PREPARATION OF ANTIMICROBIAL FILMS FROM AGRICULTURAL BIOMASS

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PREPARATION OF ANTIMICROBIAL FILMS FROM AGRICULTURAL BIOMASS

submitted by **AYŞE GİZEM SEBER** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. İnci Eroğlu _____
Head of Department, **Biotechnology**

Prof. Dr. Ufuk Bakır _____
Supervisor, **Chemical Engineering Dept., METU**

Prof. Dr. Zümrüt Begüm Ögel _____
Co-Supervisor, **Food Engineering Dept., METU**

Examining Committee Members:

Prof. Dr. Gürkan Karakaş _____
Chemical Engineering Dept., METU

Prof. Dr. Ufuk Bakır _____
Chemical Engineering Dept., METU

Prof. Dr. Zümrüt Begüm Ögel _____
Food Engineering Dept., METU

Assoc. Prof. Dr. Ayşe Gül Gözen _____
Biological Sciences Dept., METU

Asst. Prof. Dr. Didem Sutay Kocabaş _____
Chemical Engineering Dept., Ege University

Date: 11.01.2010

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by the rules and conducts, I have fully cited and referenced all materials and results that are not original to this work.

Name, Last name: Ayşe Gizem Seber

Signature:

ABSTRACT

PREPARATION OF ANTIMICROBIAL FILMS FROM AGRICULTURAL BIOMASS

Seber, Ayşe Gizem

M.Sc., Department of Biotechnology

Supervisor : Prof. Dr. Ufuk Bakır

Co-Supervisor: Prof. Dr. Zümrüt Begüm Ögel

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Mainly used food packaging materials are petro-chemical based polymers which present environmental problems since they are not biodegradable and ecologically sustainable. In this study, biodegradable biofilms are produced from xylan, extracted from cotton stalk which is an agricultural biowaste without nutritional value. Antimicrobial property was given to the biofilms with either titanium dioxide sol-gel coatings or titanium dioxide powder addition into the biofilm forming solutions. The antimicrobial activities of biofilms were tested against *Escherichia coli*. Among two different sol-gels coated and at different temperatures dried biofilms, BWX and CSX-50 biofilms treated at 120°C and coated with SiO_2/TiO_2 showed 88±1% and 75±2% antimicrobial activities, respectively. Same samples treated at the same conditions but coated with non-SiO_2 added TiO_2 sol-gel yielded 63±3% and 63±2% antimicrobial activities, respectively after 2 h black light illumination. So, it was determined that the highest photocatalytic antimicrobial property was achieved with SiO_2/TiO_2 coated biofilms. Moreover different concentrations of TiO_2 powder were integrated into xylan based biofilms and

100% photocatalytic inactivation was gathered at 5% (w/w) TiO_2 addition achieved at both biofilms at the end of 90 min black light illumination.

Biodegradability properties of the biofilms were investigated in soil burial test during 180 days and 10% (w/w) TiO_2 powder added CSX-50 biofilms were recorded to be 91% biodegradable where non-powder added blank biofilms was found to be 95% biodegradable.

Keywords: Xylan, Cotton Waste, Antimicrobial, Biodegradable Film, Titanium Dioxide Photocatalyst.

ÖZ

TARIMSAL ATIKLARDAN ANTİMİKROBİK FİLMLER HAZIRLAMA

Seber, Ayşe Gizem

Yüksek Lisans, Biyoteknoloji Bölümü

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Genellikle gıda paketlemesinde kullanılan petro-kimyasal materyeller biyobozunur ve ekolojik bakımdan sürdürülebilir olmamalarından dolayı çevresel sorunlara neden olmaktadır. Bu çalışmada, besinsel işlevi olmayan bir tarım atığı olan, pamuk sapından özütlenen ksilan ile biyobozunur biyofilmler üretilmiştir. Buna ek olarak, biyofilmlere, titanyumdioksit sol-jeli kaplama veya TiO_2 tozunu biyofilm oluşturma solüsyonuna ekleme yöntemleri ile antimikrobik özellik verilmiştir. *Esherischia coli*'ye karşı biyofilmlerin antimikrobik aktiviteleri test edilmiştir. İki sol-jel metodu arasında, farklı sıcaklıklarda kurutularak hazırlanmış biyofilmlerde, 120°C'de SiO_2/TiO_2 sol-jeli ile kaplanmış ticari ksilandan elde edilen biyofilmler siyah ışık kaynağı altında 2 saat aydınlatma sonucunda % 88±1, pamuk sapından özütlenerek elde edilen ksilandan oluşturulmuş biyofilmler ise %75±2 antimikrobik aktivite göstermiştir. SiO_2 eklenmemiş TiO_2 sol-jeli ile kaplanmış BWX biyofilmler aynı koşullar altında % 63±3, CSX-50 biyofilmler ise % 63±2 antimikrobik özellik göstermiştir. Sonuç olarak, en yüksek antimikrobik aktivite değeri SiO_2 eklenmiş TiO_2 sol-jeli kaplanmış biyofilmlerde elde erişilmiştir. Buna ek olarak, farklı konsantrasyonlarda TiO_2 tozu eklenen örneklerde %100

fotokatalitik inaktivite 90 dakika siyah ışık aydınlatması sonucunda % 5 TiO_2 tozu (toz/biyofilm katı ağırlığı) eklenen örneklerde gözlemlenmiştir.

Biyofilmlerin biyobozunurluk özellikleri 180 gün boyunca toprağa gömülerek değerlendirilmiştir ve % 10 (toz/biyofilm katı ağırlığı) oranında TiO_2 tozu eklenmiş CSX-50 biyofilmlerin % 91 oranında, TiO_2 tozu eklenmemiş CSX-50 biyofilmlerin ise % 95 oranında biyobozunur oldukları bulunmuştur.

Anahtar Kelimeler: Ksilan, Pamuk Atığı, Antimikrobik, Biyobozunur Film, Titanyum Dioksit Fotokatalisti.

TO MY BELOVED FAMILY

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A. niger	*Aspergillus niger*
BWX	Birchwood xylan
CSX	Cotton stalk xylan
cfu	Colony forming unit
E. coli	*Escherichia coli*
h	hour
LB	Luria Bertani
min	minute
ml	milliliter
µl	microliter
sec	second
U/ml	unit/milliliter
w/w	weight/weight

CHAPTER 1

INTRODUCTION

1.1 Biological Polymers as Food Packaging Materials

Food packaging, originally conceived to secure proper preservation of food in a container by protecting it from the influence of the environmental elements, is currently an important discipline in the area of food technology to ensure better protection, more efficient quality preservation, and enhanced safety. The characteristics of the packaging films like chemical structure, molecular weight, crystallinity and the processing conditions of the polymers used are of great importance (Chandra and Rustgi, 1998).

Mainly used food packaging materials are petro-chemical based polymers like polyolefins, polyesters, polyamides and polyethylene which are mostly preferred due to their availability in large quantities at low cost, favorable functionality characteristics like good tensile and tear strength, good barrier properties to O_2 and aroma compounds and heat sealability, but on the other hand, they have a very low water vapor transmission rate and most significantly they are totally non-biodegradable leading to ecological problems (Tharanathan, 2003).

As the today's society demand has shifted to the minimally processed, easily prepared, ready-to-eat food products and eco-friendly materials, the food

processors have to offer new potential solutions in film concept like biodegradable films (Tharanathan, 2003).

According to the ASTM D 6400-04, a biodegradable plastic is a degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi, and algae. During degradation the polymer is first converted to its monomers, and then these monomers are mineralized. Most polymers are too large to pass through cellular membranes, so they must first be depolymerized to smaller monomers before they can be absorbed and biodegraded within microbial cells. The initial breakdown of a polymer can result from a variety of physical and biological forces (Swift, 1997). Heating/cooling, freezing/thawing, or wetting/drying are the physical forces that can cause mechanical damage such as the cracking of polymeric materials (Kamal and Huang, 1992). At the depolymerization step, two categories of enzymes are actively involved: extracellular and intracellular depolymerases (Doi, 1990; Gu *et al*., 2000). During degradation, extracellular enzymes from microorganisms break down complex polymers yielding smaller molecules of short chains, e.g., oligomers, dimers, and monomers, that are smaller enough to pass the semi-permeable outer bacterial membranes. Then the smaller molecules are absorbed into microbial cells and mineralized by being utilized as carbon and energy sources. The products of this process, apart from adenosine triphosphate (ATP), are gasses, (e.g., CO_2, CH_4, N_2, H_2), water, salts and minerals, and biomass (Bastioli, 2005).

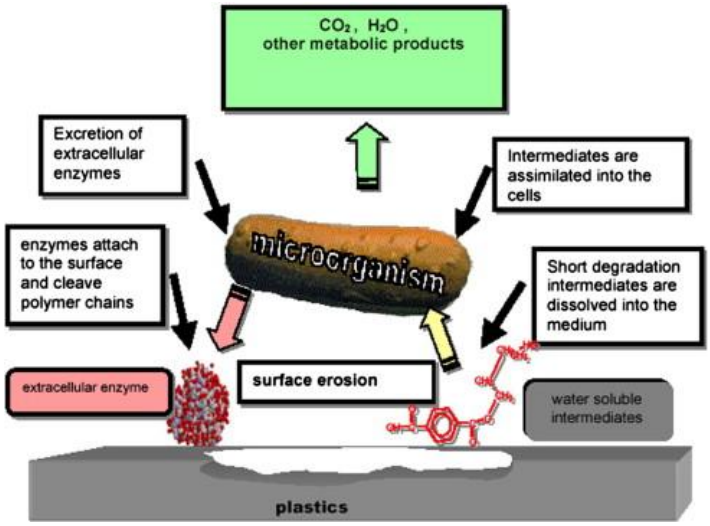


Figure 1.1 General mechanism of plastic biodegradation under aerobic conditions (Mueller, 2003).

Biodegradable polymers or biopolymers are natural polymers obtained from agricultural products or animals. Biodegradability and renewability are the main advantages of natural biopolymers over synthetic polymers. They are considered attractive alternatives for the petro-chemical non-biodegradable plastics because they are abundant, inexpensive, annually renewable, especially in countries where landfill is the main agricultural sector and eco-friendly (Rhim and Perry, 2007).

Naturally occurring biopolymers have different sources. Some are from animals like collagen, gelatin; from marine sources like chitin and chitosan; from microbial sources like polylactic acid, polyhydroxy alkanoates; and some are from agricultural feed stock like lipids, proteins and polysaccharides (Tharanathan, 2003).

1.2 Protein Films

Proteins are commonly used film-forming materials. They are macromolecules with specific amino acid sequences and molecular structures. The secondary, tertiary, and quaternary structures of proteins can be easily modified by heat denaturation, pressure, irradiation, mechanical treatment, acids, alkalis, metal ions, salts, chemical hydrolysis, enzymatic treatment, and chemical cross-linking. The most distinctive characteristics of proteins compared to other film-forming materials are conformational denaturation, electrostatic charges, and amphiphilic nature. Many factors can affect the conformation of proteins, such as charge density and hydrophilic-hydrophobic balance. These factors can ultimately control the physical and mechanical properties of prepared films and coatings. Protein film-forming materials are derived from many different animal and plant sources, such as animal tissues, milks, eggs, grains, and oilseeds (Krochta, 2002).

Corn zein comprises a group of prolamins found in corn endosperm. It accounts for 50% or more of total endosperm protein, occurring in small and compact bodies embedded in the glutelin protein matrix and distributed mainly in the outer layers of the endosperm (Dombrink-Kurtzman and Bietz, 1993). Zein is one of a few proteins, such as collagen and gelatin, used commercially as an edible coating. Zein coatings are used as oxygen, lipid, and moisture barriers for nuts, candies, confectionery and other foods (Andres, 1984), and for controlled ingredient release and protection in pharmaceutical tablets (Gennadios and Weller, 1990).

Soy protein used in the food industry is classified as soy flour, concentrate or isolate, based on the protein content. Agents of flavors microencapsulating and pharmaceuticals, coatings of fruits, vegetables, and cheese are the applications of soy protein isolate (Petersen *et al.*, 1999). Protective soy protein isolate coatings could also be used on certain food products, such as meat pies and

high-moisture-low-sugar cakes, which require films that are highly permeable to water vapor (Gennadios *et al*., 1993b).

Wheat gluten is the major storage protein in wheat (Weber, 2001). The very high molecular weight and diversity of their fractions are features of wheat gluten (WG) proteins that can be utilized to make films with novel functional properties, such as selective gas-barrier properties and rubber-like mechanical properties. WG-based materials are homogeneous, transparent, mechanically strong, and relatively water resistant. They are biodegradable, biocompatible, and edible when food-grade additives are used (Guilbert *et al*., 2002). It is possible to combine WG with other materials in order to optimize film properties. Films made from WG and lipids can combine the water vapor resistance of the lipids with the relatively good mechanical properties of the WG. Lipids can either be incorporated in film forming solutions to obtain composite films or deposited as a layer onto the surface of preformed WG films to obtain bi-layer films. The effects of lipids incorporated into WG film forming solutions can vary from destruction of the protein network to improvement of the mechanical and barrier properties of the cast films. These effects are complex and are dependent on the nature and structure of the lipids and on the chemical interactions between the lipids and the protein.

There have been studies in which bi-layer films composed of a WG film and a modified PE film. The films prepared shown the combination of the gas selectivity of the WG films and the excellent mechanical, moisture resistance properties of the PE films. Studies showed that the tensile strength and elastic modulus values of bi-layer films made of polyethylene maleic anhydride (PGMA) and WG were not affected by relative humidity, unlike those of WG films. Also, the gas and water vapor permeability values of the PGMA-WG films were considerably reduced as compared to those of control WG films (Pérez-Pérez, 1997). Application of WG-based materials can be envisioned for the coating of seeds, pills, and foodstuffs, and for making cosmetic masks, polishes or drug capsules (Guilbert *et* al., 2002).

Whey proteins are soluble proteins present in milk serum after caseinate is coagulated during the cheese processing. Whey proteins represent around 20% of total milk proteins (Brunner, 1977), and contain five main proteins: α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), immunoglobulin, and proteose-peptones. The formation of edible films and coatings composed of whey protein is also possible. When appropriately processed, whey proteins produce transparent, flavorless and flexible edible films similar to films produced from caseinates (Gennadious, 1994).

Casein, milk protein is used as surface coating material in paper and textile industries (Somanathan *et al*., 1992). Edible films based on milk proteins were reported to be flavorless, tasteless, and flexible, and, depending on the formulation, they varied from transparent to translucent (Chen, 1995).

Collagen, being a constituent of skin, tendon, and connective tissues, is a fibrous protein and represents about 30% of the total mass of the body (Gustavon, 1956). Hydrolysis of collagen results in gelatin. Edible coatings made with gelatin reduce the migration of moisture, oxygen and oil. Collagen is the most commercially successful edible protein film in the meat industry, in the production of edible sausage casings. Collagen films have an excellent oxygen barrier at 0% relative humidity, but the oxygen permeability increases rapidly with increasing relative humidity (Lieberman and Gilbert, 1973). To improve the mechanical properties, to reduce the solubility, and to improve the thermal stability of these films different cross-linking chemical agents have been used like carbodimide, microbial transglutarninase, and glutaraldehyde (Jones and Whitmore, 1972; Takahashi *et al*., 1999; Taylor *et al*., 2002). Gelatin is known to form clear, flexible, strong, and oxygen-impermeable films when cast from aqueous solutions in the presence of plasticizers (Gennadios *et al*.**,** 1994). Industrial applications of gelatin include capsule coating and microencapsulation. Edible coatings with gelatin reduce oxygen, moisture and oil migration, or can carry antioxidant or antimicrobial agents (Krochta and De

Mulder-Johnson, 1997) but due to their hydrophilic nature they have poor water barrier property.

1.3 Lipid Films

The use of lipids in coatings has long been used in the food industry to protect the quality and extend the shelf life of foods. First, waxes and oil were used alone but nowadays, they are often blended with solvents, plasticizers, emulsifiers, resins and surfactants. Currently, lipids are used as edible films and coatings for meat, poultry, seafood, fruits, vegetables, grains, candies, heterogeneous and complex foods, or fresh, cured, frozen, and processed foods. Lipid coatings have been effective at reducing moisture uptake or loss, and minimizing or delaying oxidative rancidity and moisture uptake in raisins and nuts, but, they lack sufficient strength to form self-supporting films.

1.4 Polysaccharide Films

The use of polysaccharides as coating materials for food protection has grown extensively in recent years. Generally, these systems are designed by taking advantage of their barrier properties against physical/mechanical impacts, chemical reactions, and microbiological invasion. In addition, the use of polysaccharides presents advantages due to their availability, low cost, and biodegradability. Their low cost is of great interest, as it leads to a reduction in the large quantities of non-biodegradable synthetic packaging materials. Furthermore, polysaccharides can be easily modified in order to improve their physiochemical properties.

Cellulose, starch, pectin, seaweed extracts, various plant and microbial gums are the biopolymers derived from agricultural feedstock, while chitin, chitosan are derived from connective tissue of crustaceans (Nisperos-Carriedo, 1994).

Polysaccharide films are used in Japan for meat products, ham and poultry packaging, before smoking and steaming processes (Labell, 1991; Stollman *et al*., 1994).

Cellulose, being a structural material of the cell walls of plants, is the most abundantly occurring natural polymer on earth. Cellulose is a simple polymer consisting of β-1-4-linked D-glucose molecules in a linear chain.

Several cellulose derivatives are widely produced commercially like carboxymethyl cellulose (CMC), methyl cellulose (MC), hydroxypropyl cellulose (HPC), and hydroxypropylmethyl cellulose (HPMC). Edible coatings, which include CMC, MC, HPC, and HPMC, have been applied to a variety of foods to provide moisture, oxygen or oil barriers and to improve better adhesion. CMC, MC, HPC or HPMC can be solubilized in aqueous or aqueous-ethanol solutions, producing films with good film-forming properties, but are soluble in water (Gennadios *et al*., 1997). Transparent, flexible, odorless, tasteless, water-soluble, and resistant to fats and oil films have been achieved with these cellulose ether composites (Kester and Fennema, 1986; Hagenmaier and Shaw, 1990; Hanlon, 1992; Nisperos-Carriedo, 1994). HPC is the least hydrophilic of the cellulose ethers (Gennadios *et al*., 1997); it is also thermoplastic so, it's capable of injection molding and extrusion. CMC is an anionic cellulose ether and forms a complex in the presence of casein, increasing the coating formulation viscosity (Keller, 1984); it retains the firmness of fruits and vegetables (Mason, 1969), preserves important flavor components of some fresh commodities (Nisperos-Carriedo and Baldwin, 1990), reduces oxygen uptake without causing carbon dioxide increase in internal fruits and vegetables (Lowings and Cutts, 1982), and improves the puncture strength of films based on caseinate (Ressouany *et al*., 1998).

Chitosan is a polysaccharide derived from chitin, and is found in abundance in the shells of crustaceans. Chitosan has been widely used for the production of edible coatings (Krochta and De Mulder-Johnston, 1997). Further interesting

properties of chitosan and chitin in relation to food packaging are their antimicrobial properties (Dawson *et al*., 1998; Coma *et al*., 2002) and their ability to absorb heavy metal ions, which can be used to diminish oxidation processes in foods catalyzed by free metals (Chandra and Rustgi, 1998). Chitosan films have good mechanical properties, being flexible and difficult to tear. Chitosan films also have moderate water vapor permeability properties, and exhibit good barriers to the permeation of oxygen (Rudrapatnam and Farooqahmed, 2003).

Seaweed extracts also are used to produce coating. Alginate is a polysaccharide derived from brown seaweed, possess good film-forming properties, but tend to be quite brittle when dry; however, they may be plasticized with glycerol (Glicksman, 1983). Also, alginate coatings are good oxygen barriers (Conca and Yang, 1993), can retard lipid oxidation in foods (Kester and Fennema, 1986), and can improve flavor, texture and batter adhesion. Alginate provides beneficial properties when applied as an edible coating to precooked pork patties (Wanstedt *et al*., 1981; Amanatidou *et al*., 2000). Such coatings increase moisture and reduce flavors due to lipid oxidation. Alginate coatings also improve adhesion between batter and product, thus reducing the loss of batter from the surface of meats and fish (Fischer and Wong, 1972).

