THE CHARACTERIZATION OF BACTERIA WITH FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

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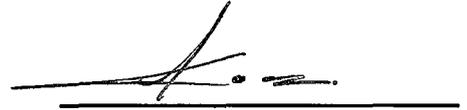THE DEGREE OF MASTER OF SCIENCE

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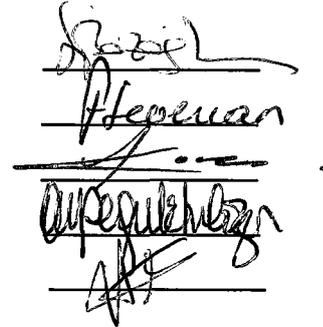Prof.Dr.Feride Severcan

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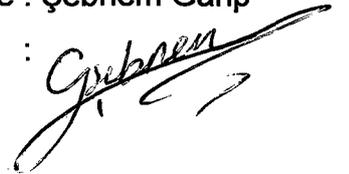
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ABSTRACT

THE CHARCTERIZATION OF BACTERIA WITH FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

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New and rapid techniques for the characterization and identification of bacteria would have an important role in clinical microbiology and in food analysis because of an increasing prevalence of infectious diseases and food poisoning.

In this work we carried out two approaches. In the first study the characterization and differentiation of mesophilic and thermophilic bacteria were investigated by using Fourier Transform Infrared (FTIR) Spectroscopic technique. In the second study, we investigated the characterization and identification of 3 *Bacillus* and *Micrococcus* species with FTIR Spectroscopy.

Our results from first approach show that there was a dramatic difference between mesophilic and thermophilic bacteria. The protein concentration was high, lipid concentration, the level of triglycerides and

the unsaturated acyl chains decreased in thermophilic bacteria. We found that in thermophilic bacteria PO_2^- groups become hydrogen bounded. In addition, our results suggest that the cellular DNA content was low in thermophilic bacteria.

Moreover there were characteristic peaks for both mesophilic and thermophilic bacteria and these peaks can be used for the differentiation of these two bacteria group. There were also some specific peaks that can be used for the differentiation of *Escherichia coli* and *Lactobacillus plantarum* at species level.

In the second approach, our results show that there were significant spectral differences between *Bacillus* and *Micrococcus* species such as the proportion of unsaturated acyl chains in triglycerides were higher in *Micrococcus* species. Moreover we observed different bands that may be explained by an acetate oxidation via the tricarboxylic acid cycle and an exopolymer formation in *Micrococcus* species. In addition to that another band similar to glycogen, may be explained by a glycogen-like storage material in *Micrococcus* species. Also there are characteristic peaks that can be used for identification of Micrococcus spp.

Key words: Mesophilic bacteria, Thermophilic bacteria, *Micrococcus, Bacillus*, characterization, FTIR.

ÖZ

FOURIER KIZILÖTESİ DÖNÜŞÜM SPEKTROSKOPİ TEKNİĞİ İLE BAKTERİ KARAKTERİZASYONU

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Besin zehirlenmesi ve salgın hastalıkların yayılmasındaki artıştan dolayı klinik mikrobiyoloji ve besin analizlerinde yeni ve hızlı bakteri karakterizasyon ve ayrım teknikleri önemli bir rol almaktadır.

Bu çalışmada iki yaklaşım ortaya koyduk. İlk çalışmada, termofilik ve mezofilik bakterilerin karakterizasyonu ve ayırımı Fourier Kızılötesi Dönüşüm (FTIR) Spektroskopi Tekniği ile incelenmiştir. İkinci çalışmada, 3 *Bacillus* ve bir *Micrococcus* türünün tanımlanmasını ve karakterizasyonunu FTIR Spektroskopisi ile inceledik.

İlk çalışmadaki termofilik ve mezofilik bakteriler arasında büyük farklılıklar gözlemlenen sonuçlarımıza göre; termofilik bakterilerin protein konsantrasyonları yüksek, lipid konsantrasyonları, trigliseridlerin seviyesi ve doymamış asil zincirleri düşüktür. Thermofilik bakterilerde PO_2^- gruplarının yaptığı hidrojen bağlarının kuvvetlendiğini bulduk. Buna ek olarak, termofilik bakterilerde hücresel DNA içeriğinin az olduğu

gözlenmiştir. Bundan başka, termofilik ve mezofilik bakterilerin ayrımında ve iki mezofilik bakterinin;

Escherichia coli ve *Lactobacillus plantarum*, karakterizasyonunda kullanılabilecek karakteristik bandlar vardır.

İkinci çalışmada sonuçlarımız göstermektedirki; *Bacillus* ve *Micrococcus* türleri arasında anlamlı spektral farklılıklar vardır. Örneğin; *Micrococcus* türlerinde trigliseridlerdeki doymamış asil zincirlerin oranı yüksektir. Bundan başka, *Micrococcus* türlerinde trikarboksilik asit döngüsündeki asetat oksidasyonu ile açıklanabilecek ve *Micrococcus* türünde exopolymer formasyonu ile açıklanabilecek farklı bandlar gözlemlenmiştir. Buna ek olarak, glikojenden kaynaklanan ve glikojen depo maddesi ile açıklanabilecek başka bir band Micrococcus türlerinde gözlemlenmiştir. Ayrıca *Micrococcus* türlerinin tanımlanmasında kullanılabilecek karakteristik bandlar vardır.

Anahtar kelimeler: Mezofilik bakteri, termofilik bakteri, *Micrococcus*, *Bacillus*, karakterizasyon, FTIR.

To my father; Yusuf GARİP

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION TO METHODS ON BACTERIAL IDENTIFICATION

Because of an increasing prevalence of infectious diseases and food poisoning, identification of bacteria is very important in clinical microbiology and in food analysis (Naumann et al., 2002). Bacterial classification and differentiation techniques are used in various fields such as clinical, environmental and food microbiology (Naumann 1998).

16S rDNA sequencing analysis is the most preferred technique for the identification of bacterial species such as *Ochrobactrum anthropi* from immunocompromised patients and nosocomial infection (Teyssier et al., 2003). Besides 16S rDNA sequencing analysis, some other techniques are used for differentiation of different bacterial species such as Denaturing Gradient Gel Electrophoresis and Sequencing (Laura et al., 2003) and PCR with DNA-DNA hybridization analysis (Scheldeman et al., 2002). For the identification of gram (+) lactic acid bacteria, another technique called RAPD-PCR is used (Elegado et al., 2004). Moreover, the post-electrophoretic detection of esterases applied in bacterial systems, that mainly provides information on the similarity of strains within the same species or subspecies according to their esterase patterns (Medina et al., 2004). Another technique that is used for the identification of bacteria is the florecence in situ hybridization (FISH) (Aman et al., 1992). Most common method used for the differentiation and identification of bacteria is selective and differential media. Selective

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detection of *Enterobacter sakazakii*, causative agent of a fatal invasive infection of neonates, with a chromogenic medium (Druggan-Forsythe-Iversen agar, DFI) is an example of this method in literature (Iversen et al., 2004).

Different from all of these traditional techniques, Fourier Transform Infrared Spectroscopy is used as an analytical tool for the identification and differentiation of bacteria because it has a lot of advantages that will be discussed later such as being a rapid and easy method (Naumann, 1998).

In the next parts of this chapter the bacterial species that were used in this study, the basic principles of infrared spectroscopy, the usage of Fourier Transform Infrared Spectroscopy in bacterial identification studies and the advantages of FTIR both in general and in microbial studies will be discussed.

1.2 INTRODUCTION ON BACTERIAL SPECIES

Life on earth exhibits an enormous adaptive capacity. Except for centers of volcanic activity, the surface of our planet is "biosphere" (Jaenicke and Böhm, 1998; Jaenicke, 2000). In quantitative terms, the limits of the biologically relevant physical variables are -40 to +115 ^{0}C (in the stratosphere and hydrothermal vents, respectively), $\leq$120 Mpa (for hydrostatic pressures in the deep sea), $a_w \approx$ 0.6 (for the activity of water in salt lakes) and $\approx$1<pH<11 (for acidic or alkaline biotopes)(Jaenicke, 1991a).

During evolution, organisms achieved viability under extreme conditions either by "escaping" or "compensating" the stress or by enhancing the stability of their cellular inventory (Jaenicke and Böhm, 1998). Temperature is one of the most important environmental factors affecting the activity and evolution of living organisms (Kristjansson,

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1989). Since all the processes of growth depend on the biochemical reactions that are effected by temperature, it has a significant role on the growth of microorganisms (Tortora et al., 1995).

Eukaryotes are unable to adapt to high temperatures, their upper limit being about 60-62 ^{0}C. The upper temperature limit for plants and animals is even lower, less than 50 ^{0}C. At temperatures above 60-62 ^{0}C, the only organisms present are procaryotes. The photosynthetic bacteria have upper temperature limits lower than those of nonphotosynthetic bacteria. The upper limit of photosynthetic bacteria is about 70-73 ^{0}C. At the higher temperatures over 100 ^{0}C, the only bacteria found are a few unusually heat adapted Archae called hyperthermophiles (Table 1)(Brock, 1985).

Although there is a continuum of organisms, from those with very low temperature optima, it is possible to broadly distinguish four groups of bacteria in relation to their temperature optima (Brock, 1985; Madigan and Naris, 1997):

Psychrophiles; with low temperature optima. They can grow at 0^{0}C, and some even as low as -10^{0}C; their upper limit is often about 25^{0}C.

Mesophiles; with midrange temperature optima. These organisms grow in the moderate temperature range, from about 20^{0}C (or lower) to 45^{0}C.

Thermophiles; with high temperature optima. They are heat loving, with an optimum growth temperature of 50^{0}C or more, a maximum of up to 70^{0}C or more and a minimum of about 20^{0}C.

Hyperthermophiles; with very high temperature optima. They have an optimum above 75^{0}C and thus can grow at the highest temperatures tolerated by an organism.

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Table 1. Upper Temperature Limits for Growth of Various Organisms (Brock, 1985)

Group	Upper Temperature Limits (^{0}C)
Animals	
Fish	38
Insects	45-50
Ostracods(crustaceans)	49-50
Plants	
Vascular plants	45
Mosses	50
Eucaryotic microorganisms	
Protozoa	56
Algae	55-60
Fungi	60-62
Procaryotes	
Bacteria	
Cyanobacteria (oxygen-producing photosynthetic bacteria)	70-73
Other photosynthetic bacteria (do not produce oxygen)	70-73
Heterotrophic bacteria (use organic nutrients)	90
Archaea	
Methane-producing bacteria	110
Sulfur-dependent bacteria	115

1.2.1 THERMOPHILIC BACTERIA

The discovery of deep-sea hydrothermal vents with dense populations of microorganisms fluorishing nearby (DeLong *et al.*, 1994) is one of the most suprising developments that have occured so far in the field of microbiology. Several members of thermophilic Archaea and Bacteria were isolated from natural and artificial environments. In many respects, the ecology of thermophilic bacteria is more complex than that of

thermophilic archaea because bacteria has more diversity in their metabolism and habitat. Many thermophilic and hyperthermophilic bacterial species have been isolated from hot environments. In the literature a total of 20 thermophilic Bacillus species are reported (Canganella, 1995). There are only two genera representing the hyperthermophilic bacteria, namely *Aquifex* and *Thermotoga* with the optimal growth at 80^0C (Prieur, 1997).

1.2.1.1. ECOLOGY OF THERMOPHILES AND HYPERTHERMOPHILES

Thermophilic procaryotes with growth temperature optima below 80^0C have been widely found in artificial thermal environments (Table 2)(Brock, 1985).

The hot water heater, domestic or industrial, usually has a temperature of $55\text{-}80^0C$ and is a favorable habitat for the growth of thermophilic procaryotes. Organisms resembling *Thermus aquaticus*, a common hot spring organism have been isolated from hot water at many installations. Electric power plants, hot industrial process water, and other artificial thermal sources probably also provide sites where thermophiles can grow (Brock, 1985).

1.2.1.2. BIOTECHNOLOGICAL AND INDUSTRIAL APPLICATIONS OF THERMOPHILES AND HYPERTHERMOPHILES

Thermophilic and hyperthermophilic microorganisms are of interest for more than just basic biological reasons. These organisms offer some major advantages for industrial and biotechnological processes, many of which run more rapidly and efficiently at high temperatures (Brock, 1985).These microorganisms have consistently attracted the curiosity of scientists from various disciplines and this interest has rapidly increased during the last decade (Aguilar A., 1996).

Table 2. Major Groups of Thermophilic and Hyperthermophilic Procaryotes (Brock, 1985)

Genus	Temperature range (^{0}C)
Bacteria	
Phototrophic Bacteria	
Cyanobacteria	55-70
	(One strain,74)
Purple bacteria	45-60
Green bacteria	40-73
Gram-positive Bacteria	
Bacillus	50-70
Clostridium	50-75
Lactic acid bacteria	50-65
Actinomycetes	55-75
Other Bacteria	
Thiobacillus	50-60
Spirochete	54
Desulfotomaculum	37-55
Gram-negative aerobes	50-75
Gram-negative anaerobes	50-75
Thermotoga/Aquifex	55-95
Thermus	60-80
Archaea	
Methanogens	45-110
Sulfur-dependent hyperthermophiles	60-113
Thermoplasma	37-60

Protein chemists, in particular, have devoted attention to these microorganisms as a source of prateins to be studied (Jaenicke *et al.*, 1996). Proteins obtained from thermophiles were expected to exhibit unusual properties, mainly an outstanding resistance to physical stres,

useful both to study protein stability and thermotolerance from an academic point of view, and for the industrial exploitation of these enzymes as biocatalysts with an extended shelf-life (Jaenicke et al., 1996; Scandurra et al., 1998).

Thermozymes are enzymes that evolved in thermophiles and hyperthermophiles (Vieille and Zeikus, 1996). Thermozymes, which are optimally active between $60^{0}C$ and $125^{0}C$, have attracted increasing attention in recent years owing to their biotechnological potential (Vieille *et al.*, 1996). They are already used in molecular biology (e.g. Taq polymerase), in addition to the detergent (e.g. proteases) and starch-processing (e.g. α-amylases, glucose isomerases) industries, and are excellent catalytic candidates for numoreous additional applications that require high stability (Vieille and Zeikus, 1996). These applications include organic syntheses, diagnostics, waste treatment, pulp and paper manufacture, and animal feed (Vieille *et al.*, 1996).

As a conclusion, they have a biotechnological significance in food, chemical and pharmaceutical industries and in environmental biotechnology (Niehaus *et al.*, 1999).

1.2.2. *LACTOBACILLUS* AND *ESCHERICHIA* SPECIES AS MESOPHILIC BACTERIA

1.2.2.1. *LACTOBACILLUS* SPP.

