of MW on living systems. In particular such work in eukaryotic microbes and other higher forms of life can be of relevance in deciphering the exact mechanism(s) behind non-thermal effects of MW.

APPENDICES

Appendix I. Media preparation

Minimal media for pectinase estimation [Atlas, 2010]

Ingredients (HiMedia)	**Amount**
K_2HPO_4	7.0 g
KH_2PO_4	2.0 g
$(NH_4)_2SO_4$	1.0 g
Sodium citrate	0.5 g
$MgSO_4.7H_2O$	0.1 g
Yeast extract	0.1 g
Pectin	10.0 g
Water	1000 ml
pH	6.8 ± 0.2

- Pectin powder was sterilized at 120º C in hot air oven for 6 hours. It was then added slowly with constant stirring to minimal media which was stored in refrigerator after autoclaving.

Minimal media for amylase estimation [Atlas, 2010]

Ingredients (HiMedia)	**Amount**
K_2HPO_4	7.0 g
KH_2PO_4	2.0 g
$(NH_4)_2SO_4$	1.0 g
Sodium citrate	0.5 g
$MgSO_4.7H_2O$	0.1 g
Yeast extract	0.1 g
Starch powder	10.0 g
Distilled Water	1000 ml
pH	6.8 ± 0.2

- Starch was added slowly and dissolved by heating at 60º C magnetic stirrer.

Media for EPS production in *X. campestris* [Katzen et al., 1998]

Ingredients	**Amount**
Tryptone	5.0 g
Yeast extract	3.0 g
$CaCl_2$	0.7 g
Water	1000

- EPS production using this media required shaking condition.

Production media for *E.coli, L. acidophilus* and *B. subtilis* [Natarajan et al., 2012].

Ingredients (HiMedia)	Amount
Lactose	10.0 g
Peptone	1.5 g
Yeast extract	1.0 g
KH_2PO_4	1.0 g
$(NH_4)_2SO_4$	7.0 g
$MgSO_4.7H_2O$	1.0 g
$CaCl_2$	0.3 g
Distilled Water	1000 ml

- Lactose was sterilized separately at 10 lb/inch^2 pressure and then added to the media.
- This media was used for estimation of growth, intracellular enzymes (Glucose-6-phosphatase, β-galactosidase) as well as intra- and extracellular proteins.

Appendix II. Buffer preparation [Nigam and Ayyagari, 2008]

- **1 M Citrate buffer**

  Stock A solution of 0.1 M citric acid (19.21 g/L) and B stock solution of 0.1 M sodium citrate (29.41 g/L) were prepared, these two solutions A and B were mixed in volume as follows:

Solution A	20.5 ml
Solution B	29.5 ml

- **TM buffer**

Ingredients	**Amount**
Tris	0.15 g
$MgSO_4$	0.03 g
Water	25 ml

- **Phosphate buffer (pH: 6.0-7.2)**

  0.2 M of stock solution A was prepared by dissolving 27.2 g KH_2PO4 in 1 litre of distilled water. Likewise, 34.8 g K_2HPO_4 was dissolved in 1 litre of distilled water to prepare 0.2 M stock solution B. Stock solutions A and B were mixed in the following ratios to get 0.1M phosphate buffer of the desired pH value:

0.2 M monobasic potassium phosphate	0.2 M dibasic potassium phosphate	pH
87.7	12.3	6.0
85.5	15.0	6.1
81.5	19.5	6.2
77.5	22.5	6.3
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6

56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2

Appropriate volumes of stocks were mixed and equal volume of distilled water was added to make a final 0.1 M concentration.

- ## Acetate buffer

  Solution A was prepared by adding 0.34 g sodium acetate trihydrate in 100 mL of distilled water. Solution B was prepared by adding 15µL of acetic acid in 10 mL distilled water. Now, 95 mL of Solution A and 5 mL of Solution B was mixed to obtain the acetate buffer of 0.025 M.

Appendix III. McFarland Standards

McFarland turbidity standards are used to standardize the approximate number of bacteria in a liquid suspension by visually comparing the turbidity of a test suspension with the turbidity of a McFarland standard. The most commonly used standard for inoculums is 0.5, representing 1.5 X 10^8 bacteria/ml approximately.