Carrageenan is derived from red seaweed. Carrageenan-based coatings have been applied for a long time to a variety of foods to carry antimicrobials or antioxidants, and to reduce moisture loss, oxidation, or disintegration.

Agar is a gum that is derived from a variety of red seaweeds. Carrageenan, antibiotics, bacteriocins or natural antimicrobial compounds can be incorporated in agar-based films which can be used in order to improve shelf life and to control pathogenic bacterial growth. Natrajan and Sheldon (1995) observed a reduction in *Salmonella typhimurium* population of 1.8 to 4.6 log cycles after 96 hours of storage at 4°C on coated fresh poultry where nisin was incorporated.

Starch, being the most abundant natural polysaccharides, is commonly used in biodegradable films. They have been utilized in the packaging and coating of food products, because of their edibility and low permeability to oxygen (Mark *et al*., 1966; Roth and Mehltretter, 1970). The overall performance of starch films and coatings is highly likely to be customizable, because of the availability of a wide variety of starches and their capacity for physical and/or chemical modifications (Ellis *et al*., 1998; Liu, 2002). Edible starch films and coatings are commonly used in bakery, confectionery, batters, and meat products (Thomas and Atwell, 1997).

Starch films usually contain amylose, amylopectin, water and other plasticizers. Normally, films containing more amylose show better performance in terms of tensile strength, elongation, and gas barrier properties. Addition of plasticizers such as glycerol and sorbitol tends to make the films weaker, but softer and more flexible; however, branched structure of amylopectin generally leads to film with poor mechanical properties like decreased tensile strenght and elongation (Tharanathan, 2003).

1.5 Food Packaging Materials from the Agricultural Biowastes

Crops and livestock is 90 % of the agricultural sector in Turkey giving rise to huge amounts of agricultural residues. These residues are usually burn in open area, used as animal feed or left in place after the harvest. If they are not treated in a controlled manner, they cause significant environmental impacts. Moreover, useful resources are wasted.

1.5.1 Cotton Stalk

Gossypium hirsutum, cotton is from Malvaceae family and is a hardwood. The average cotton production in the world is approximately 20 million tons and

Turkey is one of the major producers of cotton along with China, India, The United States, Greece, Pakistan and Brazil.

The production of cotton in Turkey is about 3 million tons per year giving rise to approximately 1.5 million tons of residues (dtm.gov.tr). Cotton stalk contain 36% of cellulose, 21% of hemicelluloses and 28% of lignin (Akpınar *et al.*, 2007).

1.5.1.1 Cellulose

Being the structural component of the primary cell wall of green plants, cellulose is the most common organic compound on the Earth. It consists of linear molecules of at least 3000 β-1-4 linked glucopyranose units. This linkage leads to a flat-ribbon arrangement maintained by intermolecular hydrogen bonding, as shown in Figure 1.2.

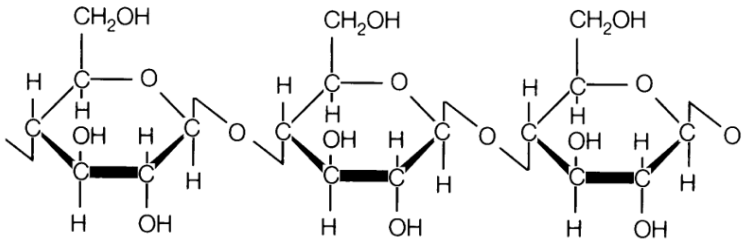


Figure 1.2 The flat-ribbon arrangement of the cellulose chain (webspace.ship.edu).

Within plant tissues cellulose molecules are aligned together to form microfibrils. Although doubt still exists as to the exact arrangement of the molecules within the microfibrils, it is clear that the chains pack together in a highly ordered manner maintained by intermolecular hydrogen bonding. It is

the stability of the ordered structure which gives cellulose both its insolubility and its strength (Coultate, 2002).

Cellulose is known to be the major constituent of paper and cardboard and of textiles made from cotton, linen, and other plant fibers.

1.5.1.2 Hemicellulose

A hemicellulose can be any of several heteropolymers (matrix polysaccharides) present in almost all plant cell walls along with cellulose. While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base as well as myriad hemicellulase enzymes. The main hemicelluloses of softwood are galactoglucomannans and arabinoglucuronoxylan while in hardwood is glucuronoxylan.

It consists of D-glucose, D-mannose, D-xylose, D-glucuronic acid, L-arabinose-4-*O*-methyl-D-glucuronic acid and D-galacturonic acid in various amounts according to the natural source. The Sugar units are mainly β-D-xylopyranose (β-D-Xyl*P*), β-D-xglucopyranose (β-D-Glc*p*), β-D-mannopyranose (β-D-Man*p*), α-Larobinofuranose (Ara*f*). They constitute the backbone structure of glucomannans and xylans. The Major hemicellulose components in softwood and hardwood (Sjostrom, E., 1993) are shown in Table 1.1.

Table 1.1 The major hemicellulose components in softwood and hardwood (Sjostrom, 1993).

Wood	Hemicellulose type	Amount (% on wood)	Composition			DP
			Units	Molar ratios	Linkage	
SW	Galacto-glucomannan	5-8	β-D-Man*p* β-D-Glc*p* α-D-Gal*p* Acetyl	3 1 1 1	1 → 4 1 → 4 1 → 6	100
	(Galacto)-glucomannan	10-15	β-D-Man*p* β-D-Glc*p* α-D-Gal*p* Acetyl	4 1 0.1 1	1 → 4 1 → 4 1 → 6	100
	Arabino-glucuronoxylan	7-10	β-D-Xyl*p* 4–O–Me–α-D-Glc*pA* α-L-Araf	10 2 1.3	1 → 4 1 → 2 1 → 3	100
HW	Glucuronoxylan	15-30	β-D-Xyl*p* 4–O–Me–α-D-Glc*pA* Acetyl	10 1 7	1 → 4 1 → 2	200
	Glucomannan	2-5	β-D-Man*p* β-D-Glc*p*	1-2 1	1→ 4 1→ 4	200

Being a source of xylitol, hemicellulose has found new applications as food additives, thickeners, emulsifiers, gelling agents, adhesives and adsorbents.

1.5.1.2.1 Xylan

Being the most common hemicelulose, xylan is considered the second most abundant biopolymer in plants (Biely, 1993). The main sugar of xylan is D-xylose. Depending on the xylan source, the structure of xylan differs from linear β-1-4-polyxylose main chains to highly branched heteropolysaccharides substituted with mainly acetyl, arabinosyl and glucuronosyl residues (Biely,

1993; Hazlewood and Gilbert, 1993). The principle xylan of hardwood, *O*-acetyl-4-*O*-methylglucuronoxylan, consists of a backbone of 1-4-glycosidic bond linked β-xylopyranose residues. Moreover, being found in the cell wall of monocots like grasses and cereals, xylan consists of linear chains of β-D-1-4-linked D-xylopyranosyl residues. Those residues can be also substituted with α-L-arabinofuranosyl at the 2-*O* and/or 3-*O* position(s) and α-D-glucuronopyranosyl or its 4-*O*-methyl derivative at the 2-*O* position (position (Brillouet, Joseleau, Utille and Lelievre, 1982; Carpita, 1996; Shibuya and Iwasaki, 1985). The structure of xylan can be seen in Figure 1.3.

In contrast to xylan from softwoods, xylan from hardwoods is very highly acetylated 4-*O*-methyl-α-D-glucuronoxylan, essentially without arabinosyl substitution. Approximatelly, we can say that nearly seven out of ten xylosyl residue carries an α-*O*-methylglucuronyl residue at *O*-2 (Evtuguin *et al*., 2003; Puls and Poutanen, 1989; Shatalov *et al.* 1999).

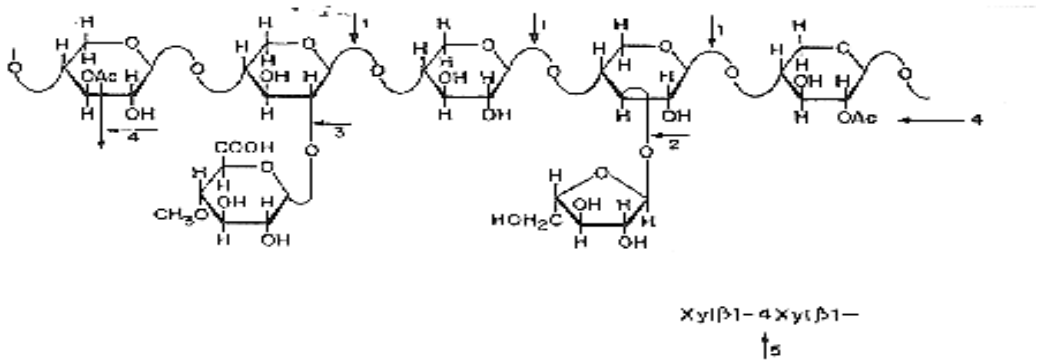


Figure 1.3 Structure of xylan and the enzyme clevage sites (Srinivasan and Rele, 1995).

The enzymes are as follow,

1. Endo-1-4-β-xylanase

2. α-L-arabinofuranosyidase

3. α-glucuronosidase

4. acetylesterase

5. β-xylosidase

Film production from xylan was seen to be possible; however pure xylan did not form continuous, self-supporting films. Mixture of extracted xylan from birchwood and aspen and also various amounts of chitosan were prepared and proved that from the 5% concentration of chitosan, the solution started to form films when dried. Self-supporting films have also prepared from the 10% concentration of chitosan content (Gabrielli and Gatenholm, 1998, Gabrielli *et al.*, 2000).

Composite film production of cotton stalk xylan was also studied. Film formation was achieved using 8-14% (w/w) xylan without complete removal of lignin during xylan isolation. Addition of about 1% (w/w lignin/xylan) was determined sufficient for film formation. Films were produced by casting-method, followed by solvent evaporation in a temperature and relative humidity controlled environment at 20°C and 40% relative humidity. The thickness and mechanical properties of the films obtained by using 8% xylan were quite low in comparison with the ones containing 10-14% xylan. The water vapor transfer rates (WVTR) decreased by increasing xylan concentration which makes the films thicker. The glycerol addition as an additional plasticizer resulted in more stretchable films having higher WVTR and lower water solubility values. As a result, film production was successfully achieved from xylan which was extracted from an agricultural waste, cotton stalk and film forming effect of lignin on pure xylan has been demonstrated (Göksu, 2005).

Xylan was also incorporated with wheat gluten films to produce biodegradable composite films. It has been seen that up to 40% (w/w) addition of xylan, the films were obtained without changing the properties of wheat gluten films. By using different types of xylan like birchwood, corncob and grass xylan and also with different concentration, the wheat gluten films showed 1 to 8 MPa tensile strength, 20 to 650% strain at break and 5 to140 MPa elastic modulus. Moreover, the film solubility was between 40-60% depending on the different xylan content and the water vapor transfer rate was not affected by the addition of xylan (Kayserilioğlu *et al*., 2003).

1.5.1.2.2 Lignin

Lignin is a complex chemical compound most commonly derived from wood, and an integral part of the secondary cell walls of plants and some algae. It is also one of the most abundant organic biopolymer on Earth. Lignin is a cross-linked racemic macromolecule with molecular masses in excess of 10,000 u. It is relatively hydrophobic and aromatic in nature. The degree of polymerisation in nature is difficult to measure, since it is fragmented during extraction and the molecule consists of various types of substructures which appear to repeat in a haphazard manner. Different types of lignin have been described depending on the means of isolation (Hon, 1996). The structure of lignin can be seen in Figure 1.4.

Lignin behaves like a thermoplastic material with a glass transition temperature that varies depending on the extraction method. So, in 1998, a German company, Tecnaro, developed a process for turning lignin into a substance, called Arboform, which behaves identically like plastic for injection molding. Therefore, it can be used in place of plastic for several applications. When the item is discarded, it can be burned just like wood. Moreover, lignin is widely used as emulsifier, dispersant, additive and binders. It is also used as an additive in the film forming solutions of pure xylan extracted from cotton stalk

and found to be a good binder to form self-supporting and continuous films (Göksu, 2005).

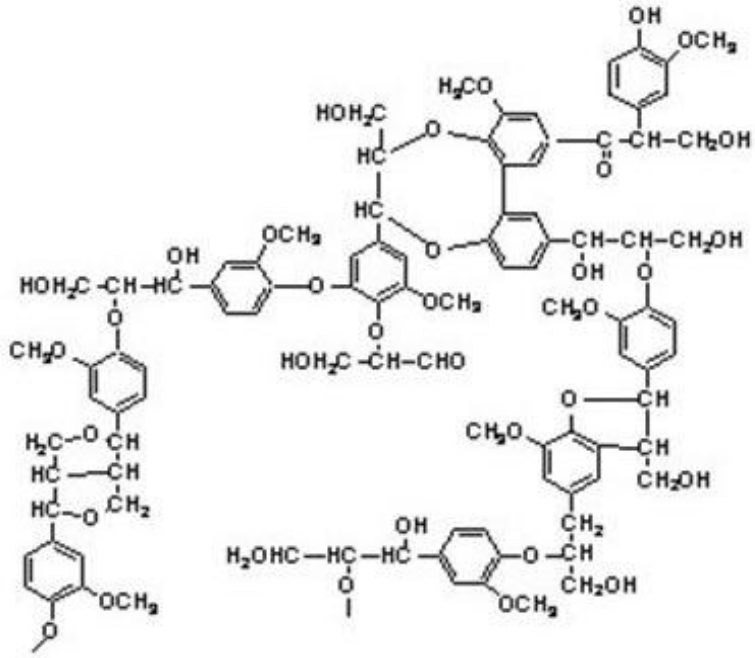


Figure 1.4 A small piece of lignin polymer (Hon, 1996).

1.6 Antimicrobial Films

Antimicrobial packaging is a system that can kill or inhibit the growth of microorganisms and thus extend the shelf life of perishable products and enhance the safety of packaged products (Han, 2000). Antimicrobial packaging can kill or inhibit target microorganisms (Han, 2000, 2003a). Among many applications such as oxygen-scavenging packaging and moisture-control packaging, antimicrobial packaging is one of the most promising innovations of active packaging technologies (Floros *et al*, 1997).

There are two ways to prepare an antimicrobial film. One involves the direct addition of the antimicrobial agent into the packaging film and the second one

involves the coating of the packaging film with an antimicrobial reagent (Cooksey, 2001).

Various antimicrobial agents could be incorporated into conventional food packaging systems and materials to create new antimicrobial packaging systems. The antimicrobial reagent is in the contact of the food product, so the reagent should be controlled as food ingredients. For non-food-grade antimicrobial reagent, it is required to immobilize the material to the packaging material and it is necessary to verify that there is no migration of the chemical from packaging materials to foods, and there is no residual free chemical after the immobilization reaction. Some mainly antimicrobial reagents are enzymes, polyamides and organic acids (Appendi and Hotchkiss, 2002).

Most commonly used antimicrobial reagents are organic acids like sorbic acid, sorbates. They are very strong antifungal agents, while their antimicrobial activities are not effective due to their different antimicrobial mechanisms so it is better to have a mixture of organic acids to have a wider antimicrobial spectrum and strength.

Fungicides are also common antimicrobial agents. Imazalil has been incorporated into the wax coating of oranges and other citrus fruits. Since fungicides are not permitted as a direct food preservative, they cannot be mixed into food ingredients or added to food-contact packaging materials as food-contact substances. Therefore, it is necessary to design antimicrobial food packaging systems when non-food-grade antimicrobial agents, such as fungicides, are used. Cast edible films have been used as carriers for antimicrobials and applied as coating onto packaging materials and foods. Examples include nisin/methylcellulose coating for polyethylene films (Cooksey, 2000; Appendini and Hotchkiss, 2002).

Nisin, a bacteriosin produced by *Lactococcus lactis* is a natural additive. This is regarded as a desirable feature for many food additives. It has GRAS

(generally recognized as safe) status for use with processed cheese, and it is particularly effective for preventing growth of *Clostridium botulinum*. Nisin is selective for G(+) bacteria but is not effective for the inhibition of G(-) bacteria (Cooksey, 2001); however when incorporated with Ethylenediamine tetra-acetic acid (EDTA), nisin shown good antimicrobial activity against the G(-) bacteria like *Listeria monocytogenes* on heat pressed corn zein films (Hoffman, 1998).

Triclosan is an inorganic antimicrobial compound mainly used in toothpaste, hand soap, cosmetics and plastics like toys and cutting-boards. A study showed that the coating made of a styrene-acrylate copolymer containing triclosan inhibited growth of *Enterococcus faecalis* (Chung, 2003).

Chitosan, derived from shells of crustaceans through repeated washes in alkaline and acid solutions is inherent antimicrobial polysaccharides including fungi, algae, and some bacteria. Chitosan has been used as a coating. Its ability to form a semi-permeable film provides to modify the internal atmosphere, as well as a decrease the transpiration lose, thus a delay the ripening of fruits and vegetables (El Ghaouth, 1991; Jiang, 2001).

The preparation of chitosan and chitosan-laminated films with other polysaccharides has been reported by various authors; these include chitosan films (Butler, 1996; Tharanathan, 1998), chitosan/pectin laminated films, and chitosan/methylcellulose films (Chen, 1996).

Chitosan films are tough, long lasting, flexible, and very difficult to tear. Most of their mechanical properties are comparable to many medium-strength commercial polymers (Butler, 1996). It was reported that chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values (Tharanathan, 1998). They have extremely good barriers characteristics for the permeation of oxygen but low vapor barrier properties (Wong, 1992).

Chitosan is also found to be an antifungal polysaccharide and recent investigations on chitosan coating of tomatoes have shown that it delayed ripening by modifying the internal atmosphere, which reduced decay (El Ghaout, 1992). It is also used as an antifungal coating for fresh fruits and vegetables. Antimicrobial films were also produced by dissolving chitosan into hydrochloric, formic, acetic, lactic and citric acid solutions (Bégin, 1999).

1.6.1 Antimicrobial Film Preparation by Photocatalytic Semiconductor Nanoparticles

Microbial contamination and growth on the surfaces are potential risks for human health. The use of aggressive chemicals such as detergents, alcohols and chlorine components for manual disinfection is not environmentally benign and is ineffective for long-term disinfection.

An effective method of disinfection is the UV radiation which requires special radiation source within UV-C (185-254 nm) band. In the meantime, the risk of intensive and direct use of UV radiation limits its use to medical and technical purposes only. Photocatalysis is an alternative to direct UV disinfection and considerable antimicrobial efficacy is possible with higher wavelengths, which are naturally present in ambient solar and artificial light. During the past 20 years, it has become an extremely well researched field due to practical interest in air and water remediation, self-cleaning surfaces, and self-sterilizing surfaces. Semiconductor photocatalysis can be more appealing than the more conventional chemical oxidation methods because semiconductors are inexpensive, nontoxic, and capable of extended use without substantial loss of photocatalytic activity. The most commonly used photocatalys, TiO_2 had been approved by the food testing laboratory by the United States Food and Drug Administration (FDA) and it is considered safe and harmless to human. It is commonly used in paint, printing ink, plastics, synthetic fibers, electronic components, food and cosmetics (Fujishima *et al*., 2000).

Large band gap semiconductors based nanoparticles like TiO_2, SnO_2 and ZnO, are suitable with their higher wavelength UV absorption (UV-A, 320-400 nm) and have found great interest on wide range of applications such as, direct splitting of water for hydrogen production, partial or complete photocatalytic oxidation of organic materials and disinfection of microorganisms (Matsunaga *et al*., 1985; Saito *et al*., 1992; Benedix *et al*., 2000; Hong *et al*., 2003; Fujishima *et al*. 2000).

Photocatalytic microbial inactivation was first reported by Matsunaga *et al*. more than 20 years ago. *Lactobacillus acidophilus, Saccharomices cerevisae, Listeria monocytogenes, Pseudomonas aeroginosa, Staphilococcus aureus, Vibrio paraheamolyticus* and *Escherichia coli* were totally inactivated with the use of TiO_2 (Wei *et al*, 1994, Matsunaga *et al*, 1995, Sunada *et al*, 1998, Kim *et al*, 2003). It is also reported by Cho *et al*. in 2004 that the OH radicals produced by the photocatalytic effect are approximately one thousand or possibly ten thousand times more effective for *E. coli* inactivation than common disinfectants such as chlorine, ozone and chlorine dioxide.