Lactobacillus is a genus of gram-positive, facultative bacteria, named as such because most of its members convert lactose and other simple sugars to lactic acid (Brock, 1985).

Many *lactobacilli* are unique among living things in requiring no iron and having extremely high hydrogen peroxide tolerance (Brock, 1985).
Moreover, many *lactobacilli* are unusual in that they operate using homofermentative metabolism (that is, they produce only lactic acid from

sugars) and are aerotolerant despite the complete absence of a respiratory chain. This aerotolerance is manganese-dependent and has been explored (and explained) in *Lactobacillus plantarum* (Archibald and Fridovich, 1981).

1.2.2.1.1. ECOLOGY

They are common and usually benign inhabitants of the bodies of humans and other animals-for example, they are present in the gastrointestinal tract and the vagina (Brock, 1985; Al Jassim *et al.*, 2005).

Many species are prominent in decaying plant material. The production of lactic acid makes their environment acidic which inhibits the growth of some harmful bacteria (Brock, 1985).

1.2.2.1.2. INDUSTRIAL USES

Lactic acid bacteria play a significant role in the food industry, as they aid in various food processes, and possess unique characteristics that allow them to be potential probiotic organisms (Vogel and Ehrmann, 1996; Dill and Oorebeck, 2002). They are used industrially for the production of yogurt, sauerkaut, pickles and other fermented foods, such as silage (Jacobsen *et al.*, 1999).

These fermented foods are a significant part of the food processing industry and are often prepared using selected strains that have the ability to produce desired products or changes efficiently (McKay and Baldwin, 1990).

The application of genetic engineering technology to improve existing strains or develop novel strains for these fermentations is an active research area (McKay and Baldwin, 1990).

1.2.2.2. *ESCHERICHIA* SPP.

1.2.2.2.1. ECOLOGY

Escherichia coli colonise the intestine of virtually all warm blooded animals from shortly after birth, where they are part of the commensal flora (Brock, 1985).

E.coli can not only survive but actively grow in many environmental waters. As *E.coli* are present in the faeces of humans and most animals their presence has been used as an indicator of faecal pollution for many years (Brock 1985).

1.2.2.2.2. PATHOGENESIS

E.coli can cause enteric infections, extraintestinal infections and animal infections (Qadri *et al.*, 2005; Riley *et al.*, 2005; Shin *et al.*, 2005 and Breitschwerdt *et al.*, 2005).

Enteric infections; there are a number of types causing human and animal intestinal diseases that include the Enteroaggregative *E.coli* (EaggEC)(Nakajima *et al.*, 2005), Enterohaemorrhagic *E.coli* (EHEC)(Crepin *et al.*, 2005), Enteroinvasive *E.coli* (EIEC)(Ahmed *et al.*, 2005; Kingombe *et al.*, 2005), Enteropathogenic *E.coli* (EPEC)(Crepin *et al.*, 2005) and Enterotoxigenic *E.coli* (ETEC)(Quadri *et al.*, 2005).

Extraintestinal infections; Uropathogenic *E.coli* (UPEC) cause urinary tract infections (Usein *et al.*, 2003; Riley *et al.*, 2005) and there are Neonatal Menigitis *E.coli* (NMEC) (Shin *et al.*, 2005).

Animal infections; apart from causing similar infections in animals as some of the human ones, there are specific animal diseases including: calf septicaemia, bovine mastitis, porcine oedema disease and

respiratory tract infections (air sac disease) in poultry (Brock, 1985; Breitschwerdt *et al.*, 2005).

1.2.2.2.3. INDUSTRIAL USES

As *E.coli* can be grown very easily on simple media and its genetic characteristics have been essentially determined. They have found extensive use as vehicles for the preparation of biological polymers, including polypeptide hormones, proteins, carbohydrates etc. (Brich and Onakunle, 2005).

1.2.2.2.4. VACCINES

Currently most of the specific *E.coli* vaccines are used for animal diseases. *E.coli* toxins have been used toxoided (made harmless but still antigenic) and used as vaccines (Brock, 1985).

There are also some studies suggesting that *E.coli* cell-wall preparations could be used as vaccines to prevent the effects of endotoxic shock in cases of gram-negative septicaemia (Xu *et al.*, 2004).

These two bacterial species (*L.plantarum* and *E.coli*) were used as a mesophilic bacteria in this study.

1.2.3. *BACILLUS* AND *MICROCOCCUS* SPECIES

1.2.3.1. *BACILLUS* SPP.

Bacillus spp. are rod-shaped, gram-positive, sporulating, aerobes or facultative anaerobes. Bacilli exhibit an array of physiologic abilities that allow them to live in a wide range of habitats, including many extreme habitats such as desert sands, hot springs and Arctic soils (Priest *et al.*, 1988). Bacilli are an extremely diverse group of bacteria that include both the causative agent of anthrax (*Bacillus anthracis*) as well as

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several species that synthesize important antibiotics (Perreten *et al.*, 2005).

In addition to medical uses bacillus spores, due to their extreme tolerance to both heat and disinfectants, are used to test the heat sterilization techniques and chemical disinfectants (Mosley *et al.*, 2005). Bacilli are also used in the detergent manufacturing industry for their ability to synthesize important enzymes (Parkar *et al.*, 2004).

Bacillus subtilis has a plant growth promoting rhizobacterium and synthesizes antifungal peptides. This ability has lead to use of *B. Subtilis* in biological control. *B.subtilis* has been shown to increase crop yields,although it is not known whether this is due to it enhances plant growth, or inhibits disease growth (Arias *et al.*, 1999).

Despite the pathogenic capabilities of some bacilli, many other species are used in medical and pharmaceutical processes. These take the advantage of bacteria's ability to synthesize certain proteins and antibiotics (Perreten *et al.*, 2005). Bacitracin and plymixin, two ingredients in Neosporin, are products of bacilli (Cao *et al.*, 2005). Innocuous Bacilli microbes are also useful for studying the virulent *bacillus* species that are closely related *B.subtilis* has multiple carbohydrate pathways representing the variety of carbohydrates found in the soil (Zamboni *et al.*, 2004).

Bacillus licheniformis is an apathogenic soil organism that is mainly associated with plant and plant materials in nature. It can be isolated from nearly everywhere due to its highly resistant endospores that are disseminated with the dust. It is toxinogenic and food poisoning in humans which has been associated with cooked meat, poultry and vegetable dishes (Veith *et al.*, 2004).

Bacillus licheniformis is used by industry to produce proteases and amylases and is also used to make the polypeptide antibiotic Bacitracin. This strain had considerable colonisation and competetive ability, and it could be used as a fertilizer or biocontrol agent without altering normal management in greenhouses (Jose *et al.*, 2004).

1.2.3.2. *MICROCOCCUS* SPP.

Micrococcus is a gram-positive, aerobic bacterium. Although *Micrococcus* rarely causes infections or problems in the body, those with compromised immune systems, such as occurs with HIV+ patients, have been known to get skin infections caused by *Micrococcus luteus* (Smith *et al.*, 1999). *Micrococcus luteus* is a natural habitant of the human skin, and can occasionally be found in the mucous membranes. The bacteria is non-pathogenic and can be spread through direct contact and is found throughout our environment (most common in dust particles), but can't survive for long in soil or in water (Brock, 1985).

In general, the only risk in immunocompromised patients where the *M.luteus* bacteria could lead to the development of pneumonia or septic shock (Smith *et al.*, 1999). However the usefullness of this organism has been researched and it has been suggested that *M.luteus* is useful for the economic production of long-chain (C21-C34) aliphatic hydrocarbons, which few bacteria other than micrococci produce. These may be useful as lubricating oils and may be substitutes for equivalent petroleum products (Bieszkiewicz *et al.*, 1998; 2002). *M.luteus* has also been used as a test organism for the assay of antibiotics in body fluids, animal feeds, milks and pharmaceuticals.

1.3. OPTICAL SPECTROSCOPY

1.3.1. BASIC PRINCIPLES OF OPTICAL SPECTROSCOPY

Spectroscopy is defined as the study of interaction of electromagnetic radiation with matter, excluding chemical effects.

When an electromagnetic wave encounters a molecule, it can be either scattered (i.e., its direction of propagation changes) or absorbed (i.e., its energy is transferred to the molecule). If the electromagnetic radiation of the light is absorbed, the molecule is said to be excited. An excited molecule can posses any one of a set of discrete amounts (quanta) of energy described by the laws of quantum mechanics. These amounts are called the energy levels of the molecule. The major energy levels are determined by the possible spatial distributions of the electrons and are called electronic energy levels, on these are superimposed vibrational levels which indicate the various modes of vibration of the molecule. All these energy levels are described by an energy-level diagram (Figure 1). The lowest electronic level is called the ground state and all others are excited states (Freifelder, 1982).

For most purposes, it is convenient to treat a molecule as if it possesses several distinct reservoirs of energy. The total energy is given by the following equation (Campbell and Dwek, 1984)

$$E_{total}=E_{transition}+E_{electronic}+E_{rotation}+E_{vibration}+E_{electron\ spin\ orientation}+E_{nuclear\ spin\ orientation}$$

Energy can reside in molecules in a number of forms, of which the most important ones, are translational, electronic, vibrational, and rotational energy. Infrared spectroscopy is based on molecular vibrations and monitors the transition between vibrational energy level (Wilson *et al.*, 1995).

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Light is a electromagnetic wave. The energy of the wave is:

$$E = h.c/\lambda = h.v$$

In which h is Planck's constant, c is the velocity of light, λ is the wavelength and v is the frequency.
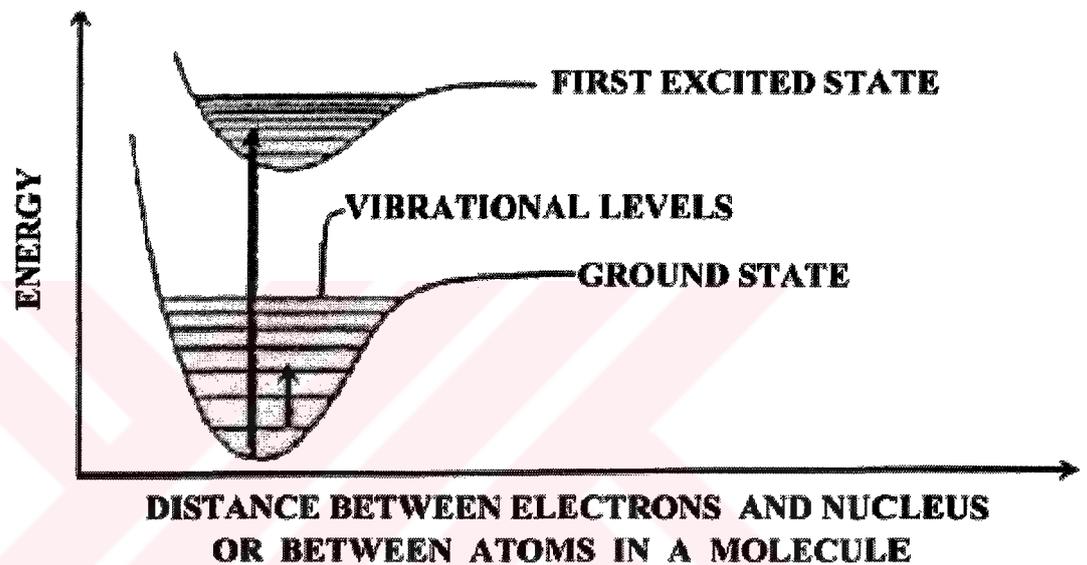
Figure 1. Typical energy-level diagram showing the ground state and the first excited state. Vibrational levels are shown as thin horizontal lines. A possible electronic transition between the ground state and the fourth vibrational level of the first excited state is indicated by the long arrow. A vibrational transition within the ground state is indicated by the short arrow (Freifelder, 1982).

1.4. INFRARED (IR) SPECTROSCOPY

1.4.1. BASIS OF INFRARED (IR) SPECTROSCOPY

IR spectroscopy is the measurement of the wavelength and intensity of the absorption of infrared light by a sample. Infrared light is energetic enough to excite molecular vibrations to higher energy levels (Campbell & Dwek., 1984 and Brey., 1984).

Infrared (IR) region is divided into three sub regions (Smith, 1999):

Region	Wavenumber range (cm^{-1})
Near	14000-4000
Middle	4000-400
Far	400-4

The vibrational levels and hence, infrared spectra are generated by the characteristic twisting, bending, rotating and vibrational motions of atoms in a molecule. All of the motions can be described in terms of two types of molecular vibrations. One type of vibration, a stretch, produces a change of bond length. A stretch is a rhythmic movement along the line between the atoms so that the interatomic distance is either increasing or decreasing. The second type of vibration, a bend, results in a change in bond angle. These are also called scissoring, rocking or wigwag motions. Each of these two main types of vibration can have variations. A stretch can be symmetric or asymmetric. Bending can occur in the plane of the molecule or out of plane; it can be scissoring, like blades of

a pair of scissors, or rocking, where two atoms move in the same directions (Figure 2)(Volland, 1999).

1.4.2. FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

In a Fourier Transform Infrared Spectrometer, a continuum source of light is used to produce light over a broad range of infrared wavelengths. Light coming from this continuum source is split into two paths using a half-silvered mirror; this light is then reflected from two mirrors back onto the beamsplitter, where it is recombined. One of these mirrors is fixed, and the second is movable. If the distance from the beamsplitter to fixed mirror is not exactly the same as the distance from the beamsplitter to the second mirror, then when two beams are recombined, there will be a small difference in the phase of the light between these two paths. Because of the "superposition principle" constructive and destructive interference exist for different wavelengths depending on the relative distances of the two mirrors from the beamsplitter.
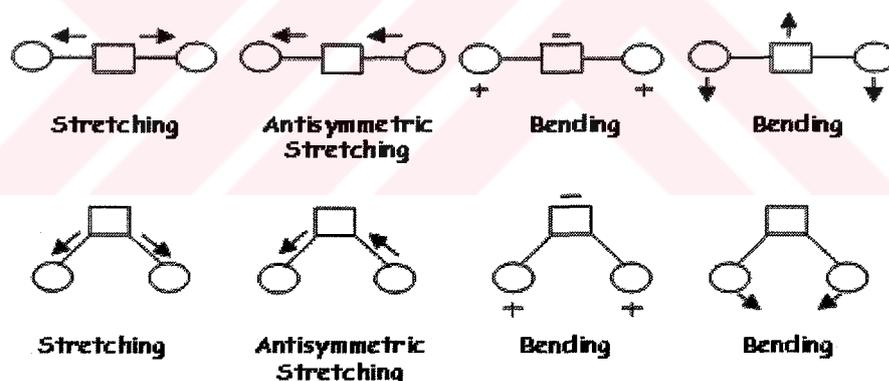
Figure 2. Types of normal vibrations in a linear and non-linear triatomic molecule.