Preparation of a 0.5 McFarland standard

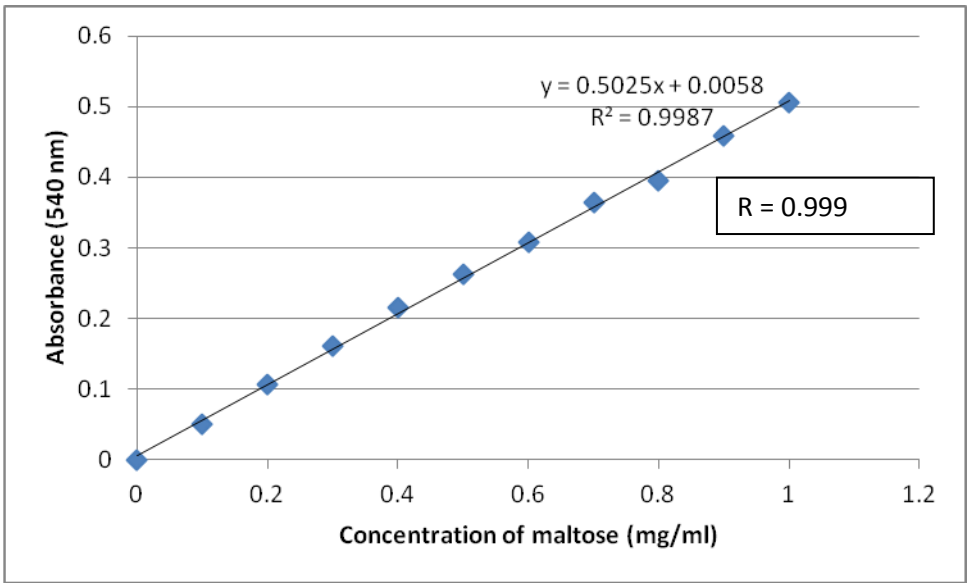
85 ml of 1% H_2SO_4 was added to a 100 ml volumetric flask. To that flask, 0.5 ml 1.175% $BaCl_2$ was added drop wise with constant swirling to the flask. The volume was made up to 100 ml with 1% H_2SO_4 optical density of the solution was set between 0.08-0.1 at 625 nm. The solution should be stored in a dark bottle at room temperature for a month.

Inoculation preparation

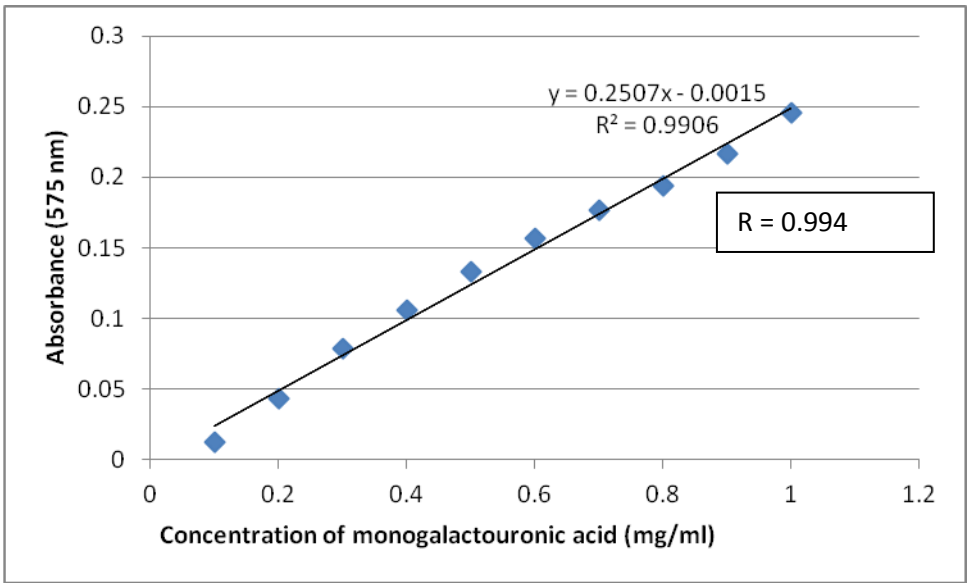
Inoculum equivalent to 0.5 McFarland was prepared by comparing turbidity of suspension of test organism with 0.5 McFarland standard.

Appendix IV. Standard plots

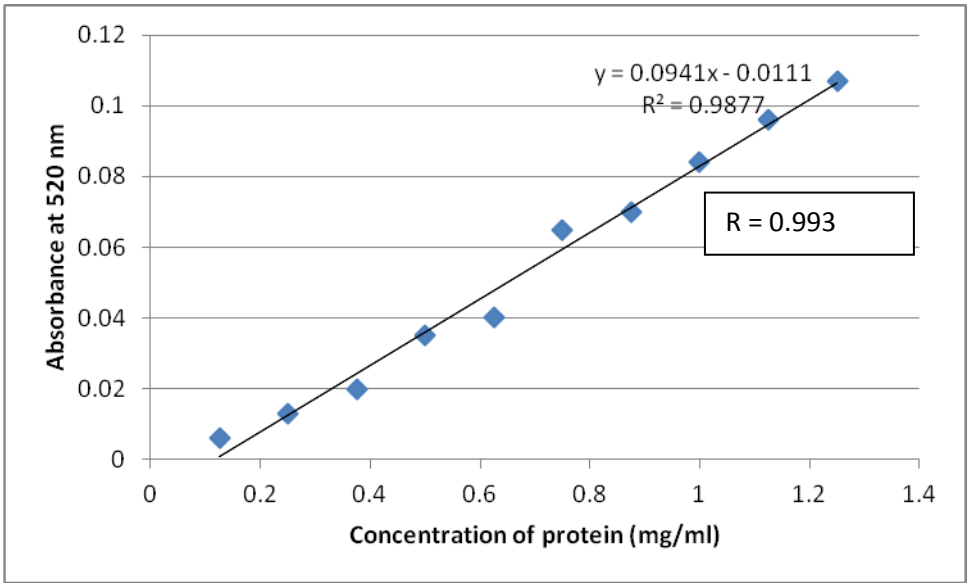
1. Standard plot of maltose:



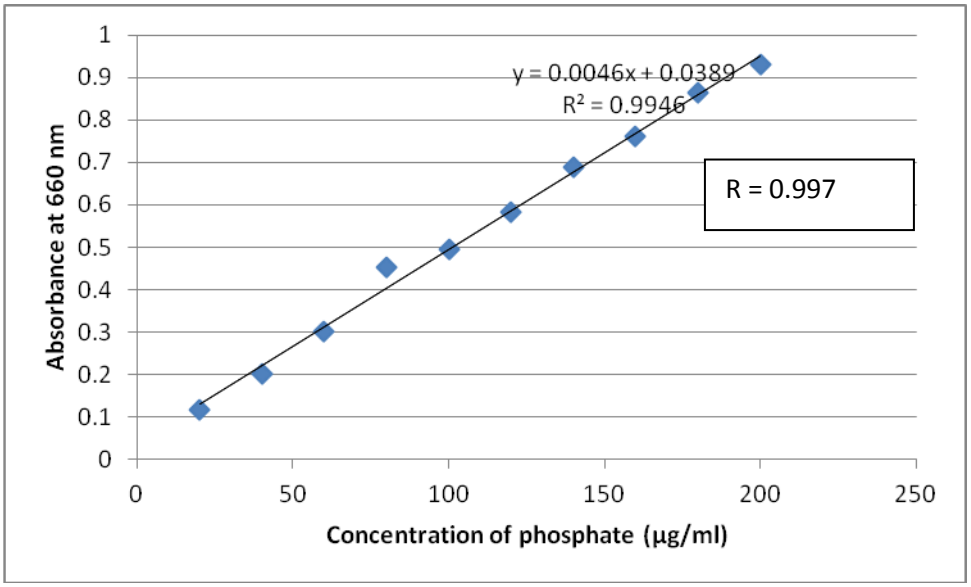
2. Standard plot of monogalactouronic acid:



3. Standard plot of protein:



4. Standard plot of inorganic phosphate:



Appendix V. Statistical Analysis

All statistical analysis and calculation was done in Microsoft® excel (part of Microsoft® office 2007).
General calculation was done in Microsoft® Excel using formula function and statistical analysis was done using "Analysis ToolPack" Add-In.

Activation of Analysis ToolPack – Add In

- Open Excel spreadsheet.
- Office button (top-left corner of Excel window) / Excel Options button – this opens the Excel Options dialog window
- Choose the Add-Ins panel
- Select Add-Ins option on the Manage drop-down list
- Click Go > to open the Add-Ins dialog window
- Check the Analysis ToolPak box.
- Click OK to close the Add-Ins dialog and return to Excel. The Data Analysis tool is now active.

t-Test:

Example:

- Effect of MW on growth of *X. campestris*

Serial No.	**Duration of MW treatment (min)**	
	0	6
1	0.554	0.568
2	0.552	0.566
3	0.550	0.567

- Open Excel data bar (in top second bar) / Data analysis option- this opens Data analysis dialog window.
- Choose the t-test: two-sample assuming equal variances- this opens new dialog window.
- Select data range, value of alpha (0.05 or 0.01) and output range.
- Click OK to close Data analysis dialog window and return to Excel and generate table as given below.

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.552	0.567
Variance	4E-06	0.000001
Observations	3	3
Pooled Variance	0.0000025	
Hypothesized Mean Difference	0	
df	4	
t Stat	-11.61895004	
P(T<=t) one-tail	0.000156785	
t Critical one-tail	2.131846782	
P(T<=t) two-tail	**0.000313571**	
t Critical two-tail	2.776445105	

Appendix VI. Measured temperature of tap water after MW treatment

After a pre-treatment of ice in the beaker for 5 min, temperature was measured after MW treatment in following cases.

- When 15 mL flat-bottom screw capped vial was placed in ice containing beaker (100 mL) for 5 min prior to MW treatment, and then the treatment was given at 90 W for 6 min, the measured temperature of the water was **10º C**.
- When 30 mL round-bottom screw capped vial was placed in ice containing beaker (250 mL) for 5 min prior to MW treatment, and then the treatment was given at 450 W for 3 min, the measured temperature of the water was **25º C.**
- When 15 mL flat-bottom screw capped vial was placed in ice containing beaker (100 mL) for 5 min prior to MW treatment, and then the treatment was given without ice at 90 W for 6 min, the measured temperature of the water was **40º C**.