The photocatalytic microbial inactivation mechanism of TiO_2 is illustrated in Figure 1.5.

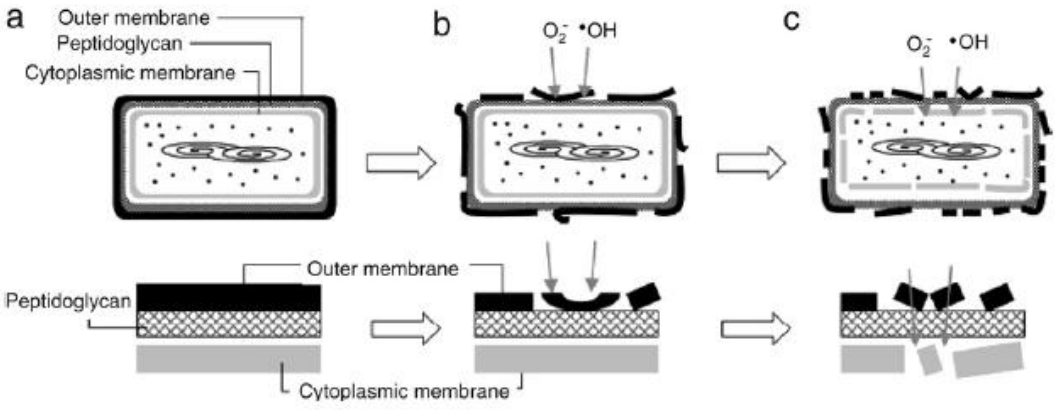


Figure 1.5 Schematic illustration of the three stages in the process of *E. coli* photocatalytic microbial inactivation on a TiO_2 film. In the lower row, part of the cell envelope is magnified (Elsevier Science S.A., 2003).

Sunada *et al*. (2003) studied the photoinactivation process of *E. coli* cells by means of AFM. They found that the photoinactivation of bacteria on the illuminated TiO_2 surface could be divided into three stages (Figure 1.5): (1) disordering of the outer membrane of bacteria cells by reactive species (•OH, H_2O_2, $•O_2^-$); (2) disordering of the inner membrane (the cytoplasmic membrane) and killing of the cell; and (3) decomposition of the dead cell. In the first stage, the outer membranes of *E. coli* cells were decomposed partially by the reactive species produced by the TiO_2 photocatalyst. During this stage, cell viability was not lost very efficiently. The partial decomposition of the outer membrane, however, changes the permeability to reactive species. Consequently, reactive species easily reach and attack the inner membrane, leading to the peroxidation of the membrane lipid. The structural and functional disordering of the cytoplasmic membrane due to lipid peroxidation led to the loss of cell viability and cell death. It was also reported that if the illumination continued for a sufficiently long time, the dead cells were found to be decomposed completely.

It has been found that when illuminated by near-UV light. TiO_2 exhibited excellent bactericidal activity, which was investigated by atomic force microscopy in conjunction with some other techniques. The results shoved that the cell death was caused by the decomposition of the cell wall and the cell membrane leading to the leakage of intracellular molecules (Lu, 2003).

TiO_2 has been used in advanced oxidation applications for the degradation of organic dyes and hazardous organic contaminants, which are one of the major pollutants in wastewaters produced from textile and other industrial processes (Konstantinou *et al*., 2004). By the solar detoxification tests, blank experiments have confirmed that loss of contaminants was only due to the photocatalytic process. Moreover, in the same study, the natural UV component of sunlight was found to be able to destroy microorganisms at a rate similar to that of illuminated TiO_2 particles under UV light. So the photocatalytic oxidation is considered to be a preferred method that the UV irradiation because OH radicals are able to destroy biological contaminants, with the oxidation of proteins, lipids or nucleic acids, thus resulting in inhibition of respiration or growth of the microorganisms (Vidal *et al*.,1999).

Semiconductors' electronic structure is characterized by a filled valance band and an empty conduction band. Due to this structure, semiconductors with their reasonable band gap energies find great attraction through this purpose. The energy difference between the lowest energy level of the conduction band and the highest energy level of the valance band is the so-called band gap energy (Erkan, 2006; Benedix *et al*., 2000).

Antimicrobial surfaces can be obtained by coating them with a semiconductor based nanoparticles giving the antimicrobial property by the photocatalytic activity.

The photocatalytic activity is based on the interaction between the photocatalytic semiconductor nanoparticles and the light, which emits energy

that exceeds the band gap energy of the photocatalytic material. This is 3.2 eV (387.5 nm in ultra-violet region). The photocatalytic reaction mechanism is initiated by the absorption of UV light, which results in the promotion of an electron from the valence band to the conduction band. When the electron is transferred to the empty conduction band, electron/hole (e^-/h^+) pairs are formed within the semiconductor materials. The components of this activated pair are capable of, respectively, reducing and oxidizing a surface-adsorbed substrate, forming on a common surface a singly oxidized electron donor and singly reduced electron acceptor (Fox *et al*., 1992).

$$TiO_2 + h\nu \longrightarrow e^- + h^+ \quad \textbf{(1.1)}$$

$$e^- + O_2 \longrightarrow \bullet O_2^- \quad \textbf{(1.2)}$$

$$h^+ + H_2O \longrightarrow \bullet OH + H^+ \quad \textbf{(1.3)}$$

$$\bullet O_2^- + H^+ + e^- \longrightarrow HO_2^- \quad \textbf{(1.4)}$$

$$2\ \bullet O_2^- + H^+ \longrightarrow HO_2^- + O_2 \quad \textbf{(1.5)}$$

$$\bullet OH + \bullet OH \longrightarrow H_2O_2 \quad \textbf{(1.6)}$$

(Hirakawa *et al*., 2001)

The electron/hole pairs move to the surface as a result of charge separation where electrons reduce oxygen to superoxide radicals ($\bullet O_2^-$) and further reaction with H_2O molecules yield OH^- ions. Both OH^- and H_2O molecules are converted to •OH radicals by reacting with the holes. Both •OH and $\bullet O_2^-$ radicals are capable of performing many selective/non-selective oxidation reactions (Erkan, 2006). Mechanism of photocatalysis is illustrated in Figure 1.6.

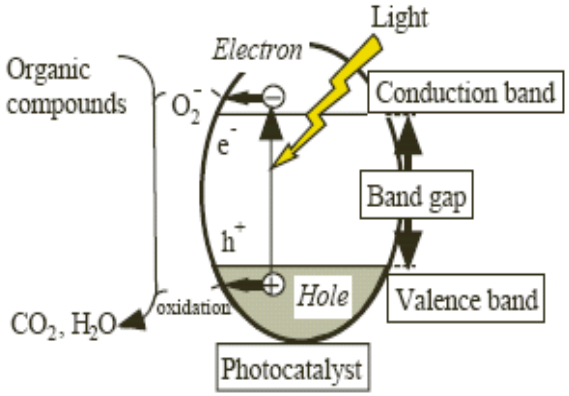


Figure 1.6 Mechanism of photocatalysis. (TitanPE Tech. Inc. Library, Shanghai).

The photocatalytic antimicrobial activity over TiO_2, SnO_2 and their Pd doped thin film samples were determined against *E. coli, Staphylococcus aureus, Saccharomyces cerevisae* and *Aspergillus niger* spores. Higher antimicrobial activity was observed with TiO_2 than SnO_2 and Pd doping was found to contribute to an increase in the activity of both semiconductors. The highest inactivation was achieved with 1% Pd/TiO_2 against *E. coli* with a 98% decrease in survival after 2 h of illumination (Erkan, 2006).

Apart from the photocatalytic properties, TiO_2 films exhibit superhydrophilicity after UV illumination. As in the case of photocatalysis, in superhydrophilicity electrons and holes are still produced, but they react in a different way. The superhydrophilicity mechanism occurs in three steps.

In the first step, as in the case of photocatalysis mechanism when the surface of TiO_2 is irradiated by UV light, an electron/hole pair is created by excitation.

$$h\nu \longrightarrow e^- + h^+ \qquad \textbf{(1.7)}$$

Then, the Ti on the TiO_2 crystal surface is reduced by a surface trapped electron and the oxygen vacancies are created.

$$e^- + Ti^{4+} \longrightarrow Ti^{3+} \quad \textbf{(1.8)}$$

$$4h^+ + 2O^{2-} \longrightarrow O_2 \quad \textbf{(1.9)}$$

(Fujishima *et al.*, 2000)

Then Ti^{3+} is oxidized by oxygen in the air and the oxygen vacancies formed, bond with H_2O (Fujishima *et al.*, 2000). The oxygen vacancies are thought to cause an increase in the absorbed OH^- group density and lead to the formation of hydrophilic regions (Mills *et al.*, 2008).

The reduced sites can be reoxidized by air and the weakly bound hydroxyl groups reactively desorb from the surface that returns to a more hydrophobic form when maintained in the dark. On the other hand, the longer the surface is illuminated with UV light, the smaller the contact angle for water becomes (Mills *et al.*, 2000). The mechanism of photoinduced superhydrophilicity of TiO_2 is exhibited in the Figure 1.7. It is desirable that the contact angle increases slowly in a dark place, and stays low for a long time. This condition is essential especially when actual use is considered; it is not always irradiated by UV light, such as sunlight in rainy or cloudy days.

It was found that the addition of SiO_2 into TiO_2 improved the superhydrophilicity of TiO_2 films kept in the dark (Carp *et al.*, 2007). According to Guan *et al.* (2005), the SiO_2 addition into TiO_2 film enhances the acidity of the composite oxide which is thought to be the main reason for improving the photocatalysis and hydrophilicity since more OH radicals can be adsorbed on the surface due to hydrophilicity.

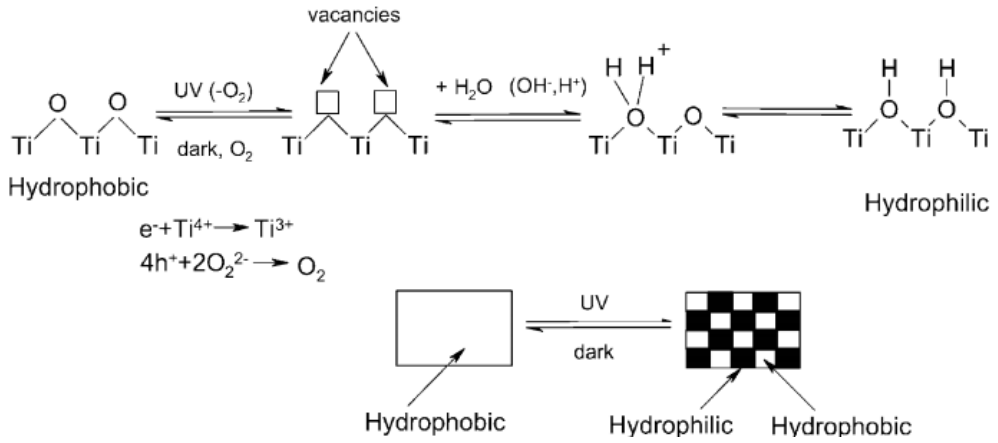


Figure 1.7 Mechanism of photoinduced superhydrophilicity of TiO_2. (Kaneko, 2003).

According to the studies of Guillard *et al*. (1999), it has been observed that one of the most important factors in achieving a high efficiency in the photocatalytic activity is the large surface area. TiO_2 has a tendency to have higher photocatalytic activity when supported on porous solids due to the increase in the effective surface area which is helpful to enhance surface reaction on TiO_2 (Guillard *et al*., 1999; Yamashita *et al*, 1996). The oxidation reactions which are effectiveness due to the surface limited reactions are improved as a result of the high surface area to volume ratios (Zhao, 2003). As a consequence, photogenerated pairs of electrons and holes can easily diffuse to the surface of the catalysts and form the active sites for the oxidation/reduction reactions. To enhance the surface area, many studies were conducted on SiO_2/TiO_2 nanocomposites which attracted more attention since many researches reported that those nanocomposites have higher photocatalytic activity than pure TiO_2 (Guan *et al.* 2003, Permpoon *et al.*, 2008, Fujishima *et al.*, 2000). Moreover, the increase in surface area achieved by the addition of SiO_2 leads an increase in the number of active sites on which the electron acceptor and donor are adsorbed and participate in the redox reaction (Fujishima *et al.*, 2000). The studies of Aguado *et al* (2002) on the removal of free cyanide; Tanaka *et al* (2002) on the photodegradation of phenol, have

found that the presence of SiO_2 has beneficial effect by generation of new active sites due to the interactions between titania and silica.

1.7 Aim of the Study

Food packaging is an important issue in food industry and studies related to food packaging are mainly concerned with better protection, more efficient quality preservation and enhanced food safety. As the consumer interest has shifted to eco-friendly, environmentally sustainable and efficient for preservation of food packaging materials, biodegradable, antimicrobial film production has gained attention in the last years.

If agricultural wastes are treated in an uncontrolled manner, they can cause significant effects on the environment. In Turkey, cotton stalk is an important agricultural waste which is mainly composed of cellulose, hemicelluloses and lignin. The literature about the xylan, the extracted part of the hemicelluloses, is very limited. However, in a recent study, xylan is extracted from hemicelluloses and used in the production of biodegradable wheat gluten films (Göksu, 2005).

Moreover, the food packaging is very important in the field of safety and health because the growth of microorganisms on the food surface is the major source of food-borne diseases and food spoilage (Kandasamy, 2007). Nisin, chitosan, triclosan, sorbic acid, sorbate have been used as antimicrobial additives in food packages.

There have been studies on the development of photocatalytic antimicrobial self-cleaning surfaces like glass, textile, ceramics and some food packaging material (petro-chemical based food packaging materials coated with the photocatalytic semiconductor based nanoparticles, TiO_2).

As there has not been any study on the production of antimicrobial biodegradable packaging films prepared by coating or adding semiconductors, there is a need for study on that field.

The aim of the study is to produce antimicrobial xylan based biofilms by the addition of TiO_2 or by coating the biofilms with TiO_2 as the photocatalytic semiconductors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Cotton stalk was brought from a local producer in Urfa, Turkey. Birchwood xylan and titanium tetraisopropoxide (TTIP), Luria-Bertani (LB) Agar, Luria-Bertani (LB) broth, barium chloride dihydrate ($BaCl_2.2H_2O$), polyvinyl alcohol (PVA), xylitol were purchased from Sigma (Germany). Titanium dioxide was bought from Aldrich (Germany). Acetic acid (CH_3COOH), ethanol (C_2H_5OH), 35% hydrochloric acid, nitric acid (HNO_3), isopropyl alcohol (C_3H_7OH), potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium borohydride ($NaBH_4$), sulfuric acid (H_2SO_4) were purchased from Merck (Germany). All the other chemicals used were analytical grade and bought either from Sigma or Merck.

2.2 Microorganisms and Growth Conditions

Esherichia coli (XL1-blue) was maintained on Luria-Bertani (LB) agar. Plates were incubated at 35°C overnight and then stored at 4°C until use. *E. coli* was cultured in Luria-Bertani (LB) broth at 35°C on a rotary shaker (Minitron) at 170 rpm overnight.

2.3 Xylan Extraction

The method used by Zilliox and Debeire (1998) was modified and followed for the xylan extraction. 100 ml of distilled water was added to 2 g of milled cotton stalk and the sample was swelled at 60°C for 16 hours in an incubator (Nüve, Turkey). The swollen sample was filtered by means of a filter paper (Whatman No. 41) and the pellet was mixed with solution containing 17 ml of 24% (w/v) KOH + 1% (w/v) $NaBH_4$ solution and stirred for 3 hours at room temperature. Afterwards, the suspension was filtered and the supernatant was mixed with 5 ml cold ethanol solution containing 10% (v/v) acetic acid. This suspension was shaken for several minutes and a centrifugation step (Sigma 3K18) was performed at 8,300 x g for 20 min to recover xylan. The pellets are dried at 60°C for 24 h and used as xylan source. In order to remove lignin completely, the volume of the washing solution was increased to 110 ml (cold ethanol solution containing 10% acetic acid).

2.4 Film Preparation

2.4.1 Xylan Based Biofilm Preparation

The biofilms were prepared by the solvent casting method according to a modified procedure of the Gröndahl *et al.* (2004). Birchwood xylan biofilms were casted from commercial birchwood xylan by dissolving 0.6 g (2.4% (w/w)) of birchwood xylan and 0.4 g (1.6% (w/w)) xylitol in 25 ml of ultra purified water and stirred by a magnetic stirrer at 90°C until complete solubilization of the polymers has been achieved. When the solubilization was observed, the biofilm solution was poured in a petri dish with diameter of 9 cm. The same procedure was performed for the preparation of the extracted cotton stalk xylan based biofilms, but for the complete solubilization, autoclaving at 120°C for 20 min was performed since extracted cotton xylan particles were not soluble in water. After, the biofilm forming solutions were poured into the

petri dishes; all solutions were dried at room conditions at a temperature of 25±5°C and a relative humidity of 35±5%.

2.4.2 Antimicrobial Xylan Based Biofilm Preparation

2.4.2.1 Preparation of Xylan Based Antimicrobial Biofilms by Sol-Gel Coating

Xylan based biofilms were coated with TiO_2 by using two different sol-gel methods.

2.4.2.1.1 Aqueous-Nitric Acid Method

0.5 ml of acetic acid was added to 100 ml of ultra purified water. 2.5 ml of titanium tetraisopropoxide (TTIP) was added slowly to this solution. 0.35 ml nitric acid (HNO_3) was also added and the solution was boiled for half an hour under a reflux condenser at 80°C. As the next step, the mixture was stirred at room conditions on a magnetic stirrer for 24 h. Biofilm samples were dipped in the sol-gel solution for 10 s and dried at room conditions for 24 h. As the final step, coated samples were calcinated at 60, 90 and 120°C for 30 min.

2.4.2.1.2 SiO_2 Added Aqueous-Nitric Acid Method

0.5 ml of acetic acid was added to 100 ml of ultra purified water. 2.5 ml of titanium tetraisopropoxide (TTIP) was added slowly to this solution. 0.35 ml nitric acid (HNO_3) was also added and the solution was boiled for half an hour under a reflux condenser at 80°C. As the next step, the mixture was stirred at room conditions on a magnetic stirrer for 2 h. and 3.2 ml of Ludox colloidal silica was added. Then, the final mixture was stirred at room conditions on a magnetic stirrer for 24 h. Biofilm samples were dipped in the sol-gel solution

for 10 s and dried at room conditions for 24 h. As the final step, coated samples were calcinated at 60, 90 and 120°C for 30 min.

2.4.2.2 Preparation of Xylan Based Antimicrobial Biofilms by the Addition of TiO_2 powder

TiO_2 powder (Aldrich, Germany) was added in different quantities to the xylan based films to investigate their photocatalytic antimicrobial activities.

2.4.2.2.1 Preparation of Birchwood Xylan Antimicrobial Biofilms by the Addition of TiO_2 powder

Biofilm forming solutions were prepared by dissolving 0.6 g (2.4% (w/w)) of birchwood xylan, 0.4 g (1.6% (w/w)) xylitol as plasticizer in order to enhance the mechanical properties of the films with the addition of 1% (w/w), 2.5% (w/w), and 5% (w/w) quantities of TiO_2 powder. The solution was autoclaved to solubilize all the polymer components, and then stirred at room temperature for 30 min before solvent casting into petri dishes with diameter of 9 cm. The films were dried at ambient condition at a temperature of 25±5˚C and a relative humidity of 35±5%.

2.4.2.2.2 Preparation of Cotton Stalk Xylan Antimicrobial Biofilms by the Addition of TiO_2 powder

Film forming solutions were prepared by dissolving 1 g (4% (w/w)) of extracted and dried xylan pellets with the addition of 1% (w/w), 2.5% (w/w), and 5% (w/w) quantities of TiO_2 powder. The solution was autoclaved to solubilize all the components at 120°C for 20 min, and then stirred at room temperature for 30 min before solvent casting into petri dishes with diameter of 9 cm. The films were dried at ambient condition at a temperature of 25±5˚C and a relative humidity of 35±5%.