One of these mirrors is fixed, and the second is movable. If the distance from the beamsplitter to fixed mirror is not exactly the same as the distance from the beamsplitter to the second mirror, then when two

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beams are recombined, there will be a small difference in the phase of the light between these two paths. Because of the "superposition principle" constructive and destructive interference exist for different wavelengths depending on the relative distances of the two mirrors from the beamsplitter.

It can be shown that if the intensity of light is measured and plotted as a function of the position of the movable mirror, the resultant graph is the Fourier Transform of the intensity of light as a function of wavenumber. In FTIR spectroscopy, the light is directed onto the sample of interest and the intensity is measured using an infrared detector. The intensity of light striking the detector is measured as a function of the mirror position, and this is then Fourier-transformed to produce a plot of intensity vs. wavenumber. (Figure 3).
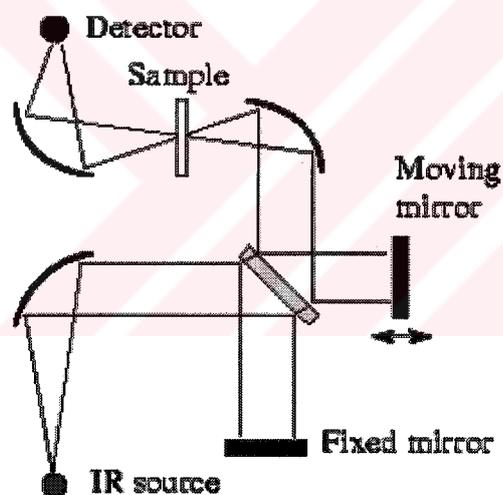
Figure 3. Shematic representation of FT-IR spectrometer

1.4.3. ADVANTAGES OF FT-IR SPECTROSCOPY

FT-IR spectroscopy produces conventional spectrum and it has several important advantages.

- The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological material can be obtained in a wide variety of environments.

- The amount of sample required is relatively small.

- FT-IR method is a rapid and sensitive technique with sampling techniques that are easy to use.

- Since a computer is already used to obtain the Fourier transform, it is easy to perform many scans to improve the signal-to-noise ratio (noise adds up as the square root of the number of scans, whereas signal adds linearly).

- The instrumentation is inexpensive compared to the cost of X-Ray diffraction, NMR, ESR, and CD spectroscopic equipment and the operation of the equipment is simple. Interpretation of the spectra is not particularly difficult and can be learned easily.

- Digital substraction (that is, point-by-point substraction of the seperate spectra by a computer) can also be used to produce good difference spectra. This method has great advantages in obtaining infrared spectra in aqueous solutions.

- The FT-IR has advantage in terms of spectral regions which originate from molecular vibrations and different molecular moieties. For instance "head group" and "hydrocarbon tails" have spectral regions for membranes.
- There is no light scattering or fluorescent effects.

- Kinetic and time-resolved studies are possible (Mantcsch et al., 1986; Mendelson and Mantsch, 1986 and Haris and Severcan, 1999).

1.4.4 INFRARED SPECTROSCOPY AS A TOOL FOR THE BACTERIA STUDIES

Last years have witnessed the emergence of new sensitive, rapid, and increasingly precise physical techniques for microbiological analysis. These new techniques range from various spectroscopic techniques such as molecular spectroscopy (including FTIR and mass spectroscopy) to the different seperation techniques like gas chromotography (GC) and high-performance liquid chromotography (HPLC) (Nelson, 1985; 1991; Fox *et al.*, 1990; Mantsch and Chapman, 1996).

In 1911 W.W.Coblentz was probably the first scientist to suggest that biological materials can profitably be analyzed by means of infrared (IR) spectroscopy. Already in the 1950s and 1960s spectroscopists have demonstarated the feasibility to identify bacteria by IR spectroscopy (Norris, 1959; Riddle *et al.*, 1956; Thomas and Greenstreet, 1954; Greenstreet and Norris, 1957; Mantsch and Chapman, 1996). Unfortunately, due to the lack of efficient computers and the weak instrumental specifications at that time, reports on bacterial characterizations by IR spectroscopy became less frequent in the 1960s and ceased in the 1970s. It is the development of modern interferometric IR spectroscopy and the availability of low-cost minicomputers that contributed greatly to revival of IR spectroscopy as a means for characterizing intact microorganisms in the last years (Naumann, 1985; Mantsch and Chapman, 1996).

Moreover, FTIR spectroscopy is a nondestructive technique and allows the rapid and simultaneous characterization of complex materials like

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bacteria (Helm *et al.*, 1991; Schallehn *et al.*, 1991; Naumann *et al.*, 1991). For example FTIR has been used to probe the structural changes resulting from growth of *Bradyrhizobium japonicum* on different media (Zeroual *et al.*, 1994), and it has also been used as an easy and safe method type, identify, and classify several bacteria at genus level, including *Listeria* spp. (Holt *et al.*, 1995), *Staphylococcus* spp., *Clostridium* spp., *Streptococcus* spp., *Legionella* spp., *Escherichia coli* (Choo-Smith *et al.*, 2001, M.A Miguel *et al.*, 2003; Naumann *et al.*, 1994), and species level, including *Pseudomonas* spp., *Bacillus* spp. (M.A Miguel *et al.*, 2003; Udelhoven *et al.*, 2000), enterococci (Kirschner *et al.*, 2001) and yeast including *Candida* spp. (Choo-Smith *et al.*, 2001). These studies show that it is possible to discriminate among various microorganisms at the genus, species, and strain level, and studies report the ability to differentiate microorganisms from varous serogroups (Helm *et al.*, 1991; Horbach *et al.*, 1988; Seltmann *et al.*, 1994).

1.4.5. ADVANTAGES OF FTIR SPECTROSCOPY FOR THE IDENTIFICATION OF BACTERIA SPECIES

Traditional methods such as polymerase chain reaction (PCR) method (based on 16S rDNA sequencing and DNA-DNA hybridization) (Scheldeman *et al.*, 2002), PCR-enzyme-linked immunosorbent assay (ELISA) (Hong *et al.*, 2003), flow cytometric technique (Holm *et al.*, 2004), fiber-based multiplex PCR assay (Adhikary *et al.*, 2004) and using selective and differential media (Horvath *et al.*, 2003) used for the identification of microorganisms can often be extremely tedious, offering results that may take time and occasions not prove totally conclusive (M.A Miguel *et al.*, 2003).

FTIR technique can be used as an analytical tool in various fields (clinical, environmental, food microbiology) for the very rapid classification, differentiation of diverse microbial species and strains (Naumann, 1998).

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The time required for the identification of pathogens is an important determinant of infection-related mortality rates of hospitalized patients. Rapid identification techniques significantly reduce mortality and cost associated with infectious diseases (Doern *et al.*, 1994). Most commercially available identification systems in routine use in hospitals are based on the physiological and nutritional characteristic of microorganisms. These systems require a pure microbial culture and a large inoculum (Miller *et al.*, 1999). In contrast, spectroscopic techniques are characterized by a minimum of sample handling: no extractions, amplifications, labeling, or staining steps of any kind are required (Maquelin *et al.*, 2003).

FTIR Spectra reflect the overall molecular composition of a sample. Since different organisms differ in overall molecular composition, their FTIR spectra will also be different. The spectra can serve as a spectroscopic fingerprints that enable highly accurate identification of microorganisms (Naumann *et al.*, 1991; Maquelin *et al.*, 2003).

1.5. AIM OF THE STUDY

Traditional methods used for the identification of various microorganisms are based on morphological characteristics, biochemical reactions, genetic properties, serological reactions and sensitivity to bacteriophage, etc. However, all of these methods can often be extremely tedious, offering results that may take time and on occasions not prove totally conclusive.

FTIR spectroscopy is a valuable technique due to its high sensitivity in detecting changes in the functional groups belonging to the components of bacteria, such as lipids and proteins. This shift in the peak positions, bandwidth, and the intensity of the bands all give valuable structural and functional information. This technique, which requires neither reagent

nor sample preparation, is non-destructive, and highly selective because of its ability to be a spectral fingerprint for molecular components.

This study aims; first,
to investigate the similarities and differences between thermophilic and mesophilic bacteria group with FTIR Spectroscopy. Although there are bacterial identification studies with FTIR in literature, there is no study that carried out with thermophilic and mesophilic bacteria for the investigation of similarities and differences by using FTIR Spectroscopy. Second, to use FTIR Spectroscopy as a rapid and suitable method to identify and characterize different bacterial species. We used 3 *Bacillus* and a *Micrococcus* species that were not used together for identification and characterization up to date.

CHAPTER 2

MATERIALS AND METHODS

2.1 ORGANISMS

Bacterial species were previously isolated from Salt Lake located in Central Turkey and identified biochemically as *Bacillus circulans* (HTG2), *Bacillus licheniformis* (HTG1) and a *Micrococcus* species (HTG7) by Firdevs Yalçın (Yalçın, 2000). *Bacillus subtilis* and *Lactobacillus plantarum* were kindly provided by Candan Gürakan (METU, Faculty of Food Engineering).

Thermophilic bacteria; *Thermoanaerobacter ethanolicus* (JW200), *Clostridium thermohydrosulfuricum* (DSM 2247) and *Thermobrachium celere* were provided by Sedat Dönmez (Ankara University, Faculty of Food Engineering).

Mesophilic bacterium *Escherichia coli* was obtained from Refik Saydam Hıfzıssıhha Merkezi (ANKARA).

2.2 SAMPLE SELECTION

2.2.1 STUDY 1

Mesophilic bacteria group was selected as a control group while comparing with thermophilic bacteria group. Two mesophilic bacteria were also compared with each other.

2.2.2 STUDY 2

Bacteria was grown processed and primary spectra were obtained in a former study (Zeyniyev *et al.*, 2003). *Micrococcus* species were selected for the experiment in order to serve as an outgroup among the Bacillus species. *B.subtilis* was included as a control group since it is a well studied representative of the genus.

2.3 GROWTH MEDIUM

2.3.1 STUDY 1

Mesophilic microorganisms (*L.plantarum* and *E.coli*) were grown in 200 ml Nutrient broth (composition of medium was given in Appendix A) in two 250 ml Erlenmeyer flasks (100 ml media in each) at 37 ^{0}C in incubator for overnight.

Three thermophilic microorganisms were obtained seperately in 60 ml TR1 media (composition of medium was given in Appendix A) in three 100 ml serum bottles. 1 ml from each culture with visible turbidity caused by massive growth were taken and transferred to Eppendorf tubes. Long term storage was done in TR1 medium including 3% glycerol at -20^{0}C.

2.3.2 STUDY 2

Microorganisms (*Bacillus* spp. and *Micrococcus* spp.) were grown in 50 ml nutrient broth in 150 ml Erlenmeyer flasks at 37^{0}C for overnight in an incubator (Zeyniyev *et al.*, 2003).

For both studies ,sterilization of the Nutrient broth was done in autoclave (Astell Scientific). Inoculations were done in flow cabinet under aseptic conditions using metal inoculation loops.

Table of the composition of TR1 Medium and Nutrient Broth were given in Appendix B.

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2.4 SAMPLE PREPARATION

All bacterial cells were collected from overnight liquid cultures (200 ml; *Escherichia coli* and *Lactobacillus plantarum*, 60 ml; each thermophilic bacteria, 50 ml; *Micrococcus* spp and each *Bacillus* spp.) by centrifugetion (Sorvall® RC5C PLUS; Rotor SS34; 10000 RPM, 10 min.). After removing supernatant, pellet was washed two times with phosphate buffer (PH 7; 1M 57.7 ml Na_2HPO_4 and 1M 42.3 ml NaH_2PO_4 dissolved in 900 ml distilled water).

2.4.1 SAMPLE PREPARATION FOR FT-IR STUDIES

After second wash in phosphate buffer, samples were lyophilized (Labconco FreeZone®, 6 liter Benchtop Freeze Dry System Model 77520). The samples then were grinded using mortar and pestle to fine particles. 1mg bacterial powder was mixed 100 mg potassium bromide (at the ratio of 1/100). KBr is most commonly used alkali halide disk which is used as a beam condensing system. It is completely transparent in the mid-infrared region (Stuart, 1997). The mixture was again lyophilized.

Pellets were prepared by establishing pressure of 100 kg/cm^2 (1200psi) in compressor (Hidropnömak) for about 6 minutes.

2.5 FT-IR STUDIES

Infrared spectra were obtained by scanning the prepared pellets with a Spectrum One Spectrometer (Perkin Elmer, Norwalk, CT, USA). Dry air has been continously purged into the spectrometer to get rid of water vapour, which disturbs the signals. Water and carbondioxide molecules in the air affect the IR spectrum. To overcome this problem the spectrum of air was recorded as background and substracted automatically by using appropriate software (Spectrum One software).

FT-IR spectra of bacteria samples were recorded in the 4000-400 cm^{-1} region at room temperature. 100 scans were taken for each interferogram at 4 cm^{-1} resolution. Athmospheric vapor was automatically subtracted from the sample spectrum via Spectrum One software of Perkin Elmer.

To make comporison between the bacterial groups, the spectra were normalized. The purpose of the normalization is to remove differences in peak heights between the spectra acquired under different conditions. It allows a point-to-point comparison to be made (Smith, 1999). The ratios of the intensities and shiftings of the frequencies were examined before the normalization process.

Spectrum One software was used for digital data processing.

2.6 STATISTICAL TESTS

The results were expressed as mean±standard deviation values. The differences in the means of the thermophilic, mesophilic bacteria and *Bacillus* spp., *Micrococcus* spp. were compared using Mann-Whitney U-test. A p value of the less than 0.05 was considered as significant ($p<0.05^*$, $p<0.01^{**}$, $p<0.001^{***}$).

CHAPTER 3

RESULTS

3.1 BAND ASSIGNMENT OF THE BACTERIA SPECTRUM

This work was carried out to characterize and to differentiate different type of bacteria that were not studied together up to date by using FT-IR spectroscopic technique.