Appendix VII. Effect of MW on growth and xanthan production in *X. campestris*

Effect of MW (90 W) on growth and EPS production in *X. campestris*

Duration of MW treatment (min)	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Amount of EPS produced (g/L) (Mean ± SD)	% change compared to control
0	0.735 ± 0.007	0.00	0.52 ± 0.04	0.00
2	0.775 ± 0.007	5.44*	0.56 ± 0.00	7.69
4	0.805 ± 0.007	9.52*	0.76 ± 0.00	46.15**
6	0.815 ± 0.007	10.88**	0.80 ± 0.04	53.84*

*$p<0.05$; **$p<0.01$

Effect of MW (90 W) on growth and EPS production in *X. campestris*

Duration of MW treatment (min)	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Amount of EPS produced (g/L) (Mean ± SD)	% change compared to control
0	0.918 ± 0.007	0.00	0.80 ± 0.04	0.00
2	0.967 ± 0.017	5.33	0.80 ± 0.04	0.00
4	1.068 ± 0.017	16.33**	1.06 ± 0.04	32.50*
6	0.926 ± 0.014	0.87	1.13 ± 0.00	41.25**

*$p<0.05$; **$p<0.01$

Effect of MW (90 W) on growth and xanthan produced by *X. campestris*

Duration of MW treatment (min)	**Growth (OD_{625}) (Mean ± SD)**	**% change compared to control**	**Amount of xanthan produced (g/L) (Mean ± SD)**	**% change compared to control**
0 (control)	1.280 ± 0.014	0.00	0.720 ± 0.28	0.00
2	1.295 ± 0.021	1.17	0.800 ± 0.28	11.11
4	1.280 ± 0.014	0.00	0.880 ± 0.80	22.22
6	1.260 ± 0.000	-1.56	1.040 ± 0.40	44.44*

*$p<0.05$; minus sign indicates a decrease over control

8. References

Aiyer P. V. (2005). Amylases and their applications. *African Journal of Biotechnology*, 4(13), 1525-1529.

Asadi A., Khavari-Nejad R. A., Soltan N., Najafi F., & Molaie-Rad A. (2011). Physiological variability in cyanobacterium *Phormidium* sp. Kützing ISC31 (Oscillatoriales) as response to varied microwave intensities. *African Journal of Agricultural Research*, 6(7), 1673-1681.

Asay B., Tebaykina Z., Vlasova A., & Wen M. (2008). Membrane composition as a factor in susceptibility of Escherichia coli C29 to thermal and non-thermal microwave radiation. *Journal of Experimental Microbiology and Immunology*, 12, 7-13.

Asraf S. S., & Gunasekaran P. (2010). Current trends of ß-galactosidase research and application. *Current Research, Technology and Education topics in Applied Microbiology and Microbial Biotechnology*.

Atlas R. M. (2010). Handbook of microbiological media (4th edition), USA: ASM press.

Banik S., Bandyopadhyay S., Ganguly S. (2003) Bioeffects of microwave—a brief review. *Bioresource Technology*, 87, 155–159.

Barnabas J., Siores E., & Lamb A. (2010). Non-thermal microwave reduction of pathogenic cellular population. International *Journal of Food Engineering*, 6(5), 1556-3758.

Becerra M., Cerdan E., Gonzalez Siso M. I. (1998) Dealing with different methods for Kluyveromyces lactis β-galactosidase purification. *Biological Procedures Online*, 1(1), 48-58.

Belyaev I. Y., Shcheglov V. S., & Alipov Y. D. (1992). Selection rules on helicity during discrete transitions of the genome conformational state in intact and X-rayed cells of *E. coli* in millimeter range of electromagnetic field. *In Charge and Field Effects in Biosystems*, 3,115-126.

Beszédes S., László Z., Horváth Z. H., Szabó G., & Hodúr C. (2011). Comparison of the effects of microwave irradiation with different intensities on the biodegradability of sludge from the dairy-and meat-industry. *Bioresource Technology*, 102(2), 814-821.

Bhattacharjee M. K., Sugawara K., Ayandeji O.T., (2009). Microwave sterilization of growth medium alleviates inhibition of *Aggregatibacter actinomycetemcomitans* by Maillard reaction products. *Journal of Microbiological Methods*, 78, 227-230.

Bollet C., Gevaudan M. J., De Lamballerie X., Zandotti C., & De Micco P. (1991). A simple method for the isolation of chromosomal DNA from gram positive or acid-fast bacteria. *Nucleic Acids Research*, 19 (8), 1955-1956.

Bose A. K., Hain Y., Lavlinskaia N., Sareen C., Pramanik B. N., Bartner P. L., ..& Heimark L. (2002). Microwave enhanced Akabori reaction for peptide analysis. *Journal of the American Society for Mass Spectrometry*, 13(7), 839-850.

Bren S. (1996) Historical introduction to EMF health effects. *Engineering in Medicine and Biology Magazine*, 15(4), 24-30.

Carta R., & Desogus F. (2010). The effect of low-power microwaves on the growth of bacterial populations in a plug flow reactor. *AIChE Journal*, 56(5), 1270-1278.

Chen W., Hang F., Zhao J. X., Tian F. W., & Zhang H. (2007). Alterations of membrane permeability in *Escherichia coli* and *Staphylococcus aureus* under microwave. *Wei sheng wu xue bao=Acta Microbiologica Sinica*, 47(4), 697.

Chernyakov G. M., Korochkin V. L., Babenko A. P., & Bigdai E. V. (1989). Reactions of biological systems of various complexity to the action of low-level EHF radiation. *Millimeter Waves in Medicine and Biology. Radioelectronica*, Moscow, 141-167.