2.5 Photocatalytic Antimicrobial Activity Test for Xylan Based Biofilms against *E. coli* Cells

E. coli cells were cultured in Luria-Bertani (LB) broth at 35°C on a rotary shaker (Minitron) at 170 rpm overnight.

An overnight *E. coli* culture of 1.5 ml/film sample was put in an Eppendorf tube and a centrifugation step was performed at 10,000 rpm for 4 min (Sigma centrifuge). Supernatant was separated and the pellet was mixed with 1 ml of peptone water and 100µl of which was the put on ¼ of film samples. Before black light illumination, the film samples were left in dark for 30 min for absorption of the bacterial culture into the biofilm samples.

The test samples were illuminated using a 20 W black light source (General Electric) with a peak wavelength of 368 nm placed at 20 cm above the biofilms for consecutive periods of time like 15, 30, 45, 60 min or only during 2 h periods. Then, serial dilution in peptone water (0.01%) was performed. Therefore for dissolving the biofilm samples, they were well mixed with 9.9 ml of peptone water from which 100µl of suspension was taken and mixed with 9.9 ml of peptone water. After vortex mixing, this suspension was diluted with peptone water for counting. 200µl of suitably diluted (10^6 dilution) microbial suspension was inoculated on Luria-Bertani (LB) agar plates and incubated at 35°C overnight. Next day, number of *E. coli* cells survived was counted by the viable colony counting method.

The effect of irradiation alone on the microorganisms was determined on uncoated and without TiO_2 powder added biofilms. The effect of sol-gel and TiO_2 powder without photocatalysis on microbial growth was measured on sol-gel coated and TiO_2 added biofilms in the dark. In addition, to determine the initial number of *E. coli* cells that was put on the biofilms, liquid cell suspension of 200µl was spread onto agar plates and incubated at 35°C.

2.6 Biodegradability Test

Forest soil, collected from Middle East Technical University, Ankara, was used as microbial sources in the biodegradation experiments. The tests were carried out in the air-tight glass flasks containing a multilayer substrate in which defined amounts forest soil (20 g) sieved at 1.8 mm which was mixed with 20 g perlite and supplemented with 25 ml of 0.1% $(NH_4)_2$ HPO_4 solution to amend the soil with nitrogen to give a C:N of between 10:1 and 20:1. Finally the mixture was sandwiched between two layers consisting of 20 g perlite wetted with 30 ml distilled water. Polymeric test specimen was placed in the core of soil layer. The vessels were kept and incubated at room temperature.

The CO_2 evolved from various samples (CSX-50 and 10% TiO_2 powder added CSX-50 containing films, BWX films, cotton-stalk, one time autoclaved soil and autoclaved cotton-stalk containing flasks, two times autoclaved soil and autoclaved cotton-stalk containing flasks, lignin containing flasks, positive control containing cellulose, negative control containing LDPE and soil containing flask as blank) was trapped in each flask by means of 100 ml of 0.05 N KOH solution contained in a beaker in the test flask. The absorbing solution was back titrated with 0.1 N HCl every 3 days by adding before titration 0.5 N $BaCl_2$ solution in one-tenth proportion with respect to the overall volume of the absorbing alkaline solution. After the addition of $BaCl_2$, the solution is centrifuged (Sigma 3K18 centrifuge) at 8,300 x g for 7 min. The supernatant was taken and titrated with the phenolphtalein indicator. The biodegradation extent of each test material was evaluated as a neat percentage (corrected for the inoculums endogenous emission from blank, i.e. soil, sample) of the overall theoretical CO_2 production calculated on the basis of the relevant carbon content in the testing sample.

The calculations are made accordingly,

By element analysis, the total organic carbon content of the test material. This allows the theoretical quantity of carbon dioxide evolution to be calculated as illustrated by the following:

Material= w% carbon; w/100 x mg of material charged **(2.1)**

= Y mg carbon charged to the flask:

$C + O_2 \rightarrow CO_2$ **(2.2)**

12 mg yield 44 mg CO_2

Y mg yields $\frac{44}{12} \times Y$ mg CO_2 **(2.3)**

Amount of net CO_2 produced is calculated by correcting for the CO_2 produced in the blank test subtracting titration from the test material titration.

$Z_n = Z_b - Z_t$ **(2.4)**

Where

Z_n = calculated ml of HCl needed to titrate the CO_2 generated solely from the test sample

Z_b = ml HCl used to titrate the blank

Z_t = ml HCl used to titrate the test sample

Then,

$KOH + CO_2 \rightarrow KHCO_3$ **(2.5)**

$KHCO_3 + KOH \rightarrow K_2CO_3 + H_2O$ **(2.6)**

KOH excess is reacted with $KHCO_3$ produced.

During the titration;

$K_2CO_3 + HCl \rightarrow KHCO_3 + KCl$ **(2.7)**

$KHCO_3 + HCl \rightarrow KCl + H_2O + CO_2$ **(2.8)**

m moles of CO_2 = m moles of HCl **(2.9)**

mg of CO_2 = 0.1 N x ml HCl x 44 **(2.10)**

The percentage of CO_2 evolved is calculated as shown below:

% CO_2 evolved= (mg CO_2 produced / mg CO_2 theoretical) x 100 **(2.11)**

CHAPTER 3

RESULTS AND DISCUSSION

In the first part of the study, antimicrobial xylan based biofilms were prepared either by coating the biofilms with TiO_2 sol-gels or adding different amounts of TiO_2 powder into the film forming solutions. Then, photocatalytic microbial inactivation rates were investigated.

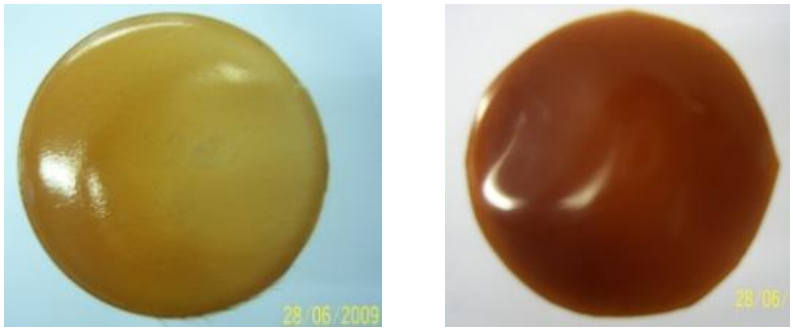
In the second part of the study, the biodegradability property of the prepared films was evaluated to determine the biodegradability extent of the xylan based biofilms and to inspect any adverse effect of TiO_2 on biodegradation.

3.1 Xylan Biofilm Preparation

Food packaging is mainly concerned with protection and containment. Antimicrobial packaging is a system that can kill or inhibit the growth of microorganisms and thus extend the shelf life of perishable products and enhance the safety of packaged products (Han, 2000). So, antimicrobial xylan films were produced either by the coating with two different TiO_2 sol-gels or by addition of TiO_2 powder into the biofilm forming solutions.

The biofilms were prepared according to solvent casting method by a modified procedure of Gröndahl *et al*. (2004). The biofilms were casted from commercial birchwood xylan by the solubilization of 0.6 g (corresponding to 2.4% (w/v)) of birchwood xylan with the addition of 0.4 g (corresponding to

1.6% (w/v)) of xylitol as plasticizer, dissolved in 25 ml of ultra purified water and the biofilm solution was poured in a petri dish with a diameter of 9 cm.



(a) **(b)**

Figure 3.1 60% (w/w) (a) BWX, (b) CSX-50 films containing 40% (w/w) xylitol.

Karamanlıoğlu (2008) produced self-supporting films from cotton stalk xylan by dissolving 10 g xylan in ultra purified water at a concentration of 10% (w/v) for 8 h at room conditions and transferring 20 g of the film formation solution into a petri dish with a diameter of 9 cm. Then, the films were dried at ambient temperature of 25±5˚C and a relative humidity of 35±5 %. If we compare two methods, we can say that autoclaving the solution at 120°C for 20 min was shortened the time significantly for the solubilization of the polymers.

Moreover, it was observed that by performing the modified Gröndahl method, the xylan required for the formation of self-supporting biofilms (2.4% (w/v)) was dramatically lowered as compared to the xylan films (10% (w/v)) prepared by Karamanlıoğlu (2008).

3.2 Photocatalytic Antimicrobial Biofilm Formations from Xylan

3.2.1 Antimicrobial Xylan Biofilm Formation by Sol-gel Coating Methods

For photocatalytic antimicrobial biofilm production, Cotton stalk xylan (CSX-50) and Birchwood xylan (BWX) biofilms were chosen considering their difference of preparation methods and their base material. CSX-50 biofilms were obtained by extracting xylan from cotton stalk whereas BWX biofilms were obtained from commercial birchwood xylan.

The xylan based biofilms were prepared according to the procedure 2.4.1, but first, as the pH of the SiO_2 added and non-added TiO_2 sol-gels were 1.34 and 1.28, respectively, the films' pH were adjusted to 7.6±0.2 in order to prevent the effect of pH which can lead to inactivation of *E. coli* cells. Then the biofilms were coated with two different TiO_2 sol-gel procedures, aqueous-nitric acid and SiO_2 added aqueous-nitric acid used sol-gels. Then, their antimicrobial activities were tested against *E. coli* cells.

Particle size of the TiO_2 photocatalyst is an important property for the photocatalytic activity since the predominant way of electron-hole pair recombination may be different depending on the particle size. The variations on the particle size can lead to great modifications in the volume and electron-hole pair's recombination. So; the higher the crystallinity, the lower the particle size and the higher the surface area, the more active sites for photocatalysis (Guan *et al.* 2005). In order to gain high antimicrobial activity, high crystallization of TiO_2 should be obtained. Recent studies showed that the higher surface area is obtained by calcination of the TiO_2 coated substrates (Fujishima *et al*., 2008).

The calcination is the thermal treatment at high temperatures between 450° and 700˚C. As the biofilms can not be calcinated at those high temperatures due to

their low melting temperature, drying is performed as thermal treatment instead of calcination.

Coated biofilms were dried at different temperatures, 60°, 90° and 120°C, to find out the optimum drying temperature required to acquire higher crystallinity and more active sites for photocatalysis to achieve maximum antimicrobial property.

Although photocatalytic antimicrobial property has been observed and studied for a very long time on different inorganic substrates such as glass and ceramics, Karamanlıoğlu (2008), was the first who worked on biofilms. Antimicrobial activity of TiO_2 has been observed on coated wheat gluten films with 20% (w/w) glycerol content as plasticizer. The biofilms were dipped in the TiO_2 sol-gel for 30 min and dried at room conditions for 24 h and the next day, they were dried at 60°C for 1 h.

In this study, the drying temperatures were also chosen according to the effect of temperature treatment on the mechanical properties of the films. During the experiments, with increase in the temperature, the biofilms were observed to become very brittle and deformed.

In contrast to the studies of Karamanlıoğlu (2008), the CSX-50 and BWX biofilms could not be coated for 30 min but only for 10 sec due to the water solubility of the biofilms. This coating time difference must also be because of the type of the polymer (gluten vs xylan) and the preparation procedure of the wheat gluten films. The wheat gluten films were produced by dissolving 10 g of wheat gluten in distilled water (10% (w/w)) with the addition of the 2 g (20% (w/w)) of glycerol as plasticizer. However, in our case, the biofilms were casted by dissolving 0.6 g of xylan in distilled water (2.4 % (w/w)) with the addition of 0.4 g (2% (w/w)) of xylitol as plasticizer.

The CSX-50 and BWX biofilms, coated with aqueous-nitric acid and SiO_2 added aqueous-nitric acid sol-gels were tested against *E. coli* cells to observe their antimicrobial inactivation properties by illumination for 2 h using a black light source (General Electric) with a peak wavelength of 368 nm placed at 20 cm above the films. During the antimicrobial property tests, the notion of control groups is also very important. If the control groups are not observed and analyzed correctly, misleading results may be obtained since cell inactivation's occurring from only the light source or the inactivation occurring in the dark may be attributed to the photocatalytic effect.

For all heat treatments, the number of viable *E. coli* cells from the biofilms samples was counted on uncoated control biofilm samples by colony counting method. The results are shown in Figure 3.2, 3.3 and 3.4 for CSX-50 biofilms and in Figure 3.5, 3.6 and 3.7 for BWX biofilms.

The antimicrobial activities of CSX-50 biofilms coated with and without SiO_2 added TiO_2 sol-gels are given in Figure 3.2. The biofilms were dried at 60°C for 30 min after coating procedure.

Observation of Figure 3.2 indicates that considerable amount of *E. coli* cells were inactivated on the coated and uncoated biofilms kept in the dark. This *E. coli* cell death in CSX-50 biofilms used as control groups can be due to a decrease in pH of the biofilms during drying. While drying, the water vaporizes and results in a decrease in the pH value, so the biofilms become more acidic. The extraction method may also be another reason for the cells' inactivation. During drying, sodium borohydride ($NaBH_4$), used in the extraction process, was thought not to be evaporated, resulting in a decrease in acidity of the biofilms as well.

After considering the *E. coli* cell deaths at the control groups, a sharp decrease in the number of viable *E. coli* cells, obtained from the biofilms coated with and without SiO_2 added TiO_2 sol-gels was noticed where no such decrease was

seen for the uncoated biofilms. This is due to the fact that the two sol-gel coatings are photocatalytically active for the bacterial inactivation.

Taken into account the cells' deaths at the control groups, CSX-50 biofilms coated with SiO_2/TiO_2 sol-gel showed 52±1% microbial inactivation whereas 33±11% inactivation was observed for biofilms coated with non-SiO_2 added TiO_2 sol-gel.

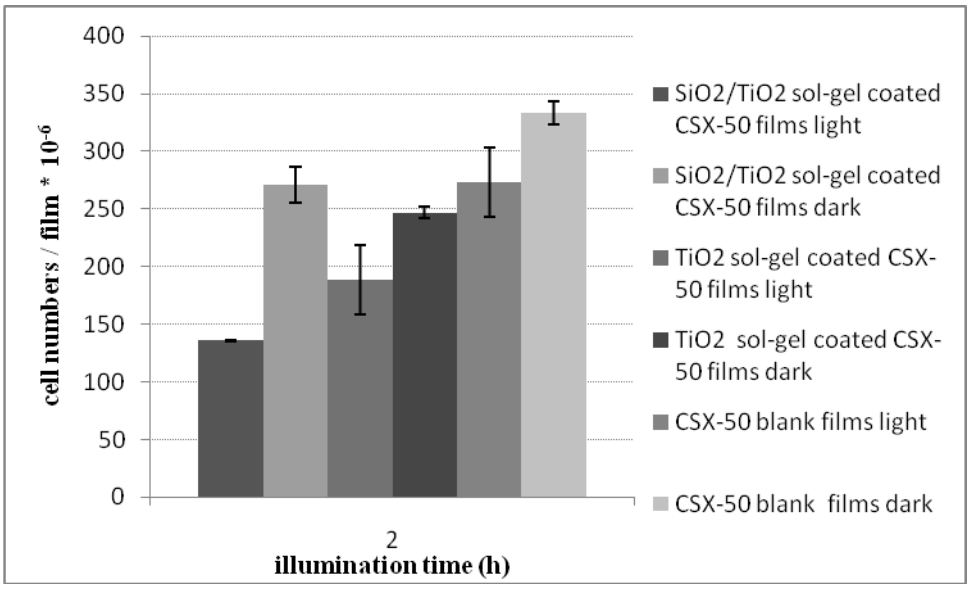


Figure 3.2 Antimicrobial property of the CSX-50 biofilms, dried at 60°C for 30 min.

Secondly, the antimicrobial activities of CSX-50 biofilms coated with and without SiO_2 added TiO_2 sol-gel are given in Figure 3.3. The biofilms were dried at 90°C for 30 min after coating procedure. The decrease in the viable *E. coli* cells' number from the coated films with two different sol-gels and from the uncoated blank biofilms kept in the dark was also observed as in the case of sol-gel coated CSX-50 biofilms dried at 60°C for 30 min. The photocatalytic activities of the TiO_2 sol-gels were recorded by comparing the *E. coli* cell death at the coated samples illuminated by the black light source and the samples kept in the dark.

After considering the cells' deaths at the control, it was deduced that biofilms coated with SiO_2/TiO_2 sol-gel were 61±3% photocatalytically active whereas 52±1% effectiveness was observed for the biofilms coated with non-SiO_2 added TiO_2 sol-gel.

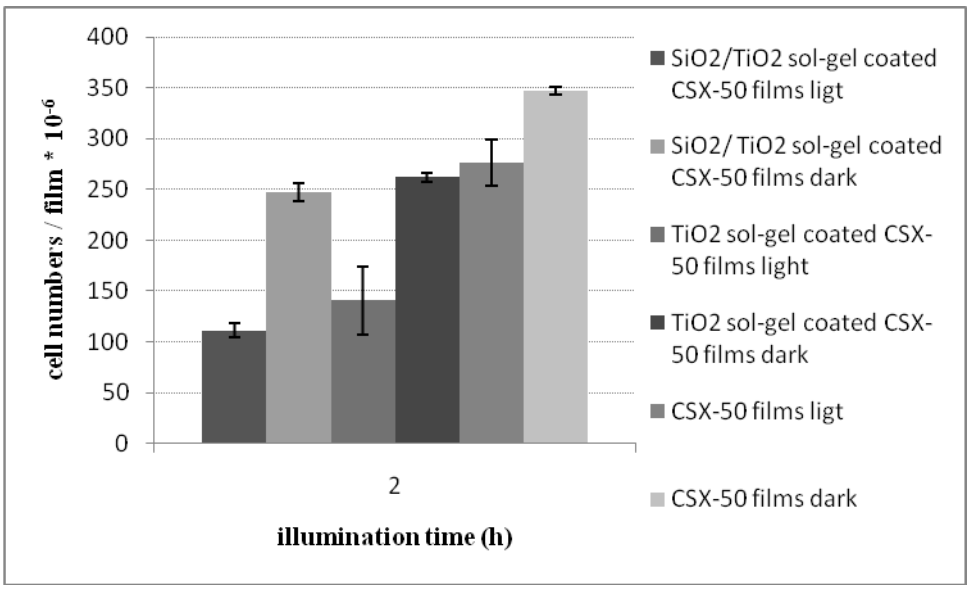


Figure 3.3 Antimicrobial property of the CSX-50 biofilms, dried at 90°C for 30 min.

Thirdly, the antimicrobial activities of CSX-50 biofilms coated with and without SiO_2 added TiO_2 sol-gels are given in Figure 3.4. In this case, the biofilms were dried at 120°C for 30 min after coating procedure. The photocatalytic activities of the TiO_2 sol-gels were again observed. Contrary to the CSX-50 biofilms dried at 60° and 90°C, it can be obviously seen that the number of viable *E. coli* cells from the coated (with two different sol-gels) and uncoated samples kept in the dark, was nearly same. It can be concluded that the death from the pH was prevented with increasing the drying temperature. In the case of SiO_2/TiO_2 sol-gel coated samples, the pH effect cannot be totally eliminated, but the *E. coli* cell death was prevented to a large extent. In this case, the number of viable *E. coli* cells from the coated with and with non-SiO_2

added TiO_2 sol-gel samples shown 75±2% and 63±2% photocatalytic microbial inactivation rates, respectively.

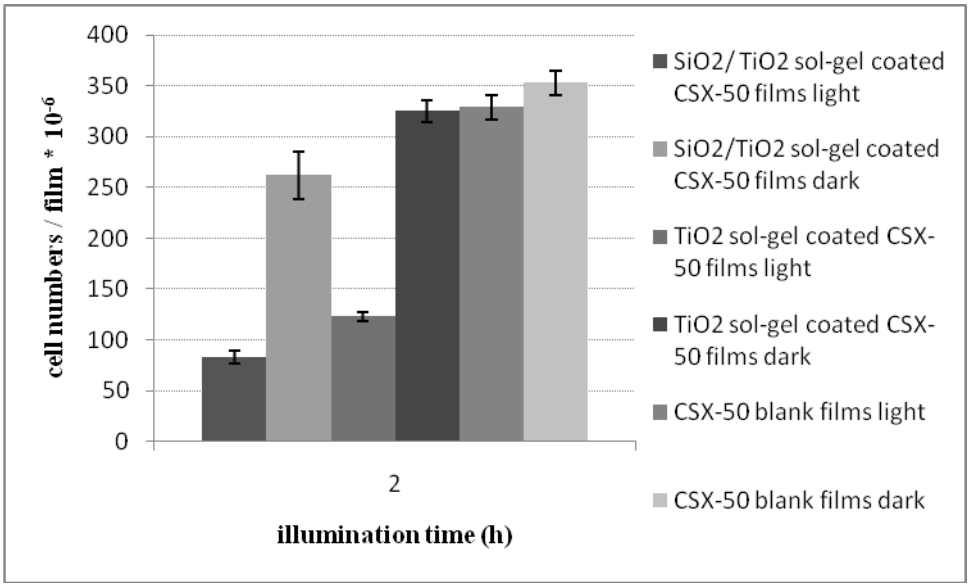


Figure 3.4 Antimicrobial property of the CSX-50 biofilms, dried at 120°C for 30 min.