Figure 4 shows the representative infrared spectrum of bacteria in the 3600-400 cm^{-1} region. The main bands were labeled in this figure and detailed spectral band assignments for bacteria were given in table 3.
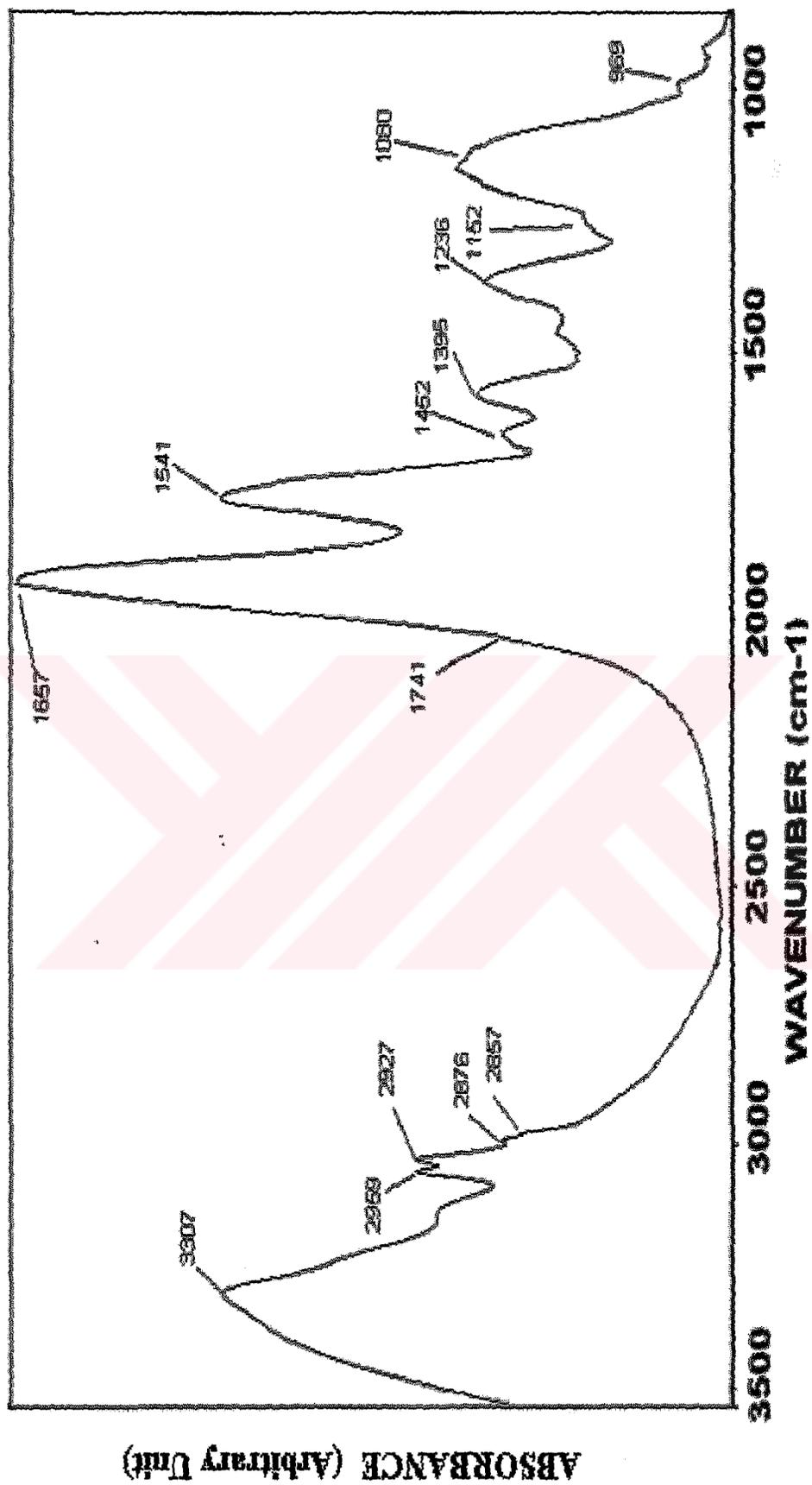
Figure 4. A general FT-IR spectrum of a bacteria.

Table 3. General band assignment of bacteria in literature (Rigas *et al.*, 1990; Naumann *et al.*, 1991; Takahashi et al., 1991; Wong et al., 1991; Wang et al., 1997; Jamin et al., 1998; Menashi et al., 1998; Jackson et al., 1998; Melin et al., 1999; Lyman et al., 1999; Chiriboga et al., 2000).

Peak numbers	Wave numbers (cm^{-1})	Definition of the spectral assignment
1	3307	N-H and O-H stretching vibration: polysaccharides, proteins
2	2959	CH_3 asymmetric stretch: mainly lipids
3	2927	CH_2 asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
4	2876	CH_3 symmetric stretch: mainly proteins, with the little contribution from lipids, carbohydrates, nucleic acids
5	2857	CH_2 symmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
6	1739-1744	Ester C=O stretch: lipid, triglycerides
7	1657	Amide I (protein C=O stretching): α helices
8	1541	Amide II (protein N-H bend, C-N stretch): α helices
9	1452	CH_2 Bending: lipids
10	1391	COO^- symmetric stretch: aminoacid side chains, fatty acids
11	1236	PO_2^- asymmetric stretching: mainly nucleic acids with the little contribution from phospholipids
12	1152	CO-O-C asymmetric stretching: glycogen and nucleic acids
13	1080	PO_2^- symmetric stretching: nucleic acids and phospholipids
14	969	$C-N^+-C$ stretch: nucleic acids

3.2 COMPARISON OF SPECTRA OF DIFFERENT BACTERIA SPECIES

3.2.1 COMPARISON OF THERMOPHILIC AND MESOPHILIC BACTERIA GROUPS

FT-IR spectral data were collected over the frequency range of 3600-400 cm $^{-1}$. In order to display the details of spectral changes, the analysis was performed in two distinct frequency ranges. The first range was between 3600-2800 cm $^{-1}$ and the second was between 1800-400 cm $^{-1}$. All mesophilic spectra which were used as control group, are overlapped. All thermophilic spectra which are different than mesophilic spectra, are also overlapped. For this reason, for the following discussions average spectra of thermophilic and mesophilic will be used.

Figure 5 shows the infrared spectra of thermophilic and mesophilic bacteria in the 3600-2800 cm $^{-1}$ region. As could be seen from this figure that the thermophilic and mesophilic spectra considerably differ in peak positions, peak heights and the widths in the 3600-2800 cm $^{-1}$ region.

Figure 6 shows the infrared spectra of the thermophilic and mesophilic bacteria in the 1800-400 cm $^{-1}$ region. As could be seen from this figure, thermophilic and mesophilic spectra have different bandwidth, intensity and frequency values of the FT-IR bands, which will be later discussed more specifically.
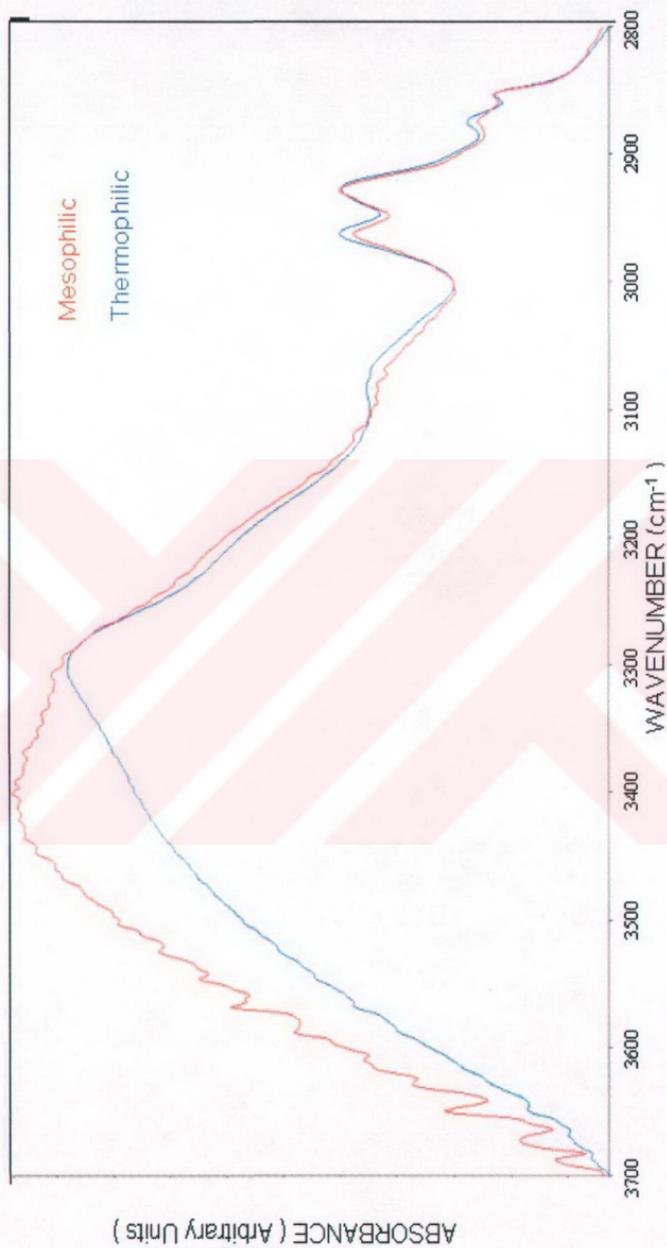
Figure 5. The infrared spectra of thermophilic and mesophilic bacteria in the 3600-2800 cm^{-1} region (the spectra were normalized with respect to the CH$_2$ asymmetric mode which is observed at 2927 cm^{-1}).

Figure 6. The infrared spectra of thermophilic and mesophilic bacteria in the 1800-400 cm^{-1} region (the spectra were normalized with respect to amide I band which is observed at 1657 cm^{-1}).

3.2.2 NUMERICAL COMPARISONS OF THE BANDS OF THERMOPHILIC AND MESOPHILIC SPECTRA

In order to take into account of possible spectral differences that could be observed between the individuals of even same bacterial group, the means and the standard deviations of the band intensities and frequencies with respect to the control (mesophilic group) were obtained for thermophilic group. The thermophilic means were compared with those of the mesophilic meansusing statistical analysis. Results were given in table 4.

3.2.3 COMPARISON OF *BACILLUS* SPP. AND *MICROCOCCUS* SPP. SPECTRA

In order to characterize the different species of bacteria with FT-IR, 3 different *Bacillus* spp. and *Micrococcus* spp. were analyzed. Bacillus *subtilis* was included as a control group since it is a well studied representative of the genus. Infrared spectra of both *Bacillus* spp. and *Micrococcus* spp. were shown in two regions of the spectra in figures 7 and 8. As it can be seen from these figures, *Micrococcus* spp. spectrum showed differences in the bandwidth and frequency values from *Bacillus* spp. spectrum. It could also be seen from figure 8 that there were characteristic peaks for different species which are labeled by arrows in 1800-400 cm^{-1} region.

Table 4. Numerical summary of the detailed differences in intensity and frequency values for thermophilic and mesophilic spectra. Mesophilic group was used as a control group and the values are the mean ± Standard Deviation for each sample.