Copty A. B., Neve-Oz Y., Barak I., Golosovsky M., & Davidov D. (2006). Evidence for a specific microwave radiation effect on the green fluorescent protein. *Biophysical Journal*, 91(4), 1413-1423.

Craciun G., Martin D., Togoe I., Tudor L., Manaila E., Ighigeanu D., Matei C. (2009). Vaccine preparation by radiation processing. *Journal of Microwave Power and Electromagnetic Energy*, 43(2), 65-70.

Crueger W., & Crueger A. (1984). Biotechnology: A textbook of industrial microbiology. *Science Technology, Inc., Madison, WI (USA). 1984.*

Czerska E. M., Elson E. C., Davis C. C., Swicord M. L., & Czerski P. (1992). Effects of continuous and pulsed 2450 MHz radiation on spontaneous lymphoblastoid transformation of human lymphocytes in vitro. *Bioelectromagnetics*, 13(4), 247-259.

Czerski P. (1975) Current views on mechanisms of interaction of microwave and radiofrequency radiation with living systems.

Czerski P. (1975) Experimental models for the evaluation of microwave biological effects. Proceedings of the IEEE, 63(11), 1540-1544.

Davis M., L. VanZandt L. (1988) Microwave response of DNA in solution: Theory. *Physics Reviews*. A, 37(3), 888–901.

Debye P. (1922). Methods to determine the electrical and geometrical structure of molecules. *Nobel Lectures, Chemistry*, 1941, 383-401.

Decareau R. V., & Kenyon E. M. (1970). Microwave energy in food processing applications. *Critical Reviews in Food Science & Nutrition*, 1(2), 199-224.

Dholiya K., Patel D., & Kothari V. (2012). Effect of low power microwave on microbial growth, enzyme activity, and aflatoxin production. *Research in Biotechnology*, 3(4), 28-34.

Diem E., Schwarz C., Adlkofer F., Jahn O., & Rüdiger H. (2005). Non-thermal DNA breakage by mobile-phone radiation (1800 MHz) in human fibroblasts and in transformed GFSH-R17 rat granulosa cells in vitro. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 583(2), 178-183.

Dreyfuss M. S., Chipley J. R. (1980) Comparison of effects of sublethal microwave radiation and conventional heating on the metabolic activity of *Staphylococcus aureus*. *Applied and Environmental Microbiology*, 39(1), 13-16.

Edwards G., C. Davis, J. Saffer, M. Swicord (1985) Microwave-field-driven acoustic modes in DNA. *Journal of Biophysics*, 47,799-807.

Elder J. A. (2003). Ocular effects of radiofrequency energy. *Bioelectromagnetics*, 24(6), S148-S161.

Fregel R., Rodrı´guez V., Cabrera V. M. (2007) Microwave improved *Escherichia coli* transformation. *Letters in Applied Microbiology*. doi:10.1111/j.1472 765X.2008.02333.x

Frey A. H. (1962). Human auditory system response to modulated electromagnetic energy. *Journal of Applied Physiology*, 17(4), 689-692.

Froissart R., Piraud M., Boudjemline A. M., Vianey-Saban C., Petit F., Hubert-Buron A., ... & Labrune P. (2011). Glucose-6-phosphatase deficiency. *Orphanet Journal of Rare Diseases*, 6(1), 1-12.

Galvin M. J., Parks D. L., & McRee D. I. (1981). Influence of 2.45 GHz microwave radiation on enzyme activity. *Radiation and Environmental Biophysics*, 19(2), 149-156.

George D. F., Bilek M. M., & McKenzie D. R. (2008). Non thermal effects in the microwave induced unfolding of proteins observed by chaperone binding. *Bioelectromagnetics*, 29(4), 324-330.

Golant M. B, Kuznetsov A. P, Boszhanova T. P (1994): Mechanisms of synchronization of the yeast cell culture by the action of EHF radiation. *Biofizika* 39, 490–495 (in Russian).

Grundler W., Keilman F., Froehlich H. (1977). Resonant growth rate response of yeast cells irradiated by weak interactions. *Physics Letters*, 62A, 463-66.

Gupta A., Naraniwal M., Kothari V. (2012). Modern extraction methods for preparation of bioactive plant extracts. International *Journal of Applied and Natural Sciences.* 1(1), 8-26.

Hankin L., & Sands D. C. (1977). Microwave treatment of tobacco seed to eliminate bacteria on the seed surface. *Phytopathology*, 67, 794-795.

Harley J. P., Prescott L. M. *Laboratory Exercises in Microbiology.* 5th edition. McGraw-Hill Publishers; 2002., pp 388.

Herrero M. A., Kremsner J. M., & Kappe C. O. (2008). Nonthermal microwave effects revisited: on the importance of internal temperature monitoring and agitation in microwave chemistry. *The Journal of Organic Chemistry*, 73(1), 36-47.

Himabindu M., Potumarthi R., Jetty A. (2007) Enhancement of gentamicin production by mutagenesis and non-nutritional stress conditions in *Micromonospora echinospora. Process Biochemistry*, 42, 1352-1356.