In order to investigate the effect of xylan as base material used in the preparation of biofilms, the commercial birchwood xylan used biofilms were also coated with and without SiO_2 added TiO_2 sol-gels. The results can be observed in Figures 3.5, 3.6 and 3.7.

The antimicrobial activities of BWX biofilms coated with and without SiO_2 added TiO_2 sol-gel are given in Figure 3.5. The biofilms were dried at 60°C for 30 min after coating procedure. Here as well, the control groups are of prime importance. As in the case of sol-gel coated CSX-50 biofilms dried at 60°C for 30 min kept in the dark, the similar behavior of *E. coli* cell death at sol-gel coated BWX control biofilms was again noticed. However, no such decrease is observed for the uncoated blank BWX biofilms. So, it can be deduced that the

pH of the extraction of CSX-50 biofilms is effective to inactivate *E. coli* cells on control, uncoated samples.

The photocatalytic inactivation activities of the two sol-gels were achieved despite the pH effectiveness of the coated BWX biofilm samples.

Taken into account the cells' inactivations at the control groups, biofilms coated with SiO_2 added TiO_2 sol-gel showed 60±2% microbial inactivation whereas 52±2% inactivation was observed for the biofilms coated with non-SiO_2 added TiO_2 sol-gel.

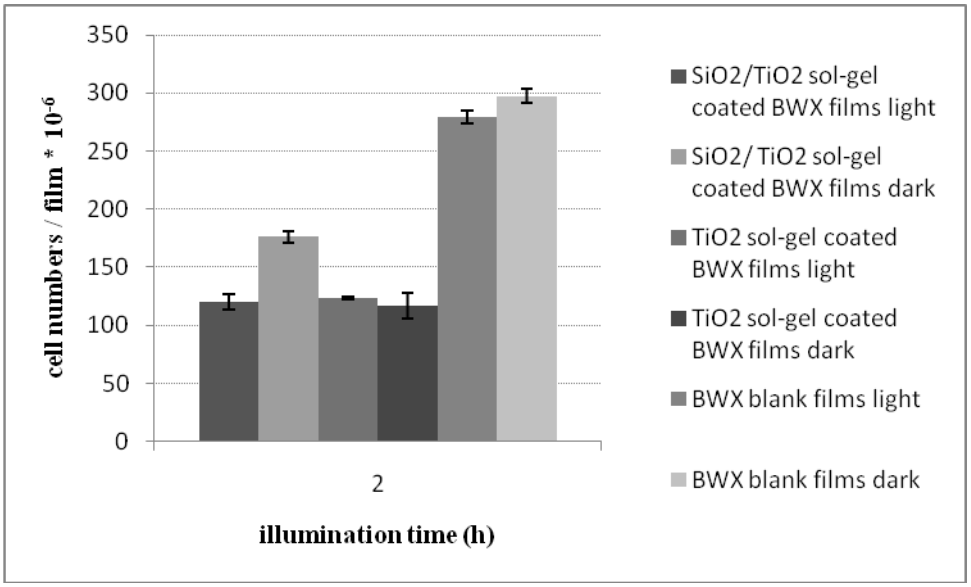


Figure 3.5 Antimicrobial property of the BWX biofilms, dried at 60°C for 30 min.

Secondly, the antimicrobial activities of BWX biofilms coated with and without SiO_2 added TiO_2 sol-gels are given in Figure 3.6. The biofilms were dried at 90°C for 30 min after coating procedure. The decrease in the number of *E. coli* cells was again noticed for the control non-SiO_2 added TiO_2 sol-gel samples kept in the dark. But, this behavior is not recorded neither for

SiO_2/ TiO_2 sol-gel coated samples nor for uncoated blank samples hold in the dark, so, the pH activity prevention is achieved.

The photocatalytic antimicrobial activities of the TiO_2 sol-gels were once more obtained by comparing the *E. coli* cell death at the coated samples illuminated by the black light source and the samples kept in the dark.

After taking into account the cells' inactivations at the control groups, it was seen that biofilms coated with SiO_2/TiO_2 sol-gel showed 64±4% microbial inactivation whereas 53±3% inactivation was observed for the biofilms coated with non-SiO_2 added TiO_2 sol-gel.

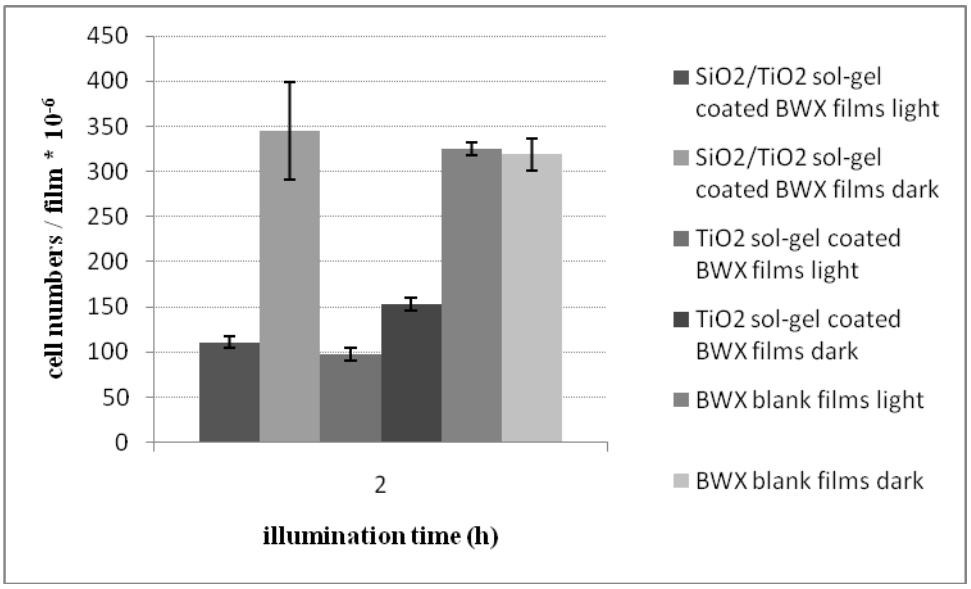


Figure 3.6 Antimicrobial property of the BWX biofilms, dried at 90°C for 30 min.

Thirdly, the antimicrobial activities of BWX biofilms coated with SiO_2 added TiO_2 sol-gel and without SiO_2 added TiO_2 sol-gel are given in Figure 3.7. In this case, the biofilms were dried at 120°C for 30 min after coating procedure. The photocatalytic activities of the TiO_2 sol-gels were again observed. In this

case, it can be obviously seen that the number of viable *E. coli* cells from the coated (with two different sol-gels) and uncoated samples kept in the dark, was nearly same. It can be concluded that the death from the pH was prevented with increasing drying temperature. Moreover, the number of viable *E. coli* cells from the SiO_2/TiO_2 sol-gel coated samples shown 88±1% microbial inactivation whereas 63±3% inactivation was observed for the non-SiO_2 added TiO_2 sol-gel coated samples.

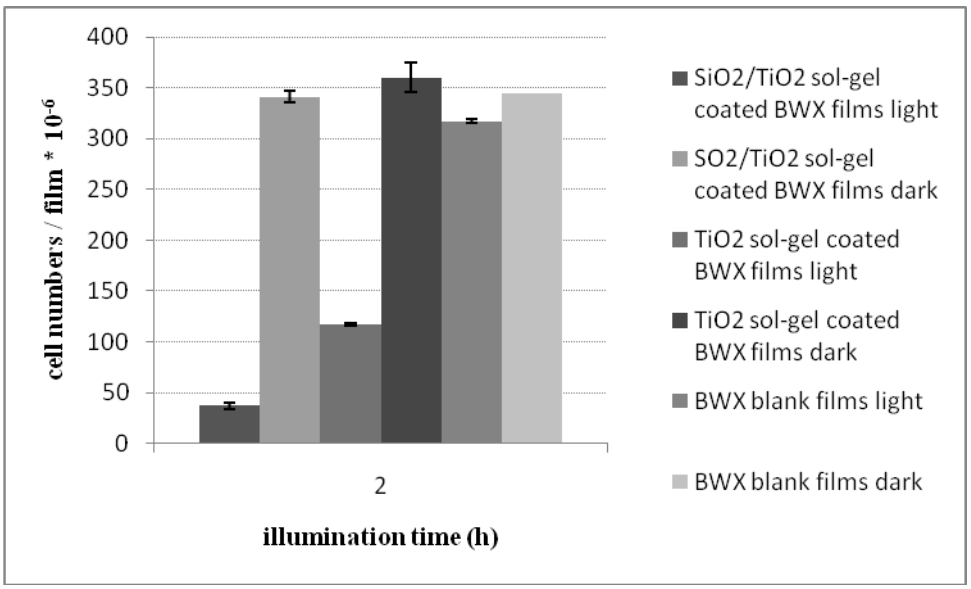


Figure 3.7 Antimicrobial property of the BWX biofilms, dried at 120°C for 30 min.

Considering the effect of the different drying temperatures on the prevention of *E. coli* inactivation from the pH of the sol-gels, the optimum heat treatment temperature is found to be 120°C for both CSX-50 and BWX biofilms.

Higher photocatalytic inactivation rates were acquired with the addition of the SiO_2 into the TiO_2 sol-gel. SiO_2 addition might cause three main changes in the titania, i.e. anatase phase stability, enhanced acidity of the film and increase in surface area.

Anatase titania has been reported to be more photocatalytically active than the rutile form (Stafford *et al*., 1993) mainly due to the structural differences of anatase and rutile. Rutile titania has a much lower specific surface area compared to that of anatase. As the specific surface area of the catalyst increases it can adsorb more water molecules. By the addition of SiO_2, the absorption of more water molecules on the surface of the anatase titania was increased, leading to higher photocatalytic activities.

Guan *et al*. (2003) and Fu *et al*. (1996) have reported that the addition of SiO_2 in TiO_2 film enhance the acidity of the composite oxide. The enhanced acidity of the film is the main reason to improve the photocatalysis. The surface with improved acidity can adsorb more OH radicals. Silicon cations, capturing tightly OH^- of adsorbed H_2O molecules, and O^{+2}, on the other hand, can easily bind with H^+ of adsorbed H_2O molecules. So, the composite surface can adsorb more hydroxyl radicals than pure TiO_2 surface. As a result, the improved acidity in photocatalysis of the SiO_2/TiO_2 composite is obtained from the increased hydroxyl groups.

According to the studies of Guillard *et al*. (1999), one of the most important factors in achieving a high efficiency in the photocatalytic activity was found to have larger surface area. TiO_2 has a tendency to have higher photocatalytic activity when supported on porous solids due to the increase in the effective surface area which is helpful to enhance surface reaction on TiO_2 (Guillard *et al*., 1999; Yamashita *et al*, 1996). The oxidation reactions (which are effectiveness due to the surface limited reactions) are improved as a result of the high surface area to volume ratios (Zhao, 2003). As a result, photogenerated pairs of electrons and holes can easily diffuse to the surface of the catalysts and form the active sites for the oxidation/reduction reactions. SiO_2 supported TiO_2 sol-gel catalysts possess larger surface areas which allow efficient UV radiation of all catalysts particles (Zhao, 2003). The studies of Aguado *et al* (2002) on the removal of free cyanide; Tanaka *et al* (2002) on the photodegradation of phenol, have found that the presence of SiO_2 has

beneficial effect by generation of new active sites due to the interactions between titania and silica.

The enhanced photocatalytic antimicrobial effect of SiO_2/TiO_2 sol-gel coated xylan based biofilms was confirmed by the results of the experiments.

3.2.2 Antimicrobial Xylan Film Formation by TiO_2 Powder Addition

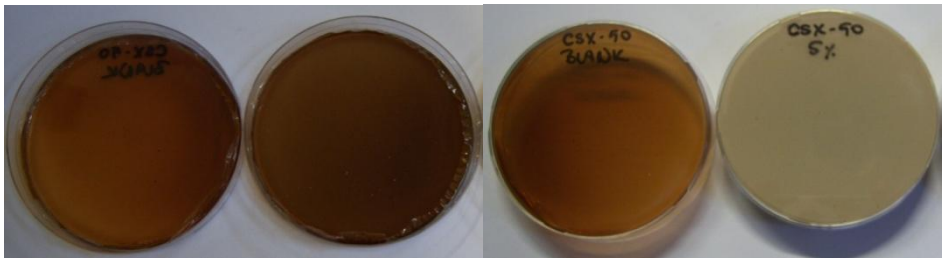
For photocatalytic antimicrobial biofilm production, CSX-50 and BWX biofilms were chosen considering their difference of preparation methods and their base materials. CSX-50 biofilms were obtained by extracting xylan from cotton stalk whereas BWX biofilms were obtained from commercial birchwood xylan.

TiO_2 powder was used to give antimicrobial property to xylan based biofilms because of its strong oxidation/reduction property which results in the oxidation of all organic compounds including cells such as microorganisms.

TiO_2 powder was added to the biofilms with various quantities and their antimicrobial properties were tested with respect to different time scales.

During the preparation of biofilms, when the biofilms were dried, TiO_2 powder precipitated at the bottom surface of the petri dishes in which biofilm solution was casted, so two different colored sided, non-homogenous biofilms were formed. The top and bottom surfaces of the produced CSX-50 biofilms are shown in Figure 3.8.

The antimicrobial photocatalytic activity tests were conducted on the bottom, white colored sides of the biofilms because the white sides were thought to have higher concentration of TiO_2 photocatalyst.



(a) **(b)** **(c)** **(d)**

Figure 3.8 CSX-50 biofilms (a) top surface of blank biofilm, (b) top surface of the 5% TiO_2 powder added biofilm, (c) bottom surface of the blank biofilm, (d) bottom surface of the 5% TiO_2 powder added biofilm.

First, the effect of TiO_2 powder concentration added in the CSX-50 biofilms' solution was tested with respect to different time scales' illumination with black light source. The ratio of TiO_2 to dry matter, which was composed of xylan and xylitol, was 1:100, 2.5:100 and 5:100 (w/w). The results are given in Figures 3.9, 3.10 and 3.11, respectively.

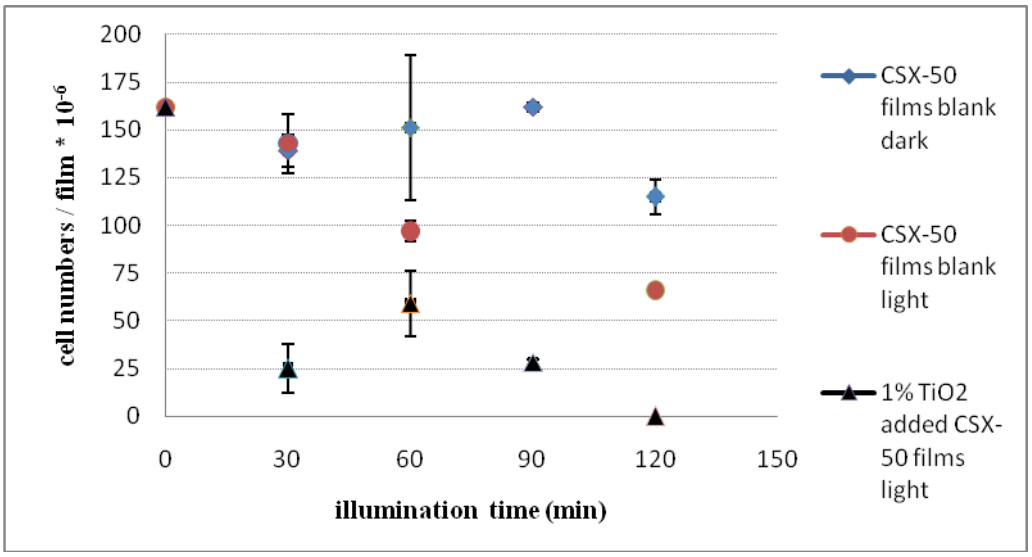


Figure 3.9 The effect antimicrobial photocatalytic activity of 1% (w/w) TiO_2 powder addition on CSX-50 biofilms at 30-60-90-120 min illumination.

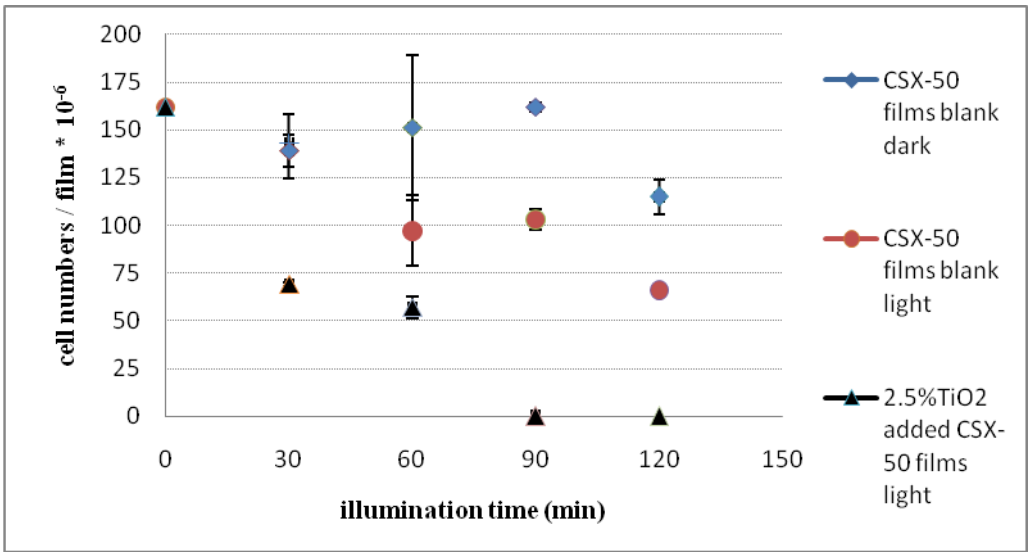


Figure 3.10 The effect antimicrobial photocatalytic activity of 2.5% (w/w) TiO_2 powder addition on CSX-50 biofilms at 30-60-90-120 min illumination.

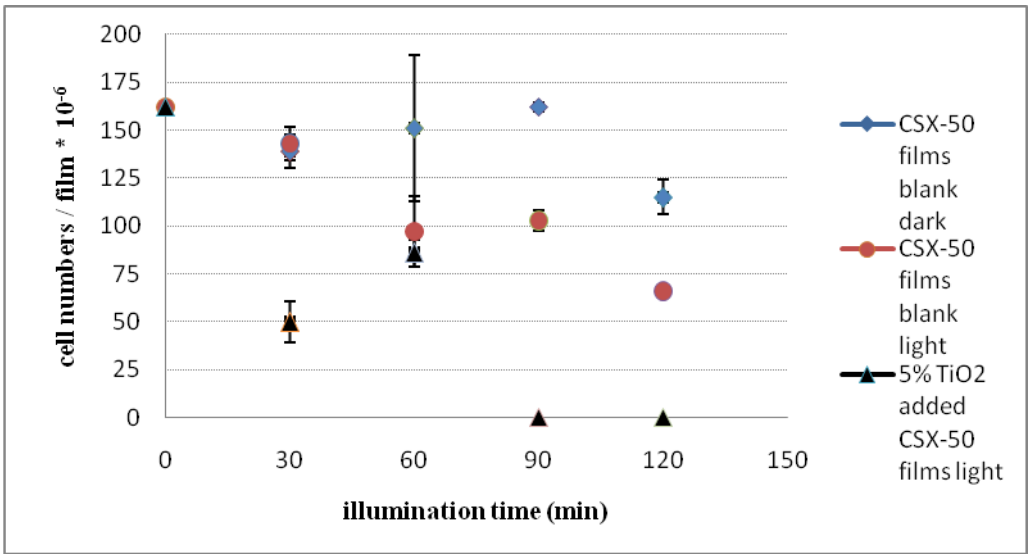


Figure 3.11 The effect antimicrobial photocatalytic activity of 5% (w/w) TiO_2 powder addition on CSX-50 biofilms at 30-60-90-120 min illumination.