Functional Groups	FREQUENCY			INTENSITY		
	Mesophilic (n=14)	Thermophilic (n=19)	p Values	Mesophilic (n=14)	Thermophilic (n=19)	p Values
CH_3 Asymmetric Stretching	2962.71 ± 0.87	2962.22 ± 0.49	< 0.001***	0.26 ± 0.04	0.39 ± 0.13	<0.001***
CH_2 Asymmetric Stretching	2930.52 ± 3.01	2928.44 ± 0.36	< 0.001***	0.27 ± 0.04	0.39 ± 0.14	<0.01**
CH_3 Symmetric Stretching	2872.80 ± 1.98	2875.27 ± 0.62	< 0.001***	0.25 ± 0.05	0.34 ± 0.11	<0.01**
CH_2 Symmetric Stretching	2853.14 ± 1.40	2854.19 ± 0.64	< 0.001***	0.25 ± 0.05	0.33 ± 0.11	<0.01**
Amide I	1666.95 ± 3.41	1653.12 ± 1.53	< 0.001***	0.30 ± 0.04	0.60 ± 0.27	< 0.001***
Amide II.	1540.83 ± 0.87	1536.79 ± 2.01	< 0.001***	0.27 ± 0.04	0.50 ± 0.21	< 0.001***
CH_2 Bending	1455.32 ± 2.00	1452.19 ± 0.73	< 0.001***	0.24 ± 0.04	0.35 ± 0.13	<0.01**
COO Symmetric Stretching	1402.83 ± 4.44	1396.45 ± 0.89	< 0.001***	0.24 ± 0.04	0.36 ± 0.12	<0.01**
PO_2 Asymmetric stretching	1232.84 ± 3.86	1236.69 ± 2.26	< 0.001***	0.25 ± 0.03	0.36 ± 0.14	<0.01**
PO_2 Symmetric stretching	1073.95 ± 4.91	1077.03 ± 1.20	< 0.001***			
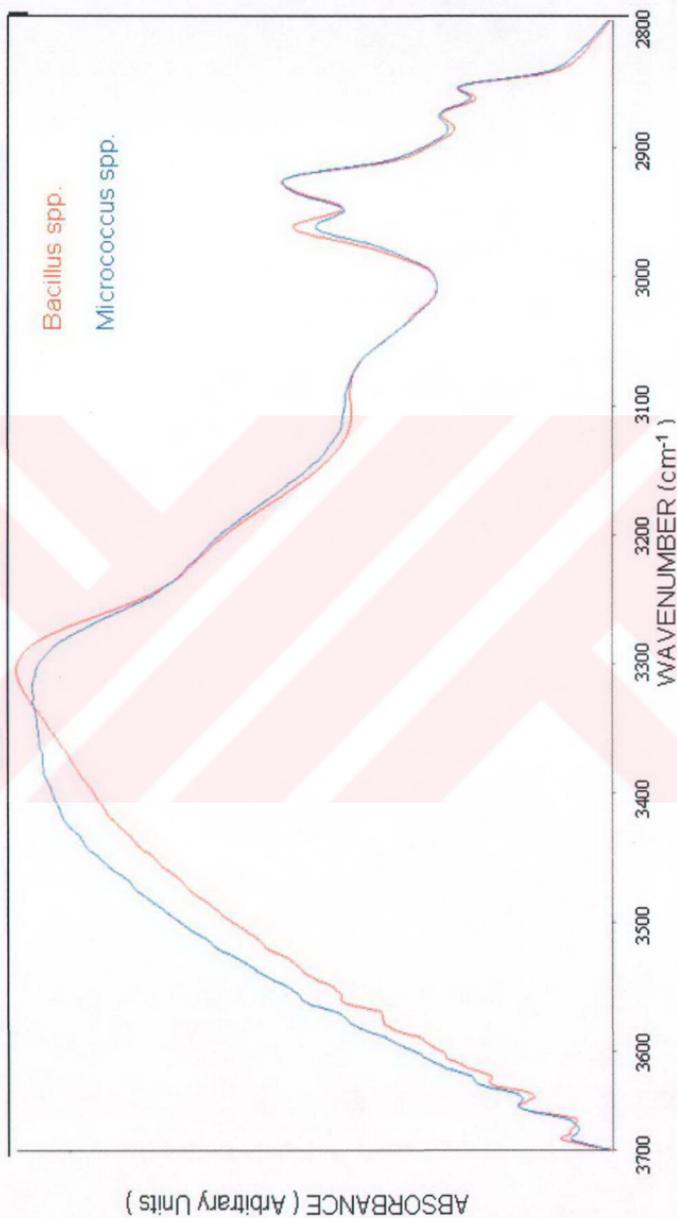
Figure 7. The infrared spectra of Bacillus spp. and Micrococcus spp. 3600-2800 cm^{-1} region (the spectra were normalized with respect to CH$_2$ asymmetric mode which is observed at 2927 cm^{-1}).
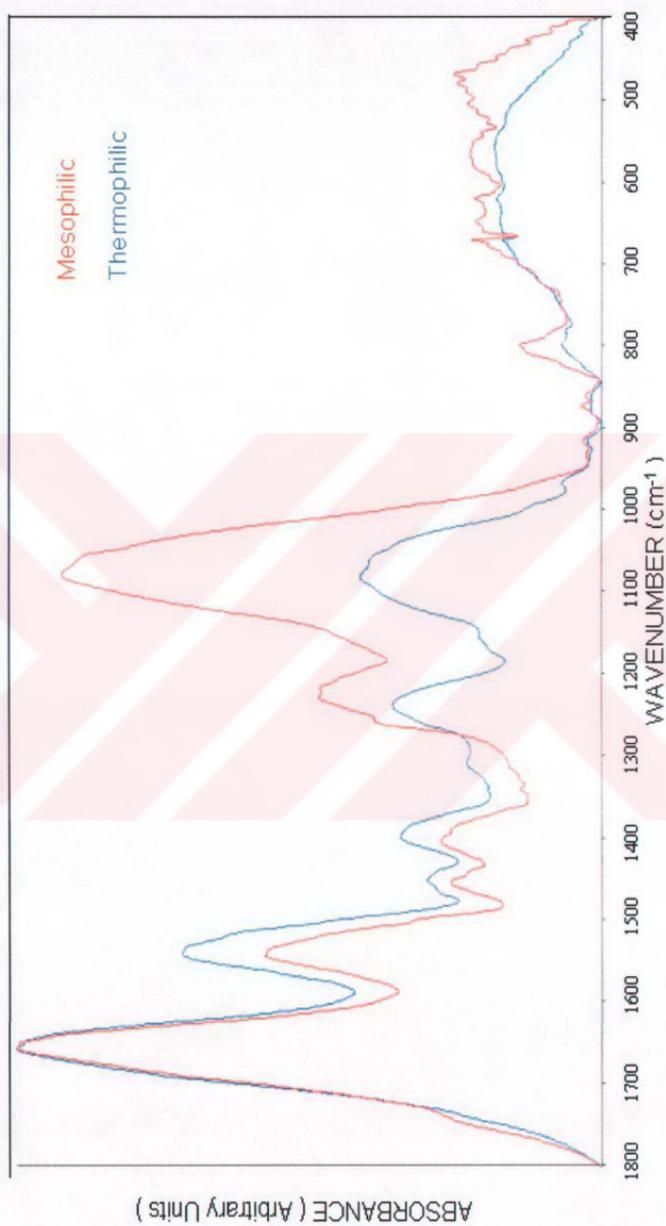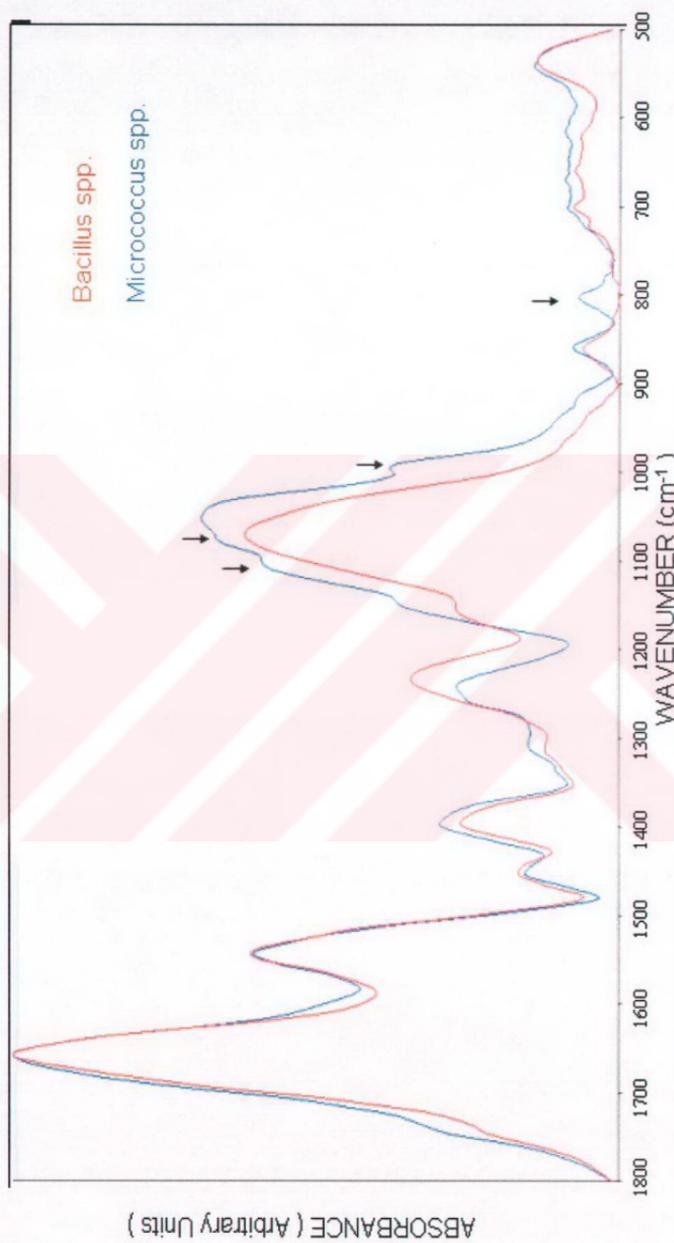
Figure 8. The infrared spectra of *Bacillus* spp. and *Micrococcus* spp. in the 1800-400 cm⁻¹ region (the spectra were normalized with respect to amide I band which is observed at 1657 cm⁻¹).

3.2.4 NUMERICAL COMPARISONS OF THE BANDS OF *BACILLUS* SPP. AND *MICROCOCCUS* SPP. SPECTRA

Spectral differences that could be observed between *Bacillus* spp. and *Micrococcus* spp. in frequency values, were compared by the help of u-test. Results were given in table 5.

The presence of significant ($p < 0.05$ - $p < 0.001$) changes in the spectra of both *E.coli* and *Lacto*bacillus *plantarum* were illustrated in the table.

Table 5. Changes in the band frequency values of various functional groups in *Bacillus* spp. (n=10) and *Micrococcus* spp. (n=5) samples.

Functional Groups : Frequency	Bacillus spp. (n=10)	Micrococcus spp. (n=5)	p Values
Ester(C=Ostrecthing)	1744.94±1.55	1738.99±3.31	<0.01**
Amide I	1657.27±1.85	1659.45±2.20	0.14
Amide II.	1542.34±1.54	1545.34±0.46	<0.01**
COO Symmetric Stretching	1396.38±2.09	1398.45±1.27	<0.05*
PO$_2$ Asymmetric Stretching	1235.51±1.74	1250.20±0.57	<0.01 **
C-O-C Symmetric stretching	1156.29±1.22	1150.96±1.48	<0.01 **
PO$_2$ Symmetric stretching	1071.57±2.61	1071.48±0.32	1.00

Values are shown as 'mean ± standard deviation'. The degree of significance was denoted as: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.3 DETAILED SPECTRAL ANALYSIS

3.3.1 COMPARISON OF THERMOPHILIC AND MESOPHILIC BACTERIA SPECTRA IN THE 3600-2800 CM $^{-1}$ REGION

The 3600-2800 cm $^{-1}$ region contains several bands. One of these bands centered at 3307 which monitors the stretching mode of OH and/or NH

and the other bands centered at 2959, 2927, 2876 and 2857 cm^{-1}, monitoring both asymmetrical CH_3 and CH_2 vibrations (Melin *et al.*, 1999 and Çakmak *et al.*, 2003).

Figure 9 shows the normalized infrared spectra of thermophilic bacteria and mesophilic bacteria samples in the 3000-2800 cm^{-1} region.

The wavenumber of the CH_3 asymmetric strecthing vibration at 2959 cm^{-1} of thermophilic bacteria (2962.22 ±0.49) was lower than mesophilic bacteria (2962.71±0.87) ($*p< 0.05$). However, the absorbtion intensity of this band was higher ($***p< 0.001$) for thermophilic bacteria spectrum compared to mesophilic bacteria spectrum. Moreover the absorbtion intensity of the CH_2 asymmetric stretching band was higher ($**p< 0.01$) for thermophilic group as was given in table 4.

Figure 9 displayed that the wavenumber of CH_3 symmetric stretching band at 2876 cm^{-1} which was higher (2875.27 ±0.62) for thermophilic bacteria when compared to mesophilic bacteria (2872.80 ±1.98) ($***p< 0.001$). Also the intensity of this band was higher ($**p< 0.01$) for thermophilic group compared to mesophilic one.

It is also observed that the intensity of the band at 2857 cm^{-1}, which is assigned as CH_2 symmetric stretching showed a higher value ($**p< 0.01$) for thermophilic bacteria and the frequency of this band was higher ($*p< 0.05$) for thermophilic bacteria spectrum compared to control group (mesophilic bacteria).
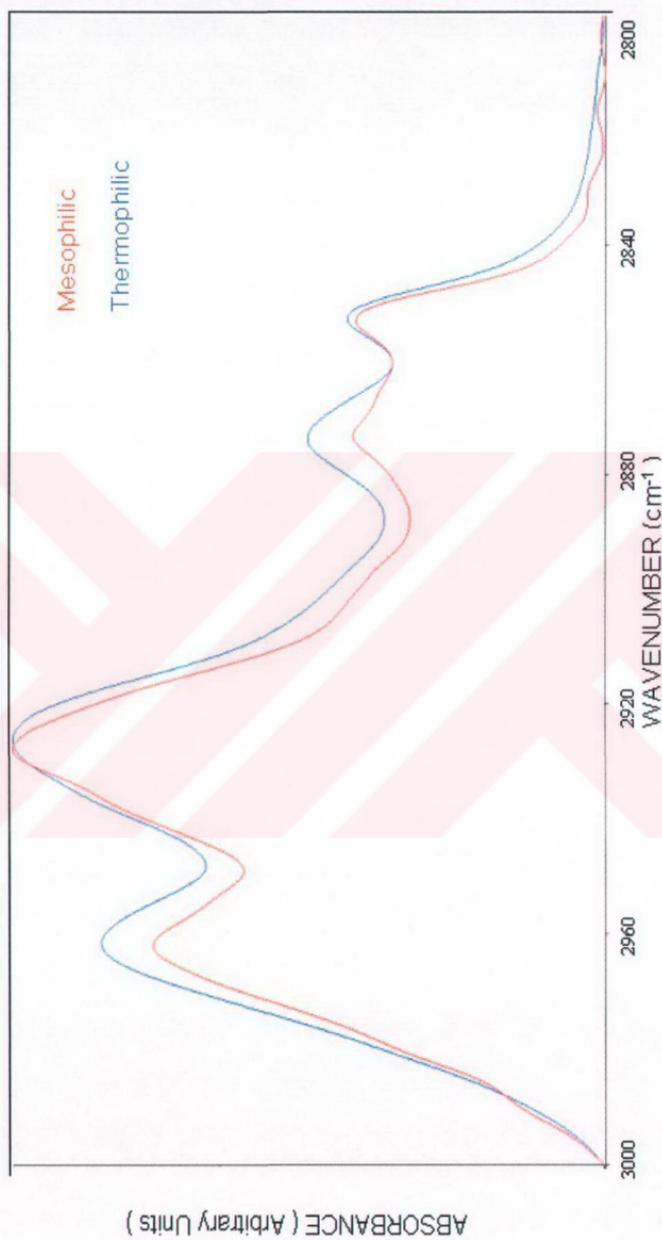
Figure 9. The infrared spectra of thermophilic and mesophilic bacteria in the 3000-2800 cm^{-1} region (the spectra were normalized with respect to CH$_2$ asymmetric mode which is observed at 2927 cm^{-1}).

The CH_2 stretching frequency reflects mainly conformational disorder and they increase with the introduction of gauche conformers in the fatty acyl chains (Rana *et al.*, 1990; Schultz *et al.*, 1991; Melin *et al.*, 1999 and Çakmak *et al.*, 2003).

The amount of proteins and lipids in the membranes is an important factor affecting the membrane structure and dynamics (Szalontai *et al.*, 2000). From the FT-IR spectrum, a precise protein-to-lipid ratio can be derived by calculating the ratio of the areas of the bands arising from lipids and proteins. It is seen from table 6 that the ratio of the area of CH_3 asymmetric stretching absorbtion to the area of CH_3 asymmetric stretching absorption to give protein-to-lipid ratio was higher (***$p<$ 0.001) for thermophilic group. The protein-to-lipid ratio of the area of the CH_3 symmetric stretching absorbtion band to the area of the CH_3 asym + CH_2 asym stretching absorbtions was also higher (***$p<$ 0.001) for thermophilic bacteria spectrum compared to mesophilic one.

Table 6. Changes in the protein to lipid band area ratios in thermophilic (n=19) and mesophilic (n=14) samples.

Functional Groups : Ratio of Peak Areas (protein to lipid)	Mesophilic (n=14)	Thermophilic (n=19)	p Values
CH3 Symmetric / CH3 Asymmetric	0.15± 0.05	0.31± 0.01	<0.001***
CH3 Symmetric / CH3 Asym.+ CH2 Asymmetric	0.07± 0.03	0.15± 0.01	<0.001***

Values are shown as 'mean ± standard deviation'. The degree of significance was denoted as: *p<0.05, **p<0.01, ***p<0.001.