Hocking B., & Westerman R. (2001). Neurological abnormalities associated with CDMA exposure. *Occupational Medicine*, 51(6), 410-413.

Hoondal G., Tiwari R., Tewari R., Dahiya N., & Beg Q. (2002). Microbial alkaline pectinases and their industrial applications: a review. *Applied Microbiology and Biotechnology*, 59(4-5), 409-418.

Husain Q. (2010). β Galactosidases and their potential applications: a review. *Critical Reviews in Biotechnology*, 30(1), 41-62.

Hyland G. J. (1998). Non-thermal bioeffects induced by low-intensity microwave irradiation of living systems. *Engineering Science and Education Journal*, 7(6): 261-269.

ICNIRP guideline (1998) for limiting exposure to time-varying electric, magnetic, and electromagnetic fields (up to 300GHz). *Health Physics*, 74(4), 494-522.

Jacob J., Chia L. H. L, Boey F. Y. C. (1995) Thermal and non-thermal interaction of microwave radiation with materials. *Journal of Materials Science*, 30, 5321-5327.

Jakubowski T., (2010) The impact of microwave radiation at different frequencies on weight of seed potato germs and crop of potato tubers. *Agricultural Engineering*, 6(124), 57-64.

Jangid R. K., Sharma R., Sudarsan Y., Eapen S., Singh G., & Purohit A. K. (2010). Microwave treatment induced mutations and altered gene expression in *Vigna aconitifolia*. *Biologia Plantarum*, 54(4), 703-706.

Jayaraman J. (2005). Laboratory manual in biochemistry. *New Age International Publishers*. 122-123.

Kapoor M., Beg Q. K., Bhushan B., Singh K., Dadhich K. S., & Hoondal G. S. (2001). Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp. MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sunn hemp (*Crotalaria juncea*) bast fibres. *Process Biochemistry*, 36(8), 803-807.

Kashyap D. R., Chandra S., Kaul A., & Tewari R. (2000). Production, purification and characterization of pectinase from a *Bacillus sp*. DT7. *World Journal of Microbiology and Biotechnology*, 16(3), 277-282.

Kashyap D. R., Vohra P. K., Chopra S., & Tewari R. (2001). Applications of pectinases in the commercial sector: a review. *Bioresource Technology*, 77(3), 215-227.

Katzen F., Ferreiro D. U., Oddo C. G., Ielmini M. V., Becker A., Pühler A., & Ielpi L. (1998). *Xanthomonas campestris* pv. Campestris gum mutants: Effects on xanthan biosynthesis and plant virulence. *Journal of Bacteriology*, 180(7), 1607-1617.

Komarova A. S., Likhacheva A. A., & Zvyagintsev D. G. (2008). Influence of microwave radiation on soil bacteria. *Moscow University Soil Science Bulletin*, 63(4), 190-195.

Kothari V., Gupta A., Naraniwal M. (2012). Comparative study of various methods for extraction of antioxidant and antibacterial compounds from plant seeds. *Journal of Natural Remedies*, 12(2), 162-173.

Kothari V., Patadia M., & Trivedi N. (2011). Microwave sterilized media supports better microbial growth than autoclaved media. *Research in Biotechnology*, 2(5), 63-75.

Kozempel M., Cook R., Scullen J., Annous B. (2000). Development of a process for detecting nonthermal effects of microwave energy on microorganisms at low temperature. *Journal of Food Processing and Preservation*, 24(4), 287-301.

Kumar M. A., Anandapandian K. T. K., & Parthiban K. (2011). Production and characterization of exopolysaccharides (EPS) from biofilm forming marine bacterium. *Brazilian Archives of Biology and Technology*, 54(2), 259-265.

Lei S. P., Lin H. C., Heffernan L., & Wilcox G. (1985). Evidence that polygalacturonase is a virulence determinant in *Erwinia carotovora*. *Journal of Bacteriology*, 164(2), 831-835.

Li Y., & Hardin I. R. (1998). Enzymatic scouring of cotton: surfactants, agitation, and selection of enzymes. *Textile Chemist and Colorist*, 30(9), 23-29.

Li J., Zhang S., Shi S., & Huo P. (2011). Mutational approach for N2-fixing and P-solubilizing mutant strains of *Klebsiella pneumoniae* RSN19 by microwave mutagenesis. *World Journal of Microbiology and Biotechnology*, 27(6), 1481-1489.

Li Y. X, Yin L. H., Qi Y. (2012) Screening of *Xanthomonas campestris* producing high-viscosity and acid-resistant xanthan gum and its fermentation process. *Food Science*, 33(9): 211-216.

Li X. H., Yang H. J., Roy B., Park E. Y., Jiang L. J., Wang D. & Miao Y. G., (2009). Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. *Microbiological Research*, 165(3), 190-198.

Lin H., Chen X., Yu L., Xu W., Wang P., Zhang X., ... & Ren N. (2012). Screening of *Lactobacillus rhamnosus* strains mutated by microwave irradiation for increased lactic acid production. *African Journal of Microbiology Research*, 6(31), 6055-6065.