According to the experimental results, with increase in the TiO_2 powder concentration, higher antimicrobial photocatalytic activation was achieved. With 2.5% (w/w) and 5% (w/w) addition of the TiO_2 powder, complete

inactivation of the microorganisms was recorded at the end of 90 min illumination under black light source. The effect of different TiO_2 powder concentration used in CSX-50 biofilms on the photocatalytic inactivation of *E. coli* cells for 30, 60, 90 and 120 min illumination times are summarized as percent inactivation rates in Tables 3.1, 3.2, 3.3 and 3.4, respectively.

Table 3.1 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of CSX-50 biofilms against *E. coli* for 30 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	30	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	CSX-50 blank biofilms kept at dark	14±5	14±5	14±5
	CSX-50 blank biofilms at light	13±2	13±2	13±2
	TiO_2 added CSX-50 biofilms at light	84±8	57±1	68±4

Table 3.2 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of CSX-50 biofilms against *E. coli* for 60 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	60	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	CSX-50 blank biofilms kept at dark	7±4	7±4	7±4
	CSX-50 blank biofilms at light	39±11	39±11	39±11
	TiO_2 added CSX-50 biofilms at light	64±9	64±3	47±5

Table 3.3 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of CSX-50 biofilms against *E. coli* for 90 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	90	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	CSX-50 blank biofilms kept at dark	0	0	0
	CSX-50 blank biofilms at light	37±3	37±3	37±3
	TiO_2 added CSX-50 biofilms at light	82±1	100	100

Table 3.4 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of CSX-50 biofilms against *E. coli* for 120 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	120	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	CSX-50 blank biofilms kept at dark	29±6	29±6	29±6
	CSX-50 blank biofilms at light	59±1	59±1	59±1
	TiO_2 added CSX-50 biofilms at light	100	100	100

Secondly, the effect of TiO_2 powder concentration added in the commercial BWX biofilms' solution was tested with respect to different time scales' illumination with black light source. The ratio of TiO_2 to dry matter, which was composed of BWX and xylitol, was 1:100, 2.5:100 and 5:100 (w/w) as in the case of CSX-50 biofilms. The results are given in Figures 3.12, 3.13 and 3.14, respectively.

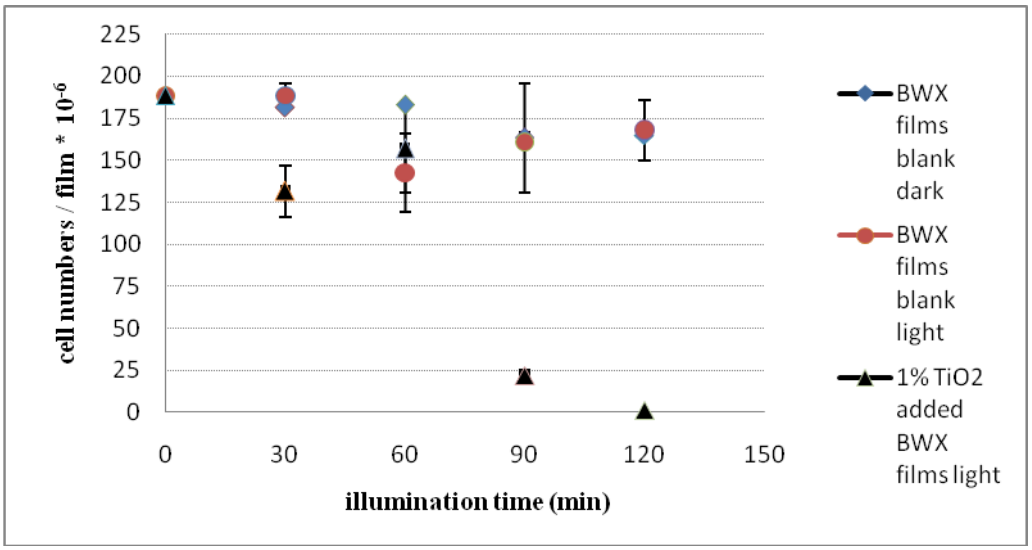


Figure 3.12 The effect antimicrobial photocatalytic activity of 1% (w/w) TiO_2 powder addition on BWX biofilms at 30-60-90-120 min illumination.

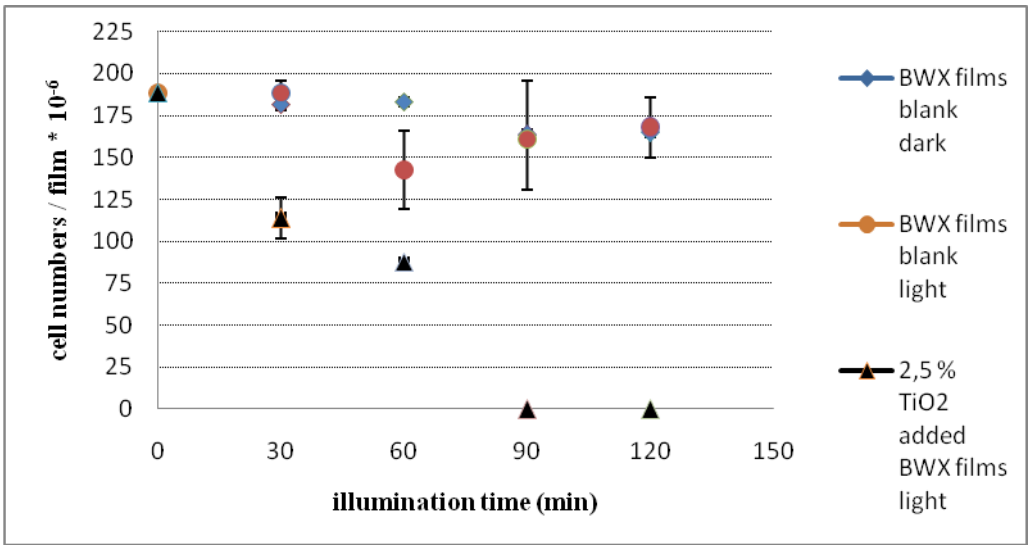


Figure 3.13 The effect antimicrobial photocatalytic activity of 2.5% (w/w) TiO_2 powder addition on BWX biofilms at 30-60-90-120 min illumination.

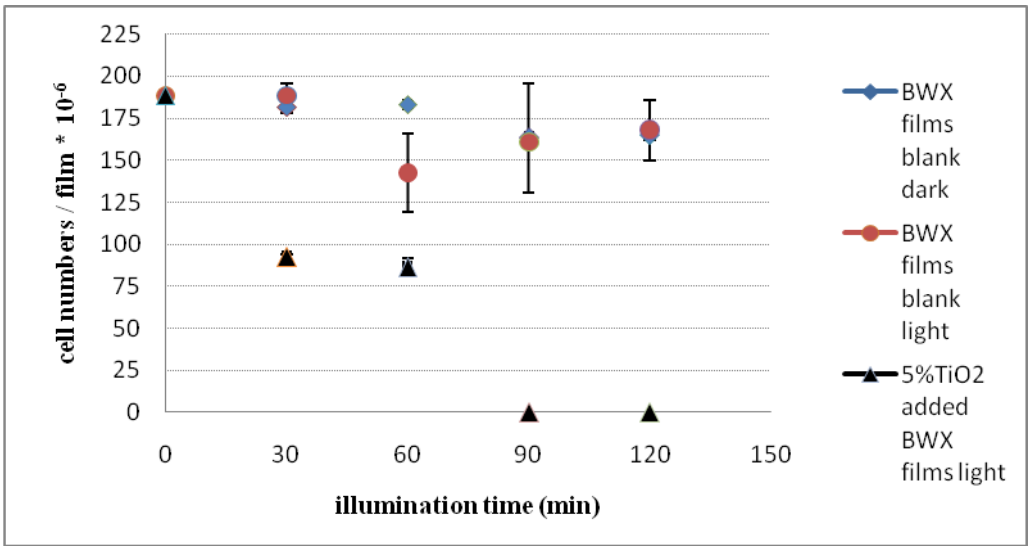


Figure 3.14 The effect antimicrobial photocatalytic activity of 5% (w/w) TiO_2 powder addition on BWX biofilms at 30-60-90-120 min illumination.

As it can be deduced, with increase in the TiO_2 powder concentration, higher antimicrobial photocatalytic activation was achieved as in the case of TiO_2 powder added CSX-50 biofilms. Like CSX-50 biofilms, with 2.5% (w/w) and 5% (w/w) addition of the TiO_2 powder, complete inactivation of the microorganisms was recorded at the end of 90 min illumination under black light source. The effect of different TiO_2 powder concentration used in BWX biofilms on the photocatalytic inactivation of *E. coli* cells for 30, 60, 90 and 120 min illumination times are given as percent inactivation rates in Tables 3.5, 3.6, 3.7 and 3.8, respectively.

Table 3.5 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of BWX biofilms against *E. coli* for 30 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	30	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	BWX blank biofilms kept at dark	3±2	3±2	3±2
	BWX blank biofilms at light	0	0	0
	TiO_2 added BWX biofilms at light	30±8	39±6	51±1

Table 3.6 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of BWX biofilms against *E. coli* for 60 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	60	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	BWX blank biofilms kept at dark	2±1	2±1	2±1
	BWX blank biofilms at light	23±12	23±12	23±12
	TiO_2 added BWX biofilms at light	17±5	53±1	54±3

Table 3.7 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of BWX biofilms against *E. coli* for 90 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	90	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	BWX blank biofilms kept at dark	11±5	11±5	11±5
	BWX blank biofilms at light	14±2	14±2	14±2
	TiO_2 added BWX biofilms at light	88±2	100	100

Table 3.8 The effect of TiO_2 powder concentration on the antimicrobial photocatalytic activity of BWX biofilms against *E. coli* for 120 min illumination.

	Illumination time (min)	**Concentration of TiO_2 powder**		
	120	**1% (w/w)**	**2.5% (w/w)**	**5% (w/w)**
% microbial inactivation	BWX blank biofilms kept at dark	12±2	12±2	12±2
	BWX blank biofilms at light	12±2	12±2	12±2
	TiO_2 added BWX biofilms at light	100	100	100

The experimental results indicate that a significant amount of photocatalytic antimicrobial activity is present for each xylan based biofilms. *E. coli* cells' inactivation in CSX-50 blank biofilms which were illuminated and used as control groups was once more observed as in the previous experiments with sol-gels coated biofilms. The reason, as discussed before, was thought to be due to the decrease in pH of the biofilms during drying. While drying, the water vaporized and resulted in a decrease in the pH value, so the biofilms became more acidic. However, this behavior was not noticed when the blank BWX biofilms were antimicrobially tested against *E. coli* cells. This might also be due to the extraction method. During drying, $NaBH_4$, used in the extraction process, was thought to be not evaporated, resulting in a decrease in the acidity of the biofilms and as BWX biofilms were prepared by using commercial birchwood xylan, there is no pH effect to inactivate *E. coli* cells. So, the tests were repeated by adjusting the pH of biofilm forming solutions at 7.5±0.5, with 5% (w/w) TiO_2 powder addition and 2 h illumination. The results were shown in Figures 3.15 and 3.16.

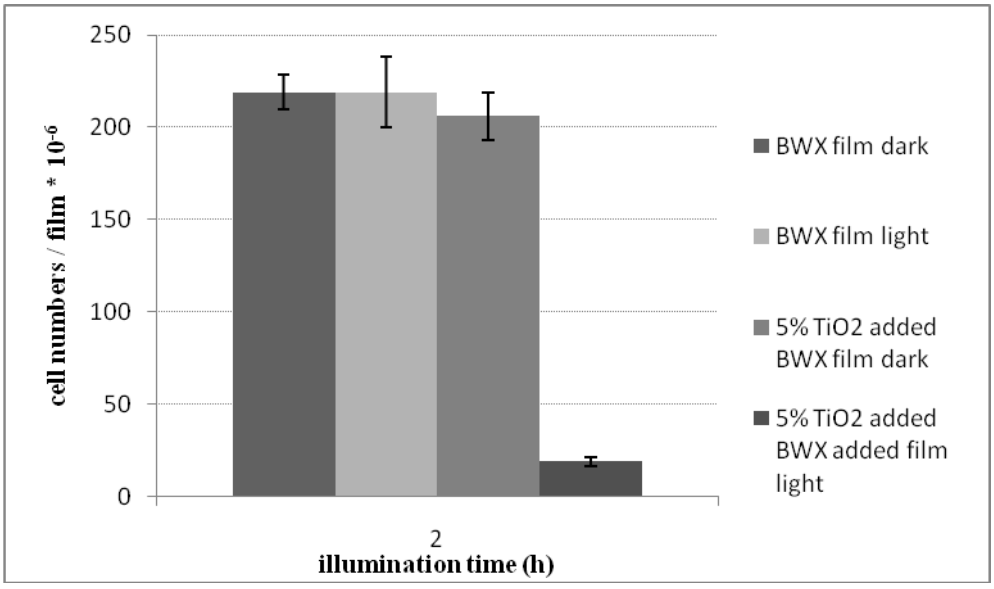


Figure 3.15 Viable *E. coli* cells units from 5% (w/w) TiO_2 powder added BWX biofilms at 2 h illumination.

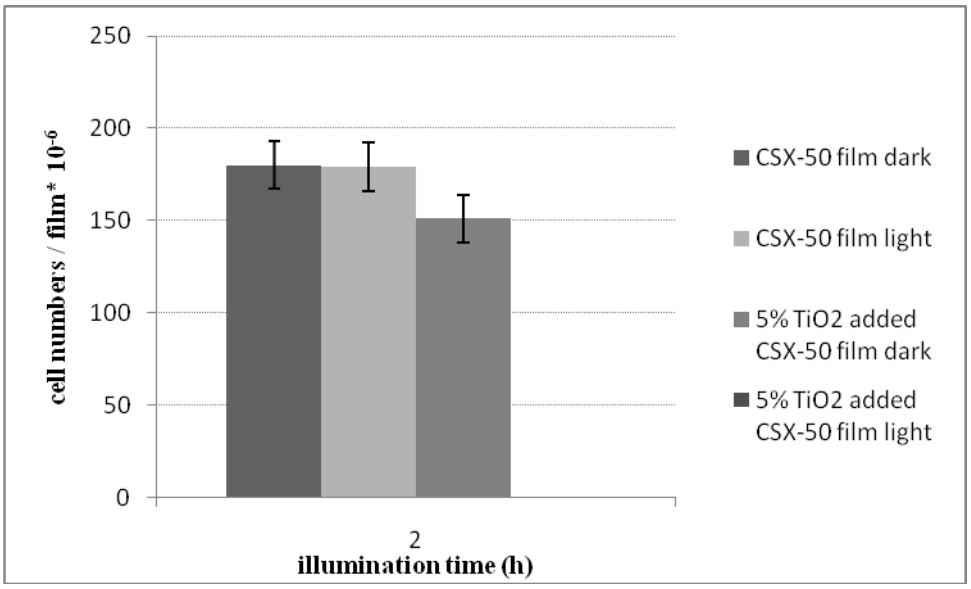


Figure 3.16 Viable *E. coli* cells units from 5% (w/w) TiO_2 powder added CSX-50 biofilms at 2 h illumination.

Comparison of TiO_2 powder added and non added biofilm samples which were both exposed to the black light source has showed that there is an obvious decrease in the survival rate of the *E. coli* cells taken from the TiO_2 powder added biofilms, whereas no such trend could be observed from the non-TiO_2-powder added control biofilms. Moreover, the *E. coli* cells survival ratio from the TiO_2 powder added biofilms and non-added biofilms which were kept in the dark and used as control groups yielded no microbial inactivation. So, it can be concluded that the biofilms containing TiO_2 powder show antimicrobial photocatalytic activity when exposed to the black light source.

BWX biofilms' initial number of *E. coli* cells was 219±19 cfu/200 ml. At the end of 2 h illumination under black light source, 5% TiO_2 powder added BWX biofilms with 2 parallels had as colony number of viable cells 19±3 cfu/200ml. Also, CSX-50 biofilms' initial number of *E. coli* cells was 179±13 cfu/200ml and as BWX biofilms, after 2 h illumination under black light source, 5% TiO_2 powder added CSX-50 biofilms with 2 parallels had as colony number of

viable cells as 0. The experiment's results showed that the photocatalytic antimicrobial effect of TiO_2 is obvious.

3.3 Biodegradation of Cotton Stalk Xylan Films

Food packaging materials which are mainly produced from petro-chemical based compounds, are leading to serious environmental and ecological problems due to their non-biodegradability. As, the consumers' awareness toward eco-friendly food packaging rises, it imposes the food packaging industry to find new solutions by focusing on the study of bio-based materials. In order to give an approach, xylan, as a natural compound, is used to produce biofilms in our researches. CSX-50 and BWX biofilm were produced to evaluate their extent of biodegradability and mineralization compared to cellulose; a naturally biodegradable compound used as positive control and LDPE; a non-biodegradable polymer used as negative control.

As stated by the ASTM D 6400-04, standard specification for compostable plastics, a biodegradable plastic is a degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae. According to the ASTM D 6400-04, the biodegradation is specified as follows;

• For homopolymers, 60% of the test sample must be converted into carbon dioxide in 180 days or less.

• For all other polymers, 90% of the test sample must be converted into carbon dioxide in 180 days or less.

The CO_2 measurement is the basis of the biodegradability testing methods used for the determination of the aerobic biodegradation in soil, as a matrix and source of inoculums, of plastic materials or residual materials after composting

according to the ASTM standards. The simple method for the CO_2 determination was the measurement of CO_2 in biodegradation experiments of organic matters by the absorption of the gas in alkaline solutions. Usually $Ba(OH)_2$ as driven by following CO_2-free air in the test cultures tightly connected with alkaline solution is used as absorber. However, in this case, previous studies shown that not all the CO_2 produced may be quantitatively entrapped, leading to the underestimation and errors at risk of accumulation in repeated determinations (Chiellini, 2003)

In our testing method, an easier procedure was conducted. Soil was reduced to a minimal required amount by partially replacing it with an inert material from horticultural application, perlite, because large amount of the CO_2 deriving from the incubation media (soil and mature compost) may affect the accuracy of the determination of CO_2 amount developed by the microbial assimilation of the test materials, since it was seen from the previous studies that, to increase the replacement of relatively large amount of natural solid matrix with inert substrates such as perlite and vermiculite was found to be more effective (Chiellini, 2003).

Evaluation of biodegradable extend within the time were carried out under equilibrium conditions by absorption in alkaline solution KOH which is directly placed inside the tightly closed test vessel. So, the developed CO_2 was determined by back titration of the excess base. This practice guarantees for precise appreciation of the CO_2 production from the background emission also in the presence of the test materials displaying low-moderate rate of degradation (Chiellini, 2003).

For the biodegradability evaluation of xylan based biofilms; first, xylitol plasticized BWX biofilms, 10% TiO_2 powder added CSX-50 biofilms and as control samples, non-TiO_2 powder added CSX-50 biofilms were prepared.

CO_2 evolution from the films was observed for 180 days to find the biodegradability degree of our samples. The CO_2 evolutions were compared with the samples of autoclaved soil and cotton-stalk, lignin; cellulose as standard positive control and LDPE as standard negative control according to the testing method in Chapter 2, section 2.6. The results are given in Figure 3.17, 3.18 and 3.19.

In the Figure 3.17, the biodegradability extent of cellulose, as a positive control, LDPE, as negative control, cotton stalk and lignin are compared.

LDPE, mainly used as raw material for most of the food packaging is found to be not biodegradable at all due to its petro-chemical structure.