3.3.2 COMPARISON OF THERMOPHILIC AND MESOPHILIC BACTERIA SPECTRA IN THE 1800-400 CM $^{-1}$ REGION

The second frequency range which is under consideration, was that of 1800-400 cm $^{-1}$ region. Figure 6 shows the 1800-400 cm $^{-1}$ region of the normalized FT-IR spectra of thermophilic and mesophilic bacteria. The band centered at 1739 cm $^{-1}$ is mainly assigned to the >C=O ester stretching vibration in phospholipids (Melin *et al.*, 1999; Çakmak *et al.*, 2003). The bands at 1657 and 1541 cm $^{-1}$ are attributable to the amide I and II vibrations of structural proteins. In addition, the intense band at 1452 cm $^{-1}$ can be assigned to the CH_2 bending mode of lipids (Manoharan *et al.*, 1993; Çakmak *et al.*, 2003). The band at 1391 cm $^{-1}$ is due to COO^{-} symmetric stretching vibration of amino acid side chains and fatty acids (Jackson *et al.*, 1998; Çakmak *et al.*, 2003). The relatively strong bands at 1236 and 1080 cm $^{-1}$ are mainly due to the asymmetric and symmetric stretching modes of phosphodiester groups in nucleic acids rather than in phospholipids, respectively (Wong *et al.*, 1991; Wang *et al.*, 1997; Menashi *et al.*, 1998; Çakmak *et al.*, 2003).

The relatively weak band at 1152 cm^{-1} is due to stretching mode of the CO-O-C groups present in glycogen and nucleic acids (Rigas *et al.*, 1990; Çakmak *et al.*, 2003). The band centered at 969 cm^{-1} is generally assigned to symmetric stretching mode of dianionic phosphate monoester of cellular nucleic acids, especially for DNA (Ci *et al.*, 1999; Chiriboga *et al.*, 2000; Çakmak *et al.*, 2003).

As it was illustrated in figure 6, the C=O ester stretching vibration band at 1739 cm^{-1} was not observed in thermophilic group which was different from that of mesophilic one, indicating a difference in packing of ester groups (Jackson *et al.*, 1998).

In protein amide groups, the band centered at 1657 cm^{-1} corresponding to the stretching C=O and bending C-N (amide I) vibrational modes of the polypeptide and protein backbone and at 1541 cm^{-1} is assigned to the bending N-H and stretching C-N (amide II) (Çakmak *et al.*, 2003). The amide I region is useful for determination of protein secondary structure. The frequency of vibration is very sensitive to changes in the nature of hydrojen bonds in the different types of protein secondary structures (Lyman *et al.*, 1998). As could be seen from figure 6 and table 4, the frequency value of the amide I band was lower (1653.12 ± 1.53) for thermophilic bacteria spectrum compared to mesophilic bacteria spectrum (1656.95 ±3.41) (***$p < 0.001$).   However the intensity of amide I was higher (***$p < 0.001$)for thetmophilic group. Both the absorbance (***$p < 0.001$) and the bandwidth (***$p < 0.001$) of amide II band (at 1541 cm^{-1}) was higher for thermophilic bacteria spectrum compared to mesophilic bacteria spectrum as seen from table 7. Moreover, the frequency of amide II band was lower for thermophilic group (1536.79 ±2.01) compared to mesophilic one (1540.83 ±0.87) (***$p < 0.001$).

Table 7. Changes in the bandwidth of various functional groups in thermophilic (n=19) and mesophilic (n=14) samples.

Functional Groups: Bandwidth	Mesophilic (n=14)	Thermophilic (n=19)	p Values
Amide A	12.1 ± 1.1	10.7 ± 0.8	< 0.001***
Amide II	3.6 ± 0.1	4.1 ± 0.3	<0.001***

Values are shown as 'mean ± standard deviation'. The degree of significance was denoted as: *p<0.05, **p<0.01, ***p<0.001.

As seen from figure 6; a higher value of the intensity for thermophilic bacteria (**p< 0.01) was observed at 1452 cm^{-1} that is due to the bending vibration of CH2 in the lipids. It could be seen from table 4 that there was a lower frequency of this band for thermophilic spectra (1452.19 ±0.73) compared to the mesophilic bacteria spectrum (1455.32 ±2.00) (***p<0.001). We also observed a higher value (**p< 0.01) of the intensity of COO$^-$ symmetric stretching vibration band at 1395 cm^{-1} that is due to amino acid side chains and fatty acids. In addition, the frequency value of this band was lower for thermophilic group (***p< 0.001) (table 4).

In the 1280-900 cm^{-1} frequency range, several macromolecules (e.g. polysaccharides and phosphate carrying compounds such as phospholipids and nucleic acids) give absorbtion bands (Melin *et al.*, 1999; Çakmak *et al.*, 2003). Study of this region revealed significant differences in the infrared spectra between thermophilic and mesophilic bacteria. Figure 6 also indicated remarkable differences in the bands

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located at 1280-900 cm^{-1}. In these spectra the strongest band are observed at 1236 cm^{-1} and 1080 cm^{-1} These bands are due to the asymmetric and symmetric phosphate stretching modes, respectively. These bands originated mainly due to the phosphodiester backbone of cellular nucleic acids (Rigas *et al.*, 1990). The intensity of the phosphate asymmetric stretching band was higher (**$p < 0.01$) and the frequency of this band was also higher for thermophilic bacteria spectrum (1236.69±2.26) compared to mesophilic bacteria spectrum (1232.84 ± 3.86) (**$p < 0.01$) as shown in figure 6. Also the frequency of phosphate symmetric stretching band showed a higher value for thermophilic bacteria spectrum (***$p < 0.001$) (table 6). The peak area ratio of bands at 1087 cm^{-1} and 1540 cm^{-1} (A1087 / A1540) is often used to illustrate the cange of the DNA / protein content of the cells (Mourant *et al.*, 2003, Zhou *et al.,* 2001; Taillandie *et al.*, 1992). A higher value for this ratio implies a higher DNA content (Mourant *et al.*, 2003, Zhou *et al.,* 2001; Taillandie *et al.*, 1992). The significant results are shown in table 8 suggesting that the cellular DNA content was lower for thermophilic bacteria.

On the other hand, in thermophilic group at 1152 cm^{-1} there was not a peak which was seen in mesophilic one, corresponding the CO-O-C asymmetric stretching.

Table 8. Changes in the DNA content to protein band area ratios in thermophilic (n=19) and mesophilic (n=14) samples.

Functional Groups: Ratio of Peak Areas (DNA to protein content)	Mesophilic (n=14)	Thermophilic (n=19)	p Values
PO_2 Symmetric / Amide II	1.35 ± 0.49	0.46 ± 0.10	< 0.001***

Values are shown as 'mean ± standard deviation'. The degree of significance was denoted as: *p<0.05, **p<0.01, ***p<0.001.

The band at 969 cm^{-1} which is due to nucleic acids was not observed in mesophilic bacteria spectrum as seen in figure 6.

In addition to that there was a characteristic peak at 467 cm^{-1} in mesophilic group.

In the 1280-900 cm^{-1} frequency range, the two mesophilic species; *E.coli* and *L. plantarum* also show significant spectral differences especially in the PO_2 asymmetric and PO_2 symmetric stretching vibrational bands which originates mainly due to in the phosphodiester backbone of cellular nucleic acids (Rigas *et al.*, 1990) as seen in figure 10.

The peak areas of these two bands were lower (**p< 0.01) in *L. plantarum* as seen in table 9. In addition to that there was a characteristic peak at 467 cm^{-1} in mesophilic group and this peak was

shifted different frequency values in two mesophilic bacteria; *E.coli* and *L.plantarum.*
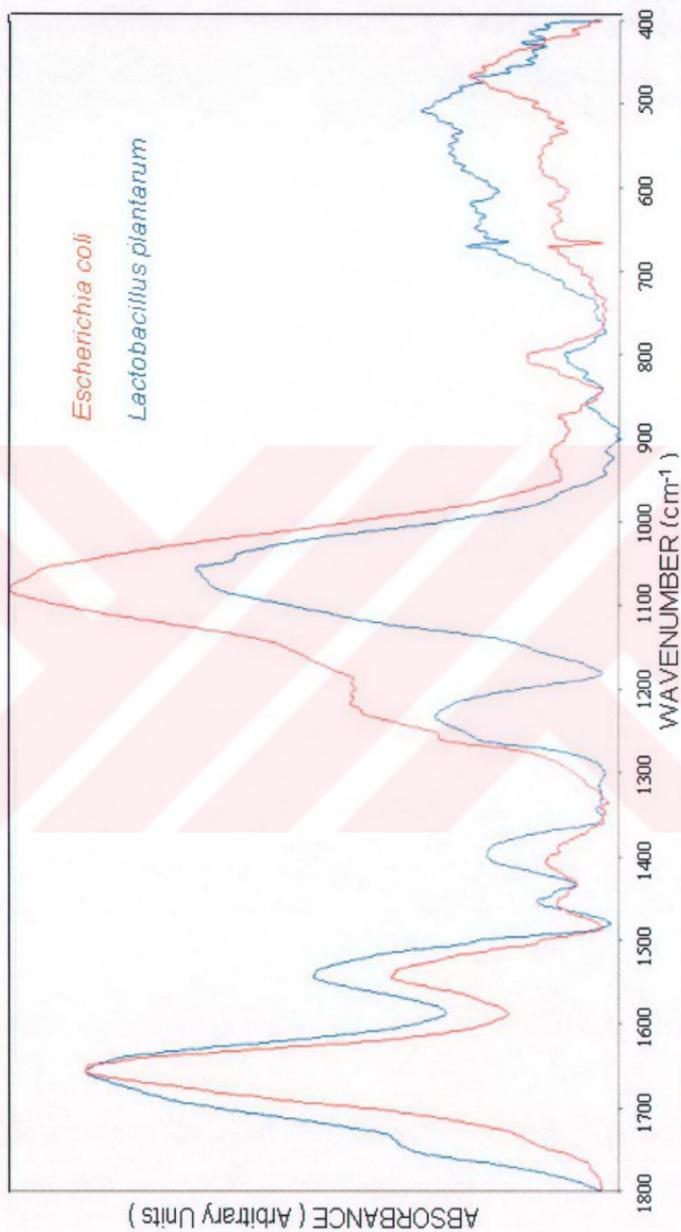
Figure 10. The infrared spectra of *Lactobacillus plantarum* and *Escherichia coli* in the 1800-400 cm⁻¹ region (the spectra were normalized with respect to amide I band which is observed at 1657 cm⁻¹).

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Table 9. Changes in the phosphate band area ratios in *L.plantarum* (n=7) and *E.coli* (n=7) samples.

Functional Groups: Peak Area	E.coli (n=7)	L.plantarum (n=7)	p Values
PO_2 Asymmetric	3.48 ± 0.74	2.35 ± 0.53	<0.01**
PO_2 Symmetric	12.05 ± 2.38	5.99 ± 1.02	<0.01**

Values are shown as 'mean ± standard deviation'. The degree of significance was denoted as: *p<0.05, **p<0.01, ***p<0.001.

3.3.3 COMPARISON OF *BACILLUS* SPP. AND *MICROCOCCUS* SPP. SPECTRA IN THE 3600-400 CM $^{-1}$ REGION

Table 5 shows changes in the frequency of the main functional groups in the 3000-2800 cm $^{-1}$ region. As seen from the table, there are significant differences between *Bacillus* spp. and *Micrococcus* spp. samples, which will be discussed in details.

The frequency of the absorbtions provides information relating to structure/ conformation and intermolecular interactions (Jackson *et al.,*1997; 1999; Toyran *et al.*, 2003).

Figure 11 shows the average spectra in the 3000-2800 cm $^{-1}$ region for both *Bacillus* spp. and *Micrococcus* spp. samples. Lipids are the major contributors in this spectral region (Mourant *et al.*, 2003). Spectra were normalized with respect to the CH_2 asymmetric stretching band. The

bands centered at 2956 cm^{-1} and 2923 cm^{-1} correspond to the stretching mode of asymmetrical CH_3 and CH_2 vibrations due to lipids. The band centered at 2876 cm^{-1} correspond to the stretching mode of symmetrical CH_3 vibrations mainly due to proteins (Melin *et al.*, 2000; Çakmak *et al.*, 2003; Toyran *et al.*, 2003).

It is seen from figure 11 that the area of the CH_3 asymmetric stretching mode was lower for *Micrococcus* spp. This means that the number of methyl groups in the acyl chains of lipids in this group was lower compared to *Bacillus* spp. (Takahashi *et al.*, 1991; Çakmak *et al.*, 2003). A higher value for the area of CH_3 symmetric stretching mode occurs, indicating a higher protein concentration in *Micrococcus* spp. (Çakmak *et al.*, 2003; Toyran *et al.*, 2003).

It is seen from table 10 that the ratio of the area of the CH_3 symmetric to CH_3 asymmetric stretching absorbtions (protein-to-lipid ratio) was higher for *Micrococcus* spp. (**p< 0.01). There was also a higher value of the protein-to-lipid ratio obtained from the ratio of the area of the CH3 symmetric stretching absorbtion band to the area of the CH_3 asym + CH_2 asym stretching absorbtions. Generally, this higher ratio suggests a high protein content, or low lipid content, or both (Jackson *et al.*, 1997; Toyran *et al.*, 2003). In our case, the low lipid content in the *Micrococcus* spp. was supported by the lower value of the area of the CH_3 asymmetric stretching vibrations. High protein content was supported by the higher value of the area of the CH_3 symmetric stretching vibration band in the *Micrococcus* spp. samples
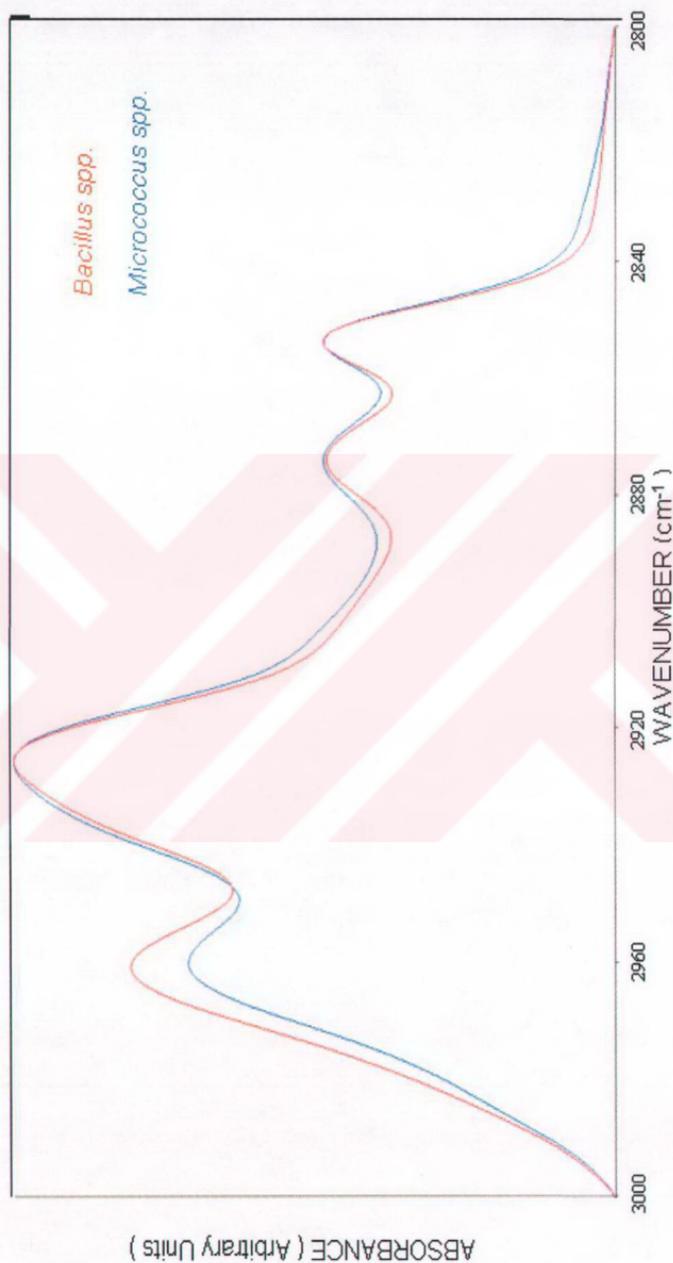
Figure 11. The infrared spectra of *Bacillus* spp. and *Micrococcus* spp. in the 3000-2800 cm^{-1} region (the spectra were normalized with respect to CH_2 asymmetric mode which is observed at 2927 cm^{-1}).