Lu S. T., Mathur S. P., Akyel Y., & Lee J. C. (1999). Ultrawide-band electromagnetic pulses induced hypotension in rats. *Physiology & Behavior*, 67(3), 463-473.

Mandal V., Mohan Y., & Hemalatha S. (2007). Microwave assisted extraction—an innovative and promising extraction tool for medicinal plant research. *Pharmacognosy Reviews*, 1(1), 7-18.

Matthews B. W. (2005). The structure of *E. coli* β-galactosidase. *Comptes rendus biologies*, 328(6), 549-556.

Moore H. A., Raymond R., Fox M., & Galsky A. G. (1979). Low-intensity microwave radiation and the virulence of *Agrobacterium tumefaciens* strain B6. *Applied and Environmental Microbiology*, 37(1), 127-130.

Morris G. and Harding S., (2009). Polysaccharides: Microbial. *Applied Microbiology: Industial.* 482-494. http://www.nottingham.ac.uk/ncmh/harding_pdfs/Paper322.pdf

Najdovski L., Draga A. Z., Kotnik V. (1991).The killing activity of microwaves on some non-sporogenic and sporogenic medically important bacterial strains. *Journal of Hospital Infection*, 19, 239-247.

Natarajan J., Christobell C., Kumar D. M., Balakumaran M. D., Kumar M. R., & Kalaichelvan P.T. (2012). Isolation and characterization of β-galactosidase producing *Bacillus* sp. From dairy effluent. *World Applied Sciences Journal*, 17(11), 1466-1474.

Nigam A. and Ayyagari A. (2008) Lab Manual in Biochemistry, Immunology, Biotechnology (2008) Tata-McGraw Hill.

Oslen C., Drake C., Bunch S. (1966). Some biological effect of microwave energy.

Otludil B., Otludil B., Tolan V., & Akbayin H. (2004). The effect of microwave on the cellular differentiation *Bacillus subtilis* YB 886 and rec derivatives YB 886 A4. *Biotechnology and Biotechnological Equipment*, 18(3), 107-112.

Otludil B., Tolan V., Otludil B. A., Demir R. (2005) The effect of microwave on plasmids amplification and cell differentiation in *Bacillus subtilis*. *Biotechnol. & Biotechnol.* Eq. 19(1), 109-114.

Özbek B., & Dadali G. (2007). Thin-layer drying characteristics and modelling of mint leaves undergoing microwave treatment. *Journal of Food Engineering*, 83(4), 541-549.

Palav T., & Seetharaman K. (2007). Impact of microwave heating on the physico-chemical properties of a starch–water model system. *Carbohydrate Polymers*, 67(4), 596-604.

Panagopoulos D. J., & Margaritis L. H. (2002). Effects of different kinds of emfs on the offspring production of insects. In P. Kostarakis (Ed.), *2 nd International Workshop on Biological effects of EMFS*, 348-42.

Pasiuga V. N., Grabina V. A., Bykov V. N., & Shkorbatov Y. G. (2007). Long-term effects of low-level microwave radiation on mutation frequency in *Drosophila*. In *Microwave & Telecommunication Technology, 2007. CriMiCo 2007. 17th International Crimean Conference* ,783-784.

Patil N. P., Patil K. P., Chaudhari B. L., & Chincholkar S. B. (2012). Production, purification of exo-polygalacturonase from soil isolate *Paecilomyces variotii* NFCCI 1769 and its application. *Indian Journal of Microbiology*, 52(2), 240-246.

Ragha L., Mishra S., Ramachandran V., & Bhatia M. S. (2011). Effects of low-power microwave fields on seed germination and growth rate. *Journal of Electromagnetic Analysis and Applications*, 3(5), 165-171.

Rebrova T. B. (1992) Effect of millimeter-range electromagnetic radiation on the vital activity of microorganisms. Millimetrovie Volni ual features. Millimetrovie Volni v *Biologii i Meditcin*, 1, 22-32, 1,37-47 (in Russian).

Ribeiro D. G., Pavarina A. C., Dovigo L. N., Palomari Spolidorio D. M., Giampaolo E. T., & Vergani C. E. (2009). Denture disinfection by microwave irradiation: a randomized clinical study. *Journal of Dentistry*, 37(9), 666-672.

Richmond V. (1969). Biological effects and health implications of microwave radiation. *Symposium proceedings*, pp. 123. Ed. Cleary

Rojavin M. A & Ziskin M. C. (1995). Effect of millimeter waves on survival of UVCexposed *Escherichia coli*. *Bioelectromagnetics,* 16, 188–196.

Rosenberg Z. M. (2006) Current trends of β-galactosidase application in food technology. *Journal of Food and Nutrition Research*, 45(2), 47-54.

Sandford P. A., Cottrell I. W., & Pettitt D. J. (1984). Microbial polysaccharides: new products and their commercial applications. *Pure and Applied Chemistry*, 56(7), 879-892.

Sarkar S., Ali S., & Behari J. (1994). Effect of low power microwave on the mouse genome: a direct DNA analysis. *Mutation Research/Genetic Toxicology*, 320(1-2), 141-147.