The mineralization profiles of samples recorded during 180 days of incubation are reported in Figure 3.17 along with the biodegradation rate of cellulose used as positive reference material. The incubation condition appeared to be satisfactory in terms of growth conditions of microorganisms during the experiment, as revealed by the high level of CO_2 production from the positive control (cellulose) after 180 days of incubation. Cellulose has shown 100% mineralization at the end of 180 days.

Cotton stalk, the agricultural biowaste from which the CSX-50 was extracted, was shown nearly the same mineralization rate with cellulose. At the end of 180 days of incubation, 98% mineralization was achieved.

The overall level of mineralization reached by lignin was quite below that comparably reached by cotton stalk and cellulose. Lignin, being a naturally occurring compound found mainly in the secondary cell wall of plants, is an amorphous, water-insoluble, three dimensional aliphatic and aromatic polymers. As the capacity of microorganisms to assimilate organic matter depends on their availability to produce the enzymes needed for degradation of

the substrates, the more complex the structure of the substrate, the more extensive and comprehensive is the enzyme system required. Because of its three-dimensional structure of aliphatic and aromatic constituents with very high molecular weight, lignin cannot be degraded by microorganisms which do not have a complex enzyme system. Moreover, microorganisms are able to use organic molecules which dissolve in water. So, being a water insoluble compound, lignin usage by microorganisms is limited. Moreover, the lignin degradable microorganisms are limited. Lignin degrading fungi known as white-rot fungi are capable of decomposing lignin in faster rate than that of any other organisms and are responsible for most of the lignin decomposition in nature (Tuomela *et al.* 2000). Lignin degrading bacteria like actinomycetes and eubacteria are also known to solubilize and modify the lignin structure extensively, but their ability to mineralize lignin is limited (Buswell and Odier, 1987). As soil used as incubation media, the naturally occurring microorganisms are limited which resulted in a slow rate of lignin degradation in our experiment.

Also, recent studies showed that lignin have a certain degree of degradation under composting conditions. The lignin degradation of plant residues which has been studied in the composting environment are summarized in Table 3.9.

The slow biodegradation of lignin is also noticed in our experiment showing only 31% mineralization at the end of 180 days. The results were also in accordance with the recent studies of the lignin degradation of plant residues, summarized in Table 3.9.

Table 3.9 Studies in which lignin degradation during composting or in soil has been reported (Tuomela *et al*. 2000).

Waste	Time/d	Compost/soil	Original lignin-content/%	Degraded lignin/%	Note	Analyzig method of lignin	Ref.
Ryegrass straw	45	Compost, 140 ml	12.15	7	25°C	Modified Klason lignin	Horwath and Elliott (1996)
				27	30 d 50°C 15 d 25°C		
Horse manure, wheat straw	47	Compost, 600 g	20.5	26	28°C	–	Waksman et al. (1939b)
				43	50°C		
				33	65°C		
				12	75°C		
Olive mill waste water, wheat straw	140	Compost, pile	–	70	Prolonged thermophilic phase of 35 d	Modified Klason lignin	Tomati et al. (1995)
Canola residue (*Brassica campestris*)	154	Soil (sample in bags)	11.5	17	Buried	Acid detergent	Franzluebbers et al. (1996)
				17	Not buried		
Wheat leaves	224	Soil (sample in bags)	5.92	99.2	Buried	H_2SO_4	Robinson et al. (1994)

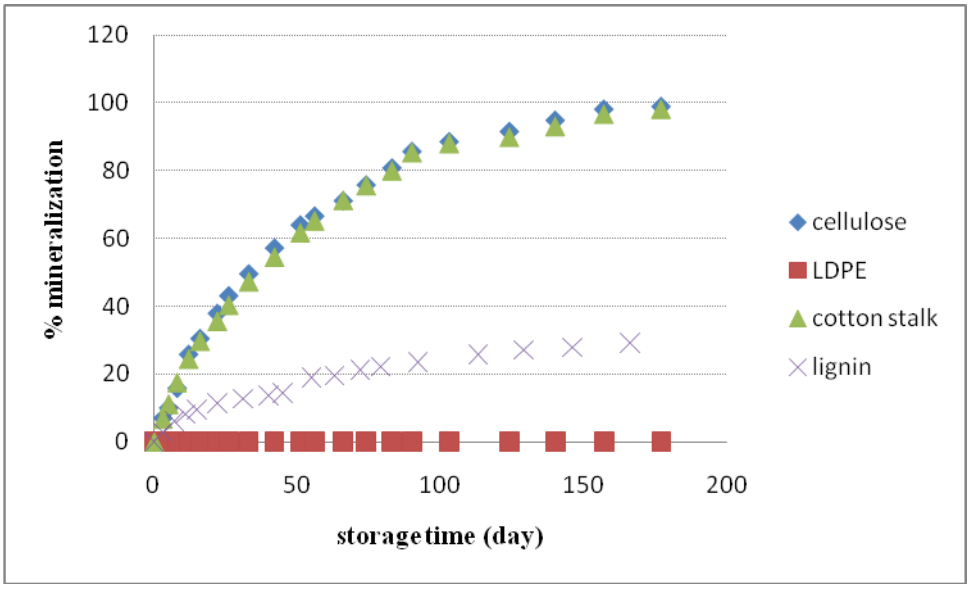


Figure 3.17 The mineralization rate of cellulose, LDPE, cotton stalk and lignin samples buried in soil containing air tight vessels.

Cotton stalk, LDPE, as negative control, autoclaved soil and cotton stalk and twice autoclaved soil and cotton stalk were compared for their biodegradability extent in Figure 3.18.

To investigate the effects of naturally occurring microorganisms in the biodegradability evolution of samples, autoclaving was performed at 121° C for 20 min for soil and cotton stalk. Autoclaving uses moist heat and pressure to kill soil organisms and typically results in a decrease in microbial biomass and microbially mediated metabolism, such as enzyme activity (Tiwari *et al*. 1988). Also, another sample was autoclaved at 121°C for 30 min, left in the room conditions until it is cooled to room temperature, then consecutively, re-autoclaved at 121°C for 20 min. According to the results, it was seen that autoclaving reduced the mineralization rate of cotton stalk due to the death of naturally occurring microbial biomass in the soil.

The once autoclaved sample buried in autoclaved soil reached 96% mineralization whereas non-autoclaved cotton stalk sample shown 98% mineralization at the end of 180 days of incubation.

By comparing the once and twice autoclaved soil and cotton stalk samples, it can be deduced that the sterilization was found to be effective in terms of destruction of microorganisms. However, complete sterilization cannot be achieved which was seen obviously from the biodegradation test results. Twice autoclaved soil and cotton stalk was found to be 80% biodegradable at the end of 180 days whereas once autoclaved soil and cotton stalk yielded 96% biodegradability.

The microbial activity on biodegradability was started after a lag phase of 1 week when microbial proliferation has thought to be started. After one time autoclaving, the sample was allowed to cool to the room temperature for a while and re-autoclaved. However, complete inactivation could not be reached

as seen for the once autoclaved sample. The heat treatment effect can be clearly seen by the biodegradability test results.

One of the reasons of the biodegradability yield in the autoclaved samples might be the enzyme activity which could remain active after autoclaving process. Although phosphatase is found to remain stable in extreme environments (Kiss *et al.* 1975, Tibett, 2002), the stability of phoshodiesterase during the autoclave-induced death of the active microbial biomass is a unique finding. Cation exchange capacity, organic matter content and pH are among the soil properties which might have contributed to enzyme stability (Tate, 2002). The observed stability might likely due to the formation of clay-phosphodiesterase complexe where the enzyme or its active sites were protected due to the adsorption onto internal and external surfaces of organic matter (Boyd and Mortland, 1990). Thus, the cation exchange and organic matter content of the soil likely stabilized the three-dimensional structure of phoshodiesterase or enabled it to refold after heat treatment. Although the mechanism of the phosphodiesterase refolding is not known in details, the current results show that the phosphodieseterase activity might contribute to the biodegradation of the cotton stalk. Another reason for the biodegradability rate recorded for autoclaved samples might be due to an accumulation of stable extracellular enzyme secreted by a number of microbial generations (Peterson and Astaf'eva, 1962). According to the studies of Kiss *et al.* (1975), accumulated enzymes were found to play an important role in nutrient cycling during the early stages of decomposition and in conditions unfavorable for the microbial proliferation. The soil used as incubation media in our experiments might serve to protect the enzyme from degradation during autoclaving process. Further studies on activities of the extracellular enzyme after heat treatment are needed to understand better their activities on the biodegradation process.

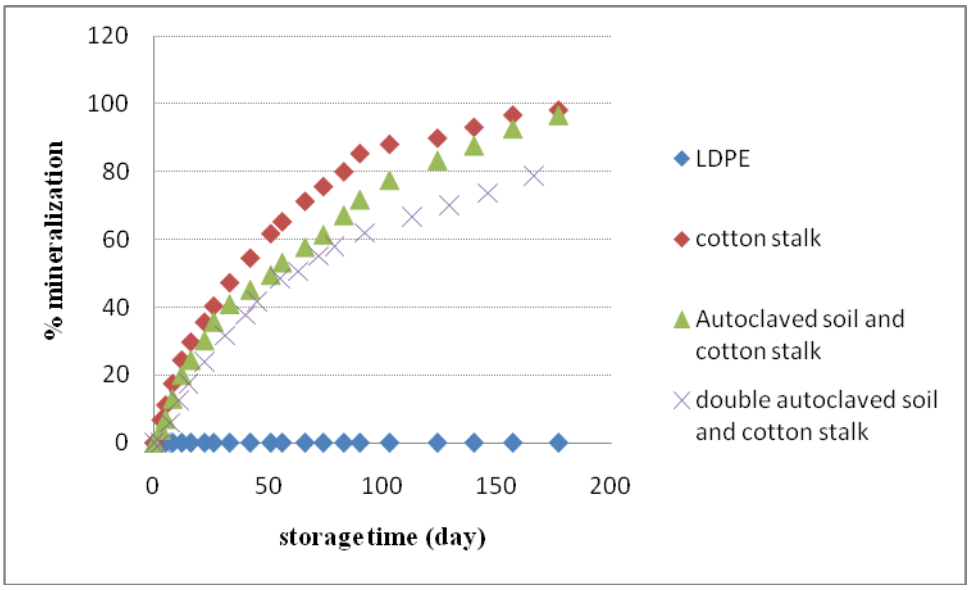


Figure 3.18 The mineralization rate of cellulose, LDPE, cotton stalk, autoclaved soil and cotton stalk and double autoclaved soil and cotton stalk samples buried in soil containing air tight vessels.

Lastly, LDPE, as negative control, commercial birchwood based xylan blank biofilms, extracted cotton stalk xylan based blank biofilms and 10% (w/w) TiO_2 added CSX-50 biofilms were evaluated to obtain their biodegradability extent and the results are shown in Figure 3.19.

The effect of TiO_2 powder addition to the CSX-50 biofilms on the biodegradation evolution was investigated. According to the test results, TiO_2 powder added CSX-50 biofilms were found to be 91% biodegradable where non TiO_2 added CSX-50 biofilms was found to be 93% biodegradable.

Moreover the effect of the difference of xylan based materials was also investigated by comparing the degree of mineralization of BWX blank biofilms and CSX-50 blank biofilms. According to the results, BWX blank biofilms were found to be 95% biodegradable where CSX-50 blank biofilms were found to be 93%. BWX biofilms were shown quite few high mineralization rates when compared with the CSX-50 biofilms' mineralization rates results. This

may be due to the TiO_2 powder added to the CSX-50 biofilms leading to prevent the enzymatic activity of naturally occurring microbial biomass found in soil.

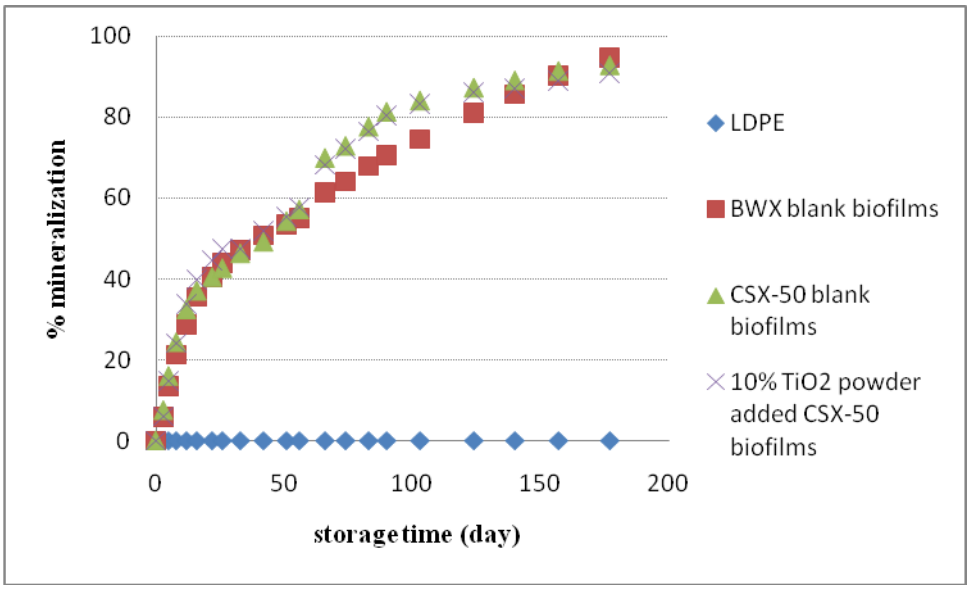


Figure 3.19 The mineralization rate of cellulose, LDPE, BWX blank biofilm, CSX-50 blank biofilm and 10% TiO_2 powder added CSX-50 biofilm samples buried in soil containing air tight vessels.

The mineralization rate in soil confirmed a fast mineralization for all samples as well as for biofilms. After 120 days, all the biofilms reached a significant amount of mineralization and appeared to be biodegradable according to the ASTM D 6400-04 standard.

As it can be concluded from the experimental results, the mineralization rate, observed on all test materials, allow considering the materials eco-compatible and valuable to be introduced on the market as replacement of non-biodegradable materials which cause growing ecological pollution.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In the first part of the study, antimicrobial xylan based biofilms were prepared either by coating the films with TiO_2 sol-gels or by adding different amounts of TiO_2 powder into the film forming solutions. SiO_2/TiO_2 coated biofilms yielded more photocatalytic activity then TiO_2 coated samples. In order to prevent the inactivation of *E. coli* cells due to the strong acidity of sol-gels, biofilms were dried at 60°, 90° and 120°C by taking account the increase in their brittleness and deformation with increase in temperature of heat treatments. With the prevention of *E. coli* cells inactivation by 120°C heat treatment, SiO_2/TiO_2 coated CSX-50 and BWX biofilms yielded 75±2% and 88±1% photocatalytic antimicrobial activities, respectively where non-SiO_2 added TiO_2 coated CSX-50 and BWX biofilms yielded 63±2% and 63±3% activities, respectively at the end of 2 h black light illumination. TiO_2 powder addition in to the film forming solutions gave significant antimicrobial effect on the CSX-50 and BWX biofilms. The experiments conducted on 1% (w/w), 2.5% (w/w) and 5% (w/w) powder added CSX-50 and BWX biofilms during 30, 60, 90,120 min black light illuminations showed 100% inactivation rates against *E. coli* cells even in 90 min.

In the second part of the study, the biodegradability test was performed on 10% (w/w) TiO_2 added CSX-50 biofilms to determine any adverse effect of TiO_2 powder on biodegradation extent. 10% (w/w) TiO_2 added CSX-50 biofilms were determined as biodegradable as non TiO_2 added CSX-50 and BWX

samples at the end of 180 days by soil burial test. Percent biodegradability rates were evaluated for different control groups and found as 100% for cellulose, 0% for LDPE, 31% for lignin, 98% for cotton stalk, 96% for one time autoclaved soil/cotton stalk, 80% for two times autoclaved soil/cotton stalk, 95% for blank CSX-50 biofilms, 93% for blank BWX biofilms and 91% for 10% (w/w) TiO_2 added CSX-50 biofilms.

For further studies, investigations may be conducted to find the relation between repeated heat treatments and the decrease in hydrophilicity of *E. coli* cells doped SiO_2/TiO_2 coated glass substrates. Different methods for sol-gel coating may be used in order to prevent the biofilms to become brittle and deformed. Also, a thicker coating on CSX-50 biofilms should be tried and effect of coating on mechanical properties of biofilms should be characterized. The TiO_2 powder precipitation may also inhibited by altering the film formation method like ultrasonication. Despite this studies consider antimicrobial biodegradable film formation from cotton stalk, different types of biowastes like corncob, wheat bran, sunflower stalk should also be investigated by means of its potential for antimicrobial biofilm production.

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APPENDIX A

PHOTOCATALYTIC ANTIMICROBIAL ACTIVITIES OF *Escherichia coli* DOPED SOL-GEL COATED GLASS SUBSTRATES

Photocatalytic antimicrobial activities and effect of repeated heat treatments on the hydrophilicity of coated glass substrates were also investigated in this thesis and the materials and methods used in this supplementary study (Koç, 2009) are given below;

A.1.1 Microorganisms and Growth Conditions

Escherichia coli (XL1-blue) was maintained on Luria-Bertani (LB) agar. Plates were incubated at 35°C overnight and then stored at 4°C until use. *E. coli* was cultured in Luria-Bertani (LB) broth at 35°C on a rotary shaker (Minitron) at 170 rpm overnight.

Aspergillus niger was maintained on the potato dextrose agar plates. The plates were incubated at 35°C for 4 days until complete sporulation occurred. Spores were transferred and homogenized by vortexing in peptone water and directly used as the spore suspension for the photocatalytic activity tests against *A. niger* spores.

A.1.2 Photocatalytic Antimicrobial Activity Test for Coated Glass Substrates against *E. coli* Cells

E. coli cells were cultured in Luria-Bertani (LB) broth at 35°C on a rotary shaker (Minitron) at 170 rpm overnight.

An overnight *E. coli* culture of 1.5 ml/sample was put in an Eppendorf tube and a centrifugation step was performed at 10,000 rpm for 4 min. Supernatant was separated and the pellet was mixed with 1 ml of peptone water and 30µl of which was the put on the glass samples. The test samples were illuminated using a 20 W black light source (General Electric) with a peak wavelength of 368 nm placed at 20 cm above the samples for 2 h and 4 h. Then, serial dilution in peptone water (0.01%) was performed. Therefore samples were well mixed with 3.0 ml of peptone water from which 100µl of suspension was taken and mixed with 9.9 ml of peptone water. After vortex mixing, this suspension was diluted with peptone water for counting. 200µl of suitably diluted (10^6 dilution) microbial suspension was inoculated on Luria-Bertani (LB) agar plates and incubated at 35°C overnight. Next day, number of *E. coli* cells survived was counted by the viable colony counting method.

The effect of irradiation alone on the microorganisms was determined on uncoated glasses. The effect of coating materials without photocatalysis on microbial growth was measured on coated samples in the dark. In addition, to determine the initial number of *E. coli* cells that was put on the glass substrates, liquid cell suspension of 200µl was spread onto agar plates and incubated at 35°C.

A.1.3 Photocatalytic Activity Test for Coated Glass Substrates against *A. niger* Spores

Aliquots (6µl) of *A.niger* spore suspensions of concentration about 2.17 x 10^8 spores/ml were first prepared before photocatalytic activity tests. *A. niger* spores were pipetted onto the sol-gel coated glass substrates and onto the control glass substrate surfaces. The test samples were illuminated using an artificial solar light source (Atlas CPS, Xe 1000 W) with 450W/m^2 light intensity. Spores inactivation was followed for 120 h with the removal of the samples at various time intervals like 72, 96 and 120 h. Spore samples were directly spread onto potato agar plates and incubated at 35°C for 46 h to determine the photocatalytic activity of the coated glass substrates against *A. niger* spores. Each evaluation was carried out at least in duplicate. Also, the effect of irradiation alone on *A. niger* spores was determined on uncoated glasses.

The effect of irradiation alone on the spores was determined on uncoated glasses. The effect of coating materials without photocatalysis on spores' growth was measured on coated samples in the dark.