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Table 10. Changes in the protein to lipid band area ratios in *Bacillus* spp. (n=10) and *Micrococcus* spp. (n=5) samples.

Functional Groups: Ratio of Peak Areas (protein to lipid)	Bacillus spp. (n=10)	Micrococcus spp. (n=5)	p values
CH_3 Symmetric stretching/ CH_3 Asymmetric stretching	0.26±0,01	0.27±0,01	<0.01**
CH_3 Symmetric stretching/ CH_3 + CH_2 Asymmetric stretching	0.12±0.01	0.13±0.01	0.10

Values are shown as 'mean ± standard deviation'. The degree of significance was denoted as: *p<0.05, **p<0.01, ***p<0.001.

The band centered at 1739 cm $^{-1}$ was mainly assigned to the >C=O ester stretching vibration in triglycerides (Nara *et al.*,2002; Voortman *et al.*,2002; Çakmak *et al.*, 2003) (Figure 8). The frecuency of the maximum of the ester C=O stretching vibration was lower for *Micrococcus* spp. (1738.99 ± 3.31) compared to *Bacillus* spp. (1744.94 ± 1.55) (**p< 0.01), indicating a difference in packing of ester groups (Jackson *et al.*, 1998). This difference in packing is presumably the result of the high proportion of unsaturated acyl chains in triglycerdides in *Micrococcus* spp. (Nara *et al.*,2002).

As can be seen from figure 12, the frequency of the maximum of amide I was lower for *Bacillus* spp. (1657.27 ± 1.85) compared to *Micrococcus* species (1659.45 ± 2.20). Since the location of amide I absorbtions was sensitive to protein conformation, these differences implied conformational canges in cell proteins (Haris and Severcan, 1999;

Jackson *et al.*, 1999). Similar to amide I band, the frequency of the maximum of amide II band was lower for *Bacillus* species (1542 ± 1.54) compared to *Micrococcus* spp. (1545.34 ± 0.46) (**p< 0.01). The band at 1391 cm $^{-1}$ is due to COO$^-$ symmetric stretching vibration of amino acid side chains and fatty acids (Jackson *et al.*, 1998; Çakmak *et al.*, 2003). It is seen from figure 8 that the frequency of COO$^-$ symmetric stretching vibration band was lower for *Bacillus* spp. (1396.38 ± 2.09) compared to *Micrococcus* spp. (1398.45 ± 1.27) (*p< 0.05), due to amino acid side chains and fatty acids (table 5).

We observed two other peaks around 1338 cm $^{-1}$ and 1313 cm $^{-1}$ in *Micrococcus* spp. which are specific in comparison to *Bacillus* spp. (figure 8). The band around 1313 cm $^{-1}$ is due to the stretching mode of carboxylic acids (Beech *et al.*, 1999). The bands located at 1236 cm $^{-1}$ and 1080 cm $^{-1}$, are due to the asymmetric and symmetric phosphate stretching modes which mainly arise from the phosphodiester backbone of cellular nucleic acids (Wong *et al.*, 1991; Wang *et al.*, 1997; Çakmak *et al.*, 2003). The contribution to these bands by phosphate residues present in membrane lipids was negligible (Wong *et al.*, 1991; Wang *et al.*, 1997; Rigas *et al.*, 1990). As seen from figure 8, while the frequency of the phosphate asymmetric band was lower for *Bacillus* spp. (1235.51 ± 1.74) compared to *Micrococcus* spp. (1250.20 ± 0.57) (**p< 0.01), the frequency of the phosphate symmetric band was higher for *Bacillus* species (1071.57 ± 2.61) compared to *Micrococcus* species (1071.48 ± 0.32) (table 5).

A shoulder at 1256 cm $^{-1}$ was observed in *Micrococcus* spp. which was different from *Bacillus* species (figure 8). It is due to the C-O stretching of O-acetyl ester bonds. The peak around 1313 cm $^{-1}$ corresponds to the stretching mode of C-O of carboxylic acids (Beech *et al.*, 1999). The band at 1152 cm $^{-1}$ is due to the stretching mode of CO-O-C groups present in glycogen and nucleic acids (Rigas *et al.*, 1990). There was an increase in the frequency of this band was higher for *Bacillus* spp.

(1156.29 ± 1.22) compared to Micrococcus one (1150.96 ± 1.48) (**p< 0.01) (table 5).

There were two shoulders at 1104 cm $^{-1}$ and 1074 cm $^{-1}$ in *Micrococcus* spp., which do not appear in *Bacillus* species as seen in figure 8. The shoulder at 1074 cm $^{-1}$ is assigned to glycogen (Jackson *et al.*, 1996) however assignment to the shoulder at 1104 cm $^{-1}$ was not found in literature.

There was another shoulder at 550 cm $^{-1}$ that appeared in the second derivative of the spectrum (data was not shown) in *Micrococcus* species.

As seen from figure 8, there was a characteristic peak at 993 cm $^{-1}$ in *Micrococcus* spp. due to nucleic acids. In addition to that there was another characteristic peak at 801 cm $^{-1}$ in *Micrococcus* species. The characteristic peaks for each species can be seen from figure 13. As seen from figure 13, the band at 960 cm $^{-1}$ was sharper and stronger in Bacillus *subtilis* than those of other bacteria spp. This band is due to symmetrical stretching mode of dianionic phosphate monoester of cellular nucleic acids, especially for DNA (Çakmak *et al.*, 2003)
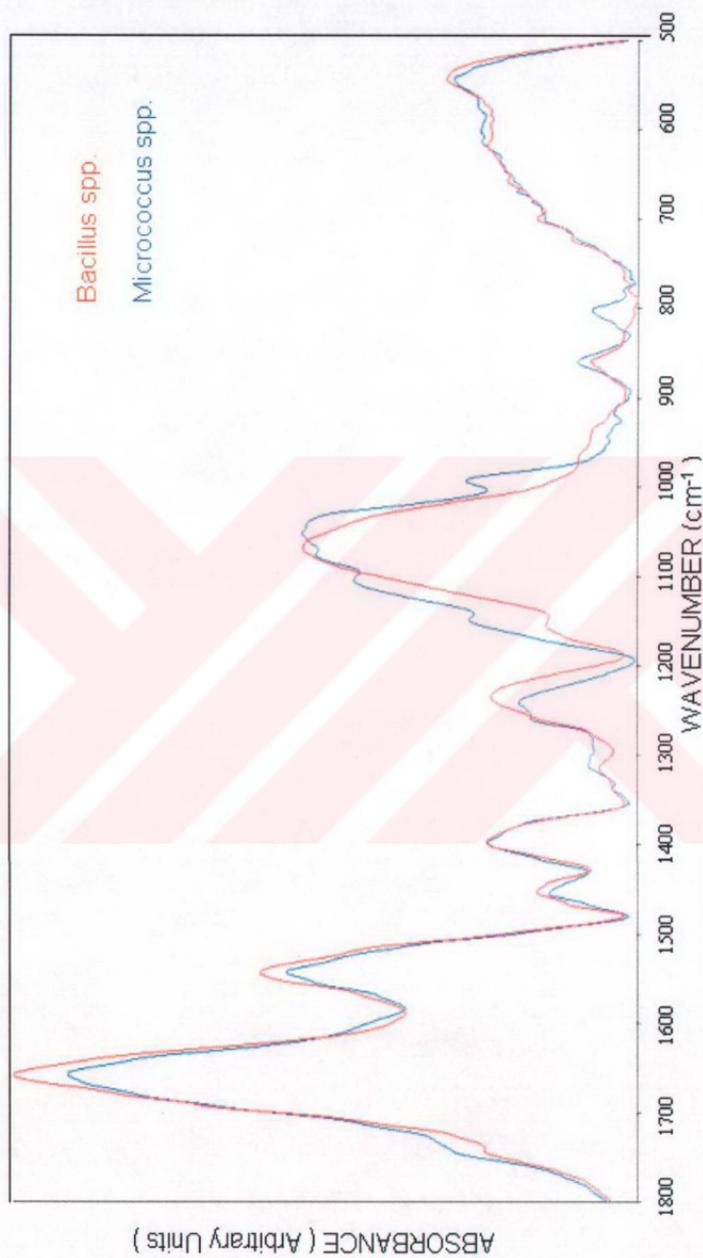
Figure 12. The infrared spectra of *Bacillus* spp. and *Micrococcus* spp. in the 1800-500 cm⁻¹ region (the spectra were normalized with respect to CH_2 asymmetric mode which is observed at 2927 cm⁻¹).
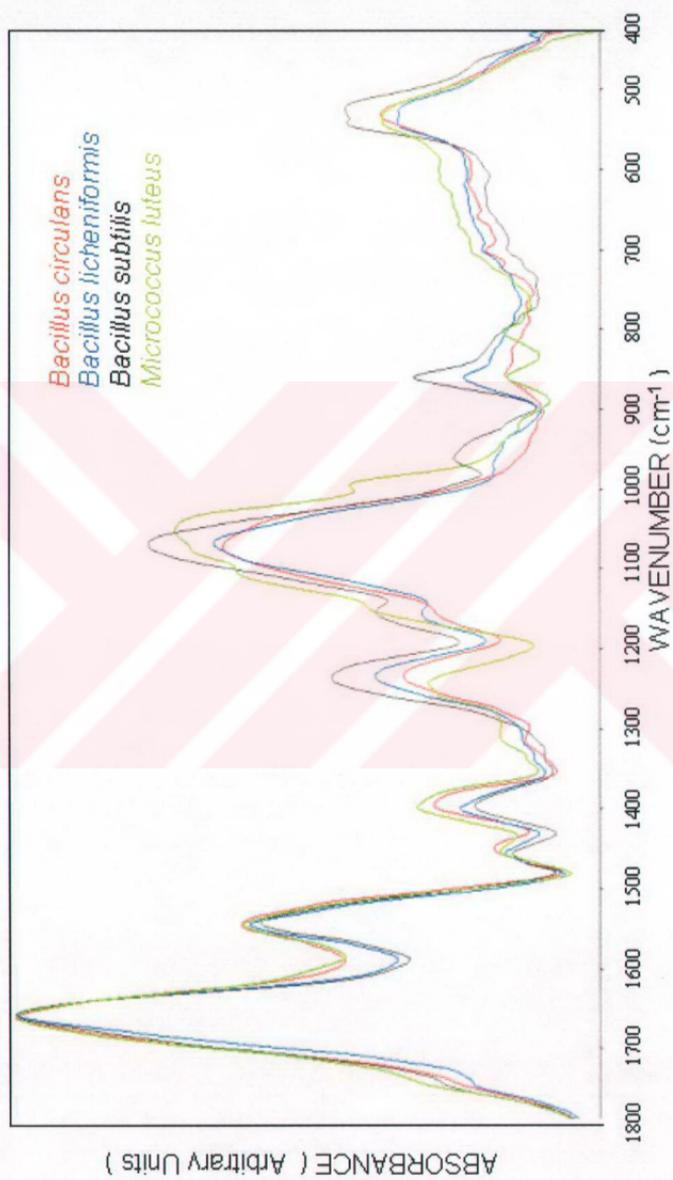
Figure 13. The infrared spectra of 3 *Bacillus* spp. and 1 *Micrococcus* spp. in the 1800-400 cm⁻¹ region (the spectra were normalized with respect to amide I band which is observed at 1657 cm⁻¹).

CHAPTER 4

DISCUSSION

The present work investigates the characterization of different bacteria groups with FT-IR spectroscopy. In first study, FT-IR technique has been used to compare spectral differences and similarities between thermophilic and mesophilic bacteria. The second study involved the characterization of three different *Bacillus* spp. and a *Micrococcus* spp. with FT-IR spectroscopy. Both studies demonstrated that different bacteria species show significant spectral differences. As seen from figure 5 and figure 6, there were differences between thermophilic and mesophilic bacteria spectrum. *Bacillus* spp. and *Micrococcus* spp. spectrum also showed significant spectral differences as seen from figure 7 and figure 8.

Table 4 and table 5 show statistically significant differences in band intensities and band frequency values between thermophilic and mesophilic bacteria and between *Bacillus* spp. and *Micrococcus* spp. Also, as seen from table 6, table 8 and table 9, we observed significant differences in peak areas and the ratio of peak areas between bacteria groups. Table 7 shows statistically significant differences in bandwidths between thermophilic and mesophilic bacteria. In addition to that, figures 9, 10, 11, 12 and 13 showed that a number of differences were apparent between the spectra of bacterial groups.

4.1 DISCUSSION ON THE COMPARISON OF THERMOPHILIC AND MESOPHILIC BACTERIA

High temperatures lead to an increase in the fluidity of the cellular membrane and to maintain optimal membrane fluidity, the composition of the membrane is altered in cells (Rothschild *et al.*, 2001). The most common change is in the ratio of saturated / unsaturated lipids via a change in fatty acyl composition (Russell *et al.*, 1998). As it can be seen from figure 9, the absorbtion intensity of the CH_3 asymmetric stretching vibration at 2959 cm^{-1} was higher in thermophilic bacteria. So we can say that the composition of acyl chains was changed in thermophilic group. The number of methyl groups in each acyl chain was higher as indicated by the higher value of the intensity of the methyl CH stretching band at 2959 cm^{-1} in the spectrum of the thermophilic bacteria compared to mesophilic bacteria (Takahashi *et al.*, 1991; Çakmak *et al.*, 2003).