Semenova M. V., Sinitsyna O. A., Morozova V. V., Fedorova E. A., Gusakov A. V., Okunev O. N., ... & Sinitsyn A. P. (2006). Use of a preparation from fungal pectin lyase in the food industry. *Applied Biochemistry and Microbiology*, 42(6), 598-602.

Shamis Y., A. Taube, N. Mitik-Dineva, R. Croft, R. Crawford, E. Ivanova (2011) Specific electromagnetic effects of microwave radiationon on *Escherichia coli. Applied and Environmental Microbiology*, 77(9), 3017–3023.

Shamis Y., Traub A., Croft R. J., Crawford R. & Ivanova E. (2012). Influence of 18GHz microwave radiation on the enzymatic activity of *Escherichia coli* lactate dehydrogenase and cytochrome c oxidase. *Journal of Physical Science and Application*, 2 (6), 143-151.

Shazman A., Mizrahi S., Cogan U., & Shimoni E. (2007). Examining for possible non-thermal effects during heating in a microwave oven. *Food Chemistry*, 103(2), 444-453.

Shckorbatov Y. G., Pasiuga V. N., Grabina V. A., Kolchigin N. N., Batrakov D. O., Kalashnikov V. V., ... & Bykov V. N. (2008). The influence of microwave radiation on the state of chromatin in human cells. arXiv preprint arXiv:0809.0559.

Shub G. M., Luniova I. O., Denisova S. G., Ostrovskii N. V. (1995) Effects of millimetre waves on bacteria in vitro and in vivo experiments. Moscow, Russia: 10th Russian Symposium , *Millimeter Waves in Medicine and Biology*, Moscow: IRE RAN, 96-97 (in Russian).

Singh S., Sharma V., Soni M. L., & Das S. (2011). Biotechnological applications of industrially important amylase enzyme. *International Journal of Pharma and Bio Sciences. India*, 2(1), 486-496.

Sokolovic D., B. Djindjic, J. Nikolic, G. Bjelakovi, D. Pavlovic, G. Kocic, D. Krstic, T. Cvetkovic, V. Pavlovic (2008). Melatonin reduces oxidative stress induced by chronic exposure of microwave radiation from mobile phones in rat brain. *Journal of Radiation Research*, 49, 579-586.

Souza P. M. D. (2010). Application of microbial α-amylase in industry-A review. *Brazilian Journal of Microbiology*, 41(4), 850-861.

Spencer R., Hafiz S., Cook C. (1985) Effect of microwave energy on the metabolism of enterobacteriaceae. *Journal of Medical Microbiology*, 19, 269-272.

Stuchly M. A., & Stuchly S. S. (1983). Industrial, scientific, medical and domestic applications of microwaves. *Physical Science, Measurement and Instrumentation, Management and Education-Reviews, IEE Proceedings* A, 130(8), 467-503.

Sun T., Jiang B., & Pan B. (2011). Microwave accelerated transglycosylation of rutin by cyclodextrin glucanotransferase from *Bacillus* sp. SK13. 002. *International Journal of Molecular Sciences*, 12(6), 3786-3796.

Tahir A., Mateen B., Univerdi S., KaraGoban O., & Zengin M. (2009). Simple method to study the mechanism of thermal and non thermal bactericidal action of microwave radiations on different bacterial species. *Journal of Bacteriology Research*, 1(5), 058-063.

Tsuji M., & Yokoigawa K. (2011). Acid resistance and verocytotoxin productivity of enterohemorrhagic *Escherichia coli* O157: H7 exposed to microwave. *Journal of Food Science*, 76(6), M445-M449.

Vela G. R., & Wu J. F. (1979). Mechanism of lethal action of 2,450-MHz radiation on microorganisms. *Applied and Environmental Microbiology*, 37(3), 550-553.

Wang A., Wang M., Wang Q., Chen F., Zhang F., Li H., ... & Xie T. (2011). Stable and efficient immobilization technique of aldolase under consecutive microwave irradiation at low temperature. *Bioresource Technology*, 102(2), 469-474.

Williams J. M. (2009) Biological Effects of Microwaves: Thermal and Non-thermal Mechanisms. http://arXiv.org/pdf/physics/0102007.

Woo I., Rhee I., Park H. (2000) Differential damage in bacterial cells by microwave radiation on the basis of cell wall structure. *Applied and Environmental Microbiology*, 66(5):2243-2247.

Yaghmaee P., T. Durance (2005) Destruction and injury of *Escherichia coli* during microwave heating under vacuum. *Journal of Applied Microbiology*, 98, 498–506.
Yu Y., & Yao K. (2010). Non-thermal cellular effects of low-power microwave radiation on the lens and lens epithelial cells. *Journal of International Medical Research*, 38(3), 729-736.

Zelentsova N. V., Zelentsov S. V., & Semchikov Y. D. (2006). On the mechanism of microwave initiated reactions. *ChemInform*, 37(8).

Zhang J., Henriksson G., & Johansson G. (2000). Polygalacturonase is the key component in enzymatic retting of flax. *Journal of Biotechnology*, 81(1), 85-89.