Growth temperature dependent changes of the lipid composition of the cytoplasmic membranes of bacteria are mainly found in the fatty acyl chain composition of the membrane lipids (Grau *et al.*, 1993; Shaw *et al.*, 1965; Svobodová *et al.*, 1988; Vossenberg *et al.*, 1999). As seen from table 4, the frequency of the symmetric CH_2 stretching band at 2857 cm^{-1} was higher for thermophilic group (2854.19 ±0.64) compared to mesophilic one (2853.14 ±1.40). Higher value of the frequency of the CH_2 symmetric stretching mode for this group indicated a reorganization of the membrane in an ordered direction to an average bilayer structure (Lewis *et al.*, 1989; Çakmak *et al.*, 2003). In addition to this difference, the intensity of this band was higher (**$p < 0.01$) in thermophilic bacteria compared to mesophilic one. These results indicated a higher concentration of fatty acyl chains in thermophilic bacteria. This was confirmed by higher value of the intensity of the CH_2 bending vibration band at 1452 cm^{-1} in thermophilic bacteria (figure 6). These results were confirmed by the study of Chan *et al.* (1971) which suggested that notable differences were that the thermophiles contained a

higher content of saturated straight- and branched-chain fatty acids. And the mesophiles were characterized by having a higher percentage of unsaturated fatty acids.

Moreover, the lipid C=O stretching vibration at 1739 cm $^{-1}$ which was observed in mesophilic group, was not observed clearly in thermophilic one. Since this band suggests an increased concentration and difference in packing of ester groups in mesophilic bacteria (Jackson *et al.*, 1998) (figure 6). This difference in packing is presumably due to the decreased proportion of unsaturated acyl chains in thermophilic bacteria. The melting points of saturated straight-chain and iso-fatty acids range from 51 to 107^0 C, whereas unsaturated, anteiso fatty acids have melting points ranging from below 0 to 40^0 C (Chan *et al.*, 1971).

According to our results it was seen that the thermophiles contain a preponderance of higher melting fatty acids. The general pattern indicated that organisms grown at higher temperatures contained a higher percentage of saturated acids with comparatively higher melting points (Chan *et al.*, 1971).

Vossenberg *et al.* (2000) studies with B.subtilis which was grown at various temperatures between its lower and upper growth temperature boundaries, 13^{0}C (mesophilic B.subtilis) and 50^{0}C (thermophilic B.subtilis). They investigated the phase transition behaviour of the lipids derived from cells grown at 13 and 50^{0}C by differential scanning calorimetry (DSC) and the viscosity of the membranes by TMA-DPH fluorescence anisotropy (*r*) and lifetime(τ_θ) measurements. They indicated that the most significant effect is a drastic decrease in the anteiso fatty acids and an increase in the iso-branched fatty acids with increasing growth temperature., that is in accordance with our results.

As it can be seen from figure 6, the intensities of amide II (1541) and COO$^-$ symmetric stretching (1391) band was higher in thermophilic group compared

to mesophilic group. These bands are mainly due to proteins (Çakmak et al., 2003). It is seen from table 6 that the ratio of the area of the CH_3 symmetric stretching absorbtion to the area of the CH_3 asymmetric stretching absorption which give protein-to-lipid ratio was higher (***$p< 0.001$) in thermophilic group. There was also a higher value (***$p< 0.001$) of the protein-to-lipid ratio of the area of the CH_3 symmetric stretching absorbtion band to the area of the CH_3 asym + CH_2 asym stretching absorbtions in thermophilic bacterial spectrum compared to mesophilic one. Generally, this higher value of the ratio suggests a high protein content, or low lipid content, or both (Jackson et al., 1997; Çakmak et al., 2003). In our case, the high protein content in thermophilic bacteria is supported by the higher value of the area of the CH_3 symmetric stretching vibrations, whilst the low lipid content is supported by the lower value of the area of the CH_3 asymmetric stretching vibration band in the thermophilic bacterial samples.

In figure 6, the strongest bands at 1238 cm^{-1} and 1080 cm^{-1} are due to the asymmetric and symmetric stretching vibration of the phosphate groups that are part of the DNA backbone (Zhou et al., 2001; Benedetti et al., 1986; Çakmak et al., 2003). The frequencies of these bands were different in two bacteria group. The frequency value of the phosphate asymmetric bandwas higher. This higher value observed in the phosphate asymmetric stretching band may be due to the higher number of hydrogen-bonded phosphodiester groups of the DNA in thermophilic bacteria (Fung et al., 1997; Wong et al., 1991; Çakmak et al., 2003). These results suggest that in thermophilic bacteria PO_2^- groups are stabilized by hydrogen bond (Wong et al., 1991; Çakmak et al., 2003).

In thermophilic microorganisms, thermostability is acquired by thermostable proteins. The amino acid composition of proteins from mesophilic and thermophilic organism is commonly assumed to reflect the mechanism of molecular adaptation to extremes of physical conditions (Bohm et al., 1994). Thermostability of a protein is a property which can not be attributed to the presence of a particular amino acid (Scandurra et al., 1998). Thermostability

seems to be a property acquired by a protein through many small structural modifications obtained with the exchange of some aminoacids (Argos *et al.*, 1979; Warren *et al.*, 1995). The advantages of the amino acid exchanges most frequently reported in thermophilic proteins are that a higher number of hydrogen bonds may be formed (Tanner *et al.*, 1996; Scandurra *et al.*, 1998). From the comparative analysis of the X-ray structures available for several families of proteins, including at least one thermophilic structure in each case, it appears that thermal stabilization is accompanied by an increase in hydrogen bonds (Querol *et al.*, 1996; Vogt *et al.*, 1997; Scandurra *et al.*, 1998). Our results confirmed by these studies, indicated that the bands which were due to the asymmetric and symmetric stretching vibration of the phosphate groups shifted in thermophilic bacteria group. The phosphate asymmetric band shifted to higher values, indicated an increase in the number of hydrogen-bonded phosphodiester groups of the DNA in molecules of thermophilic bacteria. Also these results suggested that in thermophilic bacteria PO_2^- groups become hydrogen bonded.

In the study of the phosphate symmetric stretching, whose band is located at 1087 cm^{-1}, the peak area ratio of bands at 1087 cm^{-1} and 1540 cm^{-1} (A1087 / A1540) is often applied to illustrate the change of the DNA / protein content of the cells (Mourant *et al.*, 2003, Zhou *et al.*, 2001; Taillandie *et al.*, 1992). A lower value of ratio implies a lower DNA content (Mourant *et al.*, 2003, Zhou *et al.*, 2001; Taillandie *et al.*, 1992). The results (table 8) might suggest that the cellular DNA content was lower for thermophilic bacteria. It was hypothesized that in thermophilic bacteria spectrum the apparent absorbance of DNA decreases and therefore the cellular DNA content was seen to be lower because the DNA is packed more tightly in thermophilic bacteria compared to mesophilic one (Wharton D. A., 2002).

As seen from figure 6, at 1152 cm^{-1} there was a peak in the CO-O-C asymmetric stretching region in mesophilic group different than thermophilic one.

The band near 965 cm $^{-1}$ is thought to originate from a C-C / C-O stretching vibration involving the characteristic deoxyribose and phosphate moiety of the DNA backbone (Zhou *et al.*, 2001; Benedetti *et al.*, 1986). This band which was observed in thermophilic bacteria spectrum, was not observed in mesophilic bacteria spectrum as seen in figure 6. In addition to that there was a characteristic peak at 467 cm $^{-1}$ in mesophilic group. Moreover, this characteristic peak at 467 cm $^{-1}$ shifted to different values in two mesophilic bacteria; *E.coli* and *L. plantarum* (figure 10). So this characteristic band can be also used in the characterization of these two bacterial species.

4.2 DISCUSSION ON THE COMPARISON OF BACILLUS AND MICROCOCCUS SPECIES

In the study of *Bacillus* spp. and *Micrococcus* spp., there were also significant spectral differences between these two bacterial groups. The band centered at 1739 cm $^{-1}$ is mainly assigned to the >C=O ester stretching vibration in triglycerides (Nara *et al.*,2002; Voortman *et al.*,2002; Çakmak *et al.*, 2003) (Figure 8). The frecuency of the maximum of the ester C=O stretching vibration was lower in *Micrococcus* spp. (1738.99 ± 3.31) compared to *Bacillus* spp. (1744.94 ± 1.55) (**p< 0.01), indicating a difference in packing of ester groups in the direction of ordering (Jackson *et al.*, 1998). This difference in packing is presumably the result of the higher proportion of unsaturated acyl chains in triglycerdides in *Micrococcus* spp. (Nara *et al.*,2002). Our result was confirmed by the study of Kaneda Toshi. (1991), which suggested that most *Bacillus* species have insignificant amounts of unsaturated fatty acids. *Bacillus* spp. appear not to require this class of fatty acids for growth, since no mutant of *B.subtilis* requiring unsaturated fatty acids has been reported (Kaneda T., 1977).

As can be seen from figure 12, the frequency of the maximum of amide I was lower in Bacillus spp. (1657.27 ± 1.85) when compared to Micrococcus spp. (1659.45 ± 2.20). As the position of amide I absorbtions was sensitive to protein conformation, these differences implied conformational canges in cell

proteins (Haris and Severcan, 1999; Jackson *et al.*, 1999). The maximum of amide II band was lower as amide I band, in *Bacillus* spp. (1542 ± 1.54) compared to *Micrococcus* species (1545.34 ± 0.46) (**p< 0.01). The band at 1391 cm $^{-1}$ is due to COO^- symmetric stretching vibration of amino acid side chains and fatty acids (Jackson *et al.*, 1998; Çakmak *et al.*, 2003). It is seen from figure 8 that there was a lower frequency value in COO^- symmetric stretching vibration band in *Bacillus* spp. (1396.38 ± 2.09) compared to *Micrococcus* spp. (1398.45 ± 1.27) (*p< 0.05), due to amino acid side chains and fatty acids (table 5).

We observed two bands around 1313 cm $^{-1}$ corresponding to the stretching mode of carboxylic acids (Beech *et al.*, 1999) and 1338 cm $^{-1}$ due to the acetic acid (Li-Fen *et al.*, 2001) (figure 8). Howard *et al.* (1954) suggested that in *Micrococcus* spp., acetate oxidized by the tricarboxylic acid cycle. So we can say that these two peaks at 1313 and 1338 cm^{-1} which were not seen in *Bacillus* spp., may be explained by an acetate oxidation in *Micrococcus* species.

A shoulder at around 1256 cm $^{-1}$ due to the C-O stretching of O-acetyl ester bonds and another shoulder at 1313 cm^{-1} that is corresponding to the stretching mode of C-O of carboxylic acids, may be explained by an exopolymer formation in *Micrococcus* spp. (figure 8). These results confirmed by the study of Nichols *et al.* (1985) which presented a spectrum obtained from a *Pseudomonas atlantica* exopolymer that showed strong and superimposed bands within the same frequencies; 1256 and 1313 cm^{-1}.

The shoulder at 1074 cm $^{-1}$ and another shoulder at 550 cm $^{-1}$ that was seen in the second derivative of the spectrum (data was not shown) are due to glycogen and may be explained by a glycogen-like storage material in *Micrococcus* spp. (Jackson *et al.*, 1996).

The characteristic peaks that are belonged to each species can be seen from Figure 13. There are characteristic peaks at 1104, 1074, 993 and 801 for

Micrococcus species. As seen from figure 13, the band at 960 cm $^{-1}$ is due to symmetrical stretching mode of dianionic phosphate monoester of cellular nucleic acids, especially for DNA (Çakmak *et al.*, 2003) was sharper and stronger in *Bacillus subtilis* than those of other bacteria spp.

CHAPTER 5

CONCLUSION

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This work includes two approaches. First study was carried out to investigate the characterization and differentiation of mesophilic and thermophilic bacteria by using FTIR Spectroscopic technique. Our results show that there was a dramatic difference between mesophilic and thermophilic bacteria, which mainly indicated that the protein concentrationwas high, lipid concentration, the level of triglycerides and the unsaturated acyl chains were low in thermophilic bacteria.

Moreover; it was also found there was a high number of hydrogen-bonded phosphodiester groups of the DNA in molecules of thermophilic bacteria. This suggest that in thermophilic bacteria PO_2^- groups become hydrogen bounded.

In addition to that our results suggest that the cellular DNA content was low in thermophilic bacteria.

There was a characteristic peak at 965 cm^{-1}, thought to originate from a C-C / C-O stretching vibration involving the characteristic deoxyribose and phosphate moiety of the DNA backbone for mesophilic bacteria. Another characteristic peak for mesophilic goup is at 467 cm^{-1} and it can be used for the differentiation of mesophilic and thermophilic bacteria. Moreover this band can be also used for the characterization of two mesophilic bacteria; *Escherichia coli* and *Lactobacillus plantarum* because it showed different frequency values for these two bacterial species.

In the second study, we investigated the characterization and identification of 3 *Bacillus* and a *Micrococcus* species. Our results show that there were significant spectral differences between these two bacterial groups. One of these differences is the increased proportion of unsaturated acyl chains in triglycerides in Micrococcus species.

Moreover we observed two bands around 1313 cm^{-1} corresponding to the stretching mode of carboxylic acids and 1338 cm^{-1} due to the acetic acid that may be explained by an acetate oxidation by the tricarboxylic acid cycle in *Micrococcus* spp.

Another band at 1256 cm^{-1} due to the C-O stretching of O-acetyl ester bonds with a shoulder at 1313 cm^{-1} that is corresponding to the stretching mode of C-O carboxylic acids, may be explained by an exopolymer formation in *Micrococcus* species.

In addition to that the shoulder at 1074 cm^{-1} and another shoulder at 550 cm^{-1} are due to glycogen and may be explained by a glycogen-like storage material in *Micrococcus* species.

There are characteristic peaks that can be used for identification at 1104, 1074, 993 and 801 cm^{-1} for *Micrococcus* spp. And the band at 960 cm^{-1} was sharper and stronger in *Bacillus subtilis* that those of other bacteria, so it can be also used for the identification of this bacteria.

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APPENDIX A

COMPOSITION OF CULTURE MEDIA

TR1 Medium

Components	Amounts
Trypticasein Soybroth	4.0g
Yeast Extract	3.0g
Malt Extract	4.0g
NaCl	3.0g

For 1 L of medium, PH adjusted to 7.0

Nutrient Broth

Components	Amounts
Meat Extract	10.0g
Peptone	10.0g
Sodium Chloride	5.0g

For 1 L of medium, PH adjusted to 7.5 - 7.6