A.1.4 Contact Angle Measurement

Contact angle measurements were performed for the characterization of the hydrophilicity of coated thin films relative to bare glass surface. Contact angles were measured by sessile drop method dropping ultra purified distilled water over the different positions of coated thin films. Water droplet contact angles, were measured using an CAM 100 system comprising a Firewire camera with 50 mm optics and 40 mm extension tube interfaced with a computer, which provided a continuous stream of images of any water droplet after its initial deposition onto the surface of the coated glass substrate under test. 5µl water droplet was deposited for each measurement by a Hamilton syringe with a 22 gauge needle.

The hydrophilicity of the coated glass substrates were investigated according to the curve fitting method using Young-Laplace equation. Completely automated calculation was performed by the software associated with the instrument, which calculates separately contact angles for both sides of the droplet. For each sample, the contact angle measurement was measured six times and the mean value was conducted. The test was repeated after consecutive heat treatments of the coated glass samples at 300°C for 15 min.

The results concerning the photocatalytic antimicrobial activities and effect of repeated heat treatments on the hydrophilicity of coated glass substrates are presented below;

A.2.1 Photocatalytic Antimicrobial Activities of Sol-gel Coated Glass Substrates

Different amounts of *E. coli* cells doped SiO_2/TiO_2 coated glass substrates prepared by Koç (2009) were studied to evaluate their photocatalytic activity against *E. coli* cells and *A. niger* spores. The multi-layered TiO_2 films on glass substrates were deposited by sol-gel dip coating technique to give an antimicrobial property by photocatalysis as it has strong oxidation/reduction property resulting in oxidation of all organic compounds to carbon dioxide and water including biological systems such as cells and viruses.

A.2.1.1 Photocatalytic Activity of Coated Glass Substrates against *E. coli* Cells

The multi-layered TiO_2 films on glass substrates were deposited by sol-gel dip coating technique. For the antimicrobial test, overnight cultured, fresh and centrifuged *E. coli* cells suspended in 30µl peptone water were put on both coated and non-coated glass substrates and illuminated for 2 h and 4 h using a

20 W black light source (General Electric) with a peak wavelength of 368 nm placed at 20 cm above the glass substrates.

Then, 3 ml of peptone water was added to the illuminated film samples as the 10^2 dilution, and this suspension was serially diluted until 10^6 dilution was achieved. Bacterial suspension of 200µl was inoculated on LB agar plates.

The number of *E. coli* cells from the samples was counted by colony counting method and the results showed that the coated glass substrates (Koç, 2009) were antimicrobial at the end of the 2 h black light illumination, moreover by increasing the illumination time to 4 h, the antimicrobial activity was increased significantly.

All results having the viable cell counts of different concentrations of *E. coli* doped SiO_2/TiO_2 coated glass substrates (Koç, 2009) after antimicrobial tests including photocatalytic inactivation are given in Tables A.1 and A.2.

Table A.1 Antimicrobial activities of coated glass substrates (Koç, 2009) against *E. coli* cells obtained after 2 h illumination period.

Photocatalysis period	***E. coli* cells counts**	**Antimicrobial activity**
2 h	**cfu**	**(%)**
Blank	504±1	-
Sample 1	353±4	30±1
Sample 2	357±18	29±4
Sample 3	350±12	30±2
Sample without dopant (negative control)	480±9	5±2

**E. coli* concentration used as dopant increases from sample 1 to sample 3.

Table A.2 Antimicrobial activities of coated glass substrates (Koç, 2009) against *E. coli* cells obtained after 4 h illumination period.

Photocatalysis period	***E. coli* cells counts**	**Antimicrobial activity**
4 h	**cfu**	**(%)**
Blank	434±8	-
Sample 1	85±12	80±3
Sample 2	120±16	72±4
Sample 3	89±10	79±2
Sample without dopant (negative control)	356±11	18±2

**E. coli* concentration used as dopant increases from sample 1 to sample 3.

According to the experimental results shown above, samples coated with *E. coli* cells doped SiO_2/TiO_2 sol-gel (Koç, 2009) have antimicrobial activities when compared to the blank, uncoated samples. Moreover, as the concentration of the *E. coli* cells used as dopant increases, the coated glass substrates were achieved an increased photocatalytic antimicrobial activity against *E. coli* cells. The negative control which was coated without dopant was observed to have a lower antimicrobial activity.

As it can be deduced, doping the semiconductor with bacterial cells as dopant leaded to an enhanced efficiency of the photocatalytic systems. The dopant affects the photocatalytic activity by modifying the surface structure, surface properties and composition of the coated glass substrates. So, in order to improve photocatalytic performance, *E. coli* doping has performed to increase the number of attainable surface activation sites by making porous microstructure (Yanhui *et al*, 2008). Previous studies have shown that organic materials of numerous structures and forms, which can be regulated and easily removed by calcination, are applied as templates for formation of porous inorganic structures with catalytic and photocatalytic properties. Moreover it was found that after combustion of residual organic components, an open

cellular structure consisting of anatase nanoparticles can be obtained (Yamamato *et al*, 2004).

During the experiments, to inactivate *E. coli* cells used for testing the antimicrobial photocatalytic activity of coated glass substrates were heat treated for sterilization purpose at a temperature lower than the calcination temperature performed during the preparation of those glass substrates. The heat treatments were performed at 300˚C for 15 min.

The results, after the second heat treatment, having the viable cell counts of different concentration of *E. coli* doped SiO_2/TiO_2 coated glass substrates are given in Table A.3.

Table A.3 Antimicrobial activities of heat treated coated glass substrates (Koç, 2009) against *E. coli* cells obtained after 4 h illumination period.

Photocatalysis period	***E. coli* cells counts**	**Antimicrobial activity**
4 h	**cfu**	**(%)**
Blank	199±30	-
Sample 1	137±7	31±3
Sample 2	127±12	36±6
Sample 3	111±13	44±6
Sample without dopant (negative control)	181±11	9±6

**E. coli* concentration used as dopant increases from sample 1 to sample 3.

When antimicrobial activities of coated glass substrates before and after heat treatment were compared against *E. coli* cells after 4 h black light illumination, a significant decrease in the inactivation rate was observed. The compared results are given in Table A.4.

Table A.4 Antimicrobial activities of coated glass substrates (Koç, 2009) before and after heat treatment against *E. coli* cells after 4 h illumination time period.

	Antimicrobial activity before heat treatment (%)	**Antimicrobial activity after heat treatment (%)**
Blank	-	-
Sample 1	80±3	31±3
Sample 2	72±4	36±6
Sample 3	79±2	44±6
Sample without dopant (negative control)	18±2	9±6

**E. coli* concentration used as dopant increases from sample 1 to sample 3.

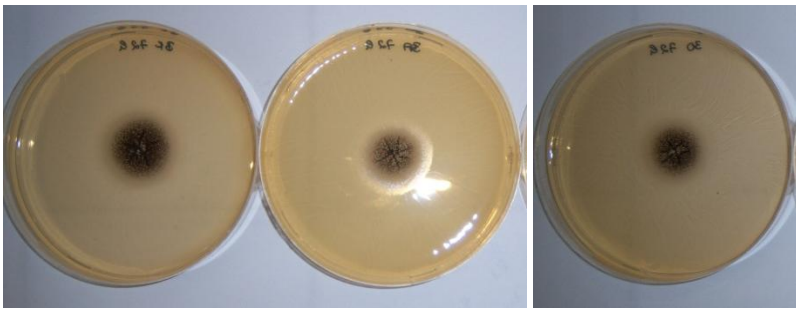
The decrease in the number of viable *E. coli* cells after the heat treatment of coated glass substrates for sterilization purpose can be due to the corrosion of the films leading to the deterioration of the open cellular structural pores or loss of hydrophilicity of the films. The results and the effect of hydrophilicity on the photocatalytic activity were mentioned in Appendix A, section A.2.2.

A.2.1.2 Photocatalytic Activity of Coated Glass Substrates against *A. niger* Spores

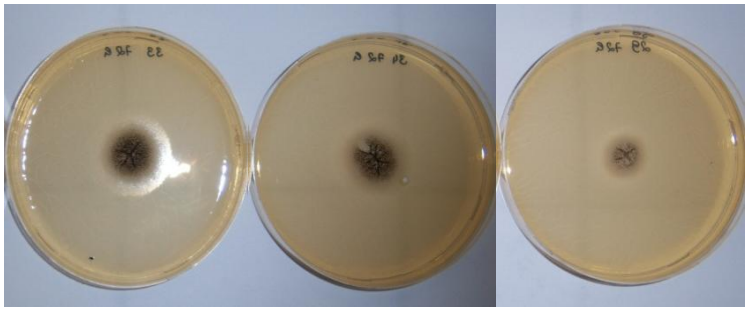
In order to characterize the photocatalytic efficiency of the films, a G(-) bacterium and a fungal spore having different cell wall structures, namely *E. coli*, as mentioned before, and *A. niger* spores, were selected. Control experiments, showing cell viabilities on uncoated glass surfaces with irradiation and on coated surfaces without irradiation have also been performed for all glass substrates. Neither the coatings nor the illumination alone has any

antimicrobial effect against the spores. *A. niger* spore coats, similar to cell walls of some microorganisms, contain chitin and glucan as the major polysaccharides (Horikosi *et al*, 1964). These spores have the capacity to survive drastic and stressful changes in their environment and to germinate after duration for long periods at difficult environments. Moreover they are extremely resistant to adverse conditions (Abrashev, 2005).

In the experiment, considering the high resistivity of *A. niger* spores, the photocatalytic activities of all surfaces against *A. niger* were investigated by prolonging the photocatalytic treatment up to 120 h at 450 W/m^2 light intensity. The results of different counts of *E. coli* cells doped SiO_2/TiO_2 coated glass substrates were given in Figures A.1, A.2 and A.3. *E. coli* cell concentration used as dopant increases from sample 1 to sample 3.

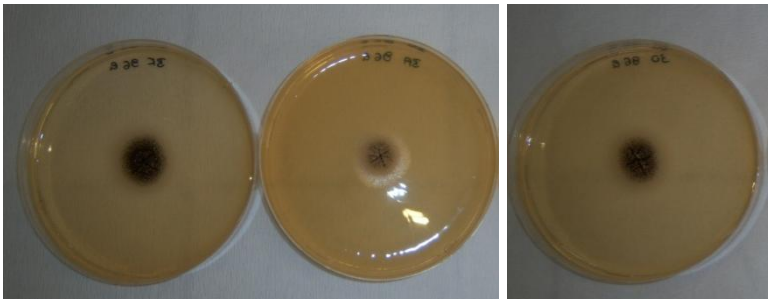


(a) **(b)** **(c)**

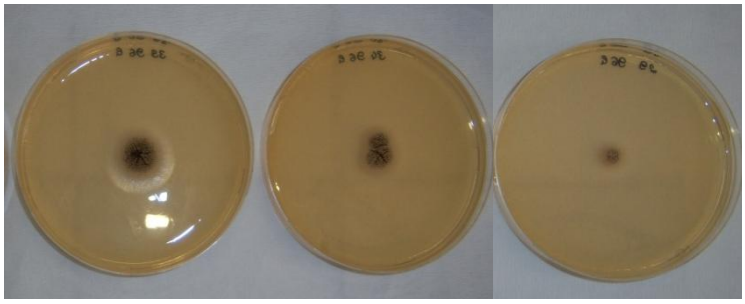


(d) **(e)** **(f)**

Figure A.1 *A. niger* spore germinations observed on coated glass substrates (Koç, 2009) illuminated at 450 W/m^2 for 72 h, after incubation for 46 h. (a) blank uncoated sample kept in the dark, (b) blank uncoated sample exposed to the light, (c) sample without dopant, (d) sample 1, (e) sample 2, (f) sample 3.



(a) **(b)** **(c)**

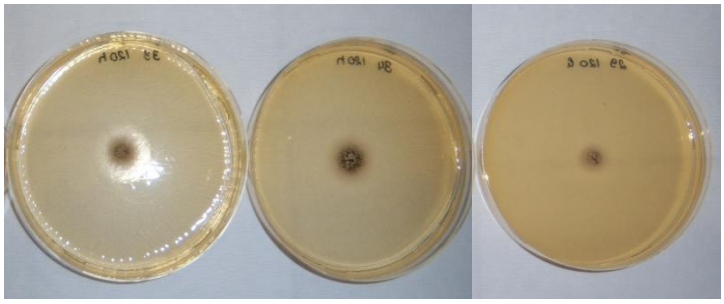


(d) **(e)** **(f)**

Figure A.2 *A. niger* spore germinations observed on coated glass substrates (Koç, 2009) illuminated at 450 W/m^2 for 96 h, after incubation for 46 h. (a) blank uncoated sample kept in the dark, (b) blank uncoated sample exposed to the light, (c) sample without dopant, (d) sample 1, (e) sample 2, (f) sample 3.

(a) **(b)** **(c)**

Figure A.3 *A. niger* spore germinations observed on coated glass substrates (Koç, 2009) illuminated at 450 W/m^2 for 120 h, after incubation for 46 h. (a) blank uncoated sample kept in the dark, (b) blank uncoated sample exposed to the light, (c) sample without dopant, (d) sample 1, (e) sample 2, (f) sample 3.



(d) **(e)** **(f)**

Figure A.3 (continued)

In the experiments, the photocatalytic activities of all the surfaces against *A. niger* spore germinations were investigated by prolonging the photocatalytic treatment up to 120 h at 450 W/m^2 light intensity. The spore development on the agar plates was checked during the incubation period to observe any changes in the spore germination. During the photocatalytic activity property against *A. niger* test, the notion of the control groups is very important. The effect of irradiation alone on *A. niger* spores was determined on uncoated glasses substrates. The effect of coating materials without photocatalysis on spore germinations was measured on coated samples in the dark. If the control groups were not observed, some deceptive results may be obtained since spore inactivation occurring from the light source or the inactivation occurring may be recognized due to the photocatalytic effect. The photocatalytic activity of the coated glass substrates were compared visually.

According to the observations, it was seen that neither the coatings nor the illumination alone has any photocatalytic effect against *A. niger* spores. As can be seen from the Figures A.1, A.2 and A.3, complete spore inactivation could not be observed. However, the TiO_2 sol-gel coating was recognized to have a retarding property on the photocatalytic inactivation against *A. niger* spores germinations when compared with the control groups.

While the glass substrates coated with different counts of *E. coli* doped SiO_2/TiO_2 sol-gel were evaluated, it was observed that the photocatalytic

activity was more effective against *A. niger* spores with the increase in the amount of the dopant. For all illumination time periods, sample 3 with the highest counts of *E. coli* dopant was found to be more photocatalytically active.

Moreover, prolonged photocatalytic treatment up to 120 h yielded a significant effect on the photocatalytic inactivations. Increase in the exposure time of the samples to the light source resulted in an increase in the retardation period for *A. niger* spore germinations.

The antimicrobial efficiency of the coated glass substrates was noticed to be higher against *E. coli* cells when compared to *A. niger* spores. This appears reasonable considering the relative strengths of cell walls. Existence of strong cell wall introduces high resistance to photocatalytic inactivation. Therefore, the higher photocatalytic efficiency was against *E. coli* presumably due to the simple and weaker cell wall structure.

A.2.2 Effect of Repeated Heat Treatments on the Hydrophilicity of Coated Glass Substrates

Recent studies have found that apart from the photocatalytic properties, TiO_2 films exhibit hydrophilicity after UV illumination. It has been reported that hydrophilicity enhances the photocatalytic activity because more OH groups can be adsorbed on the surface due to hydrophilicity (Guan *et al*., 2005). Not only the improved hydrophilic property but also improved photocatalysis have found for SiO_2/TiO_2 composite films (Permpoon *et al*., 2007). According to Guan *et al*. (2005), the addition of SiO_2 in TiO_2 film can enhance the acidity of the composite oxide which is thought to be the main reason for improving the photocatalysis and hydrophilicity, so the surface improved acidity can adsorb more OH radicals.

However, during the experiments for testing the antimicrobial photocatalytic activity of coated glass substrates against *E. coli*, samples were reused followed by a sterilization process, performed in order to remove *E. coli* cells used during the photocatalytic antimicrobial tests. The sterilization process was conducted by heat treatment of each sample at 300˚C for 15 min. After each heat treatment, a decrease in the hydrophilicity of the coated glass substrates was observed. Hydrophilicity of coated glass substrates were tested according to the contact angle measurement. The results of the coated glass substrates were given in Figures A.4, A.5, A.6 and A.7. *E. coli* cells' concentration used as dopant increases from sample 1 to sample 3.

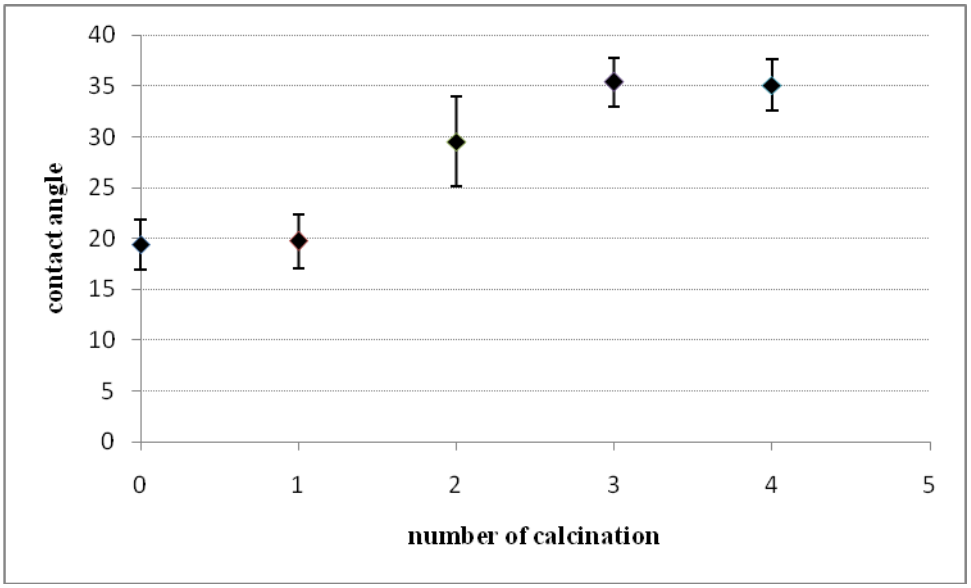


Figure A.4 Contact angle measurements for undoped, control sample after 5 repeated calcinations.

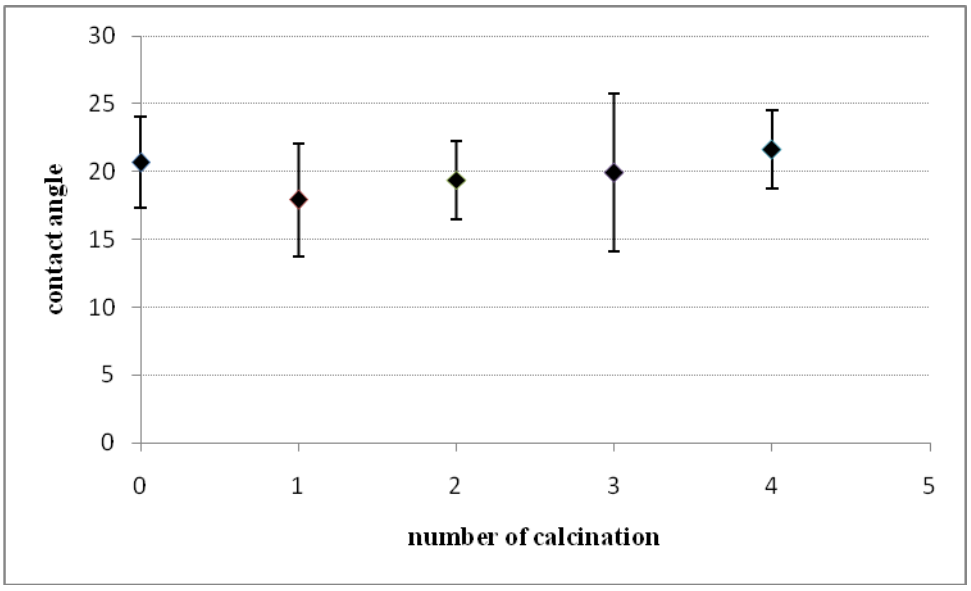


Figure A.5 Contact angle measurements for sample 1 after 5 repeated calcinations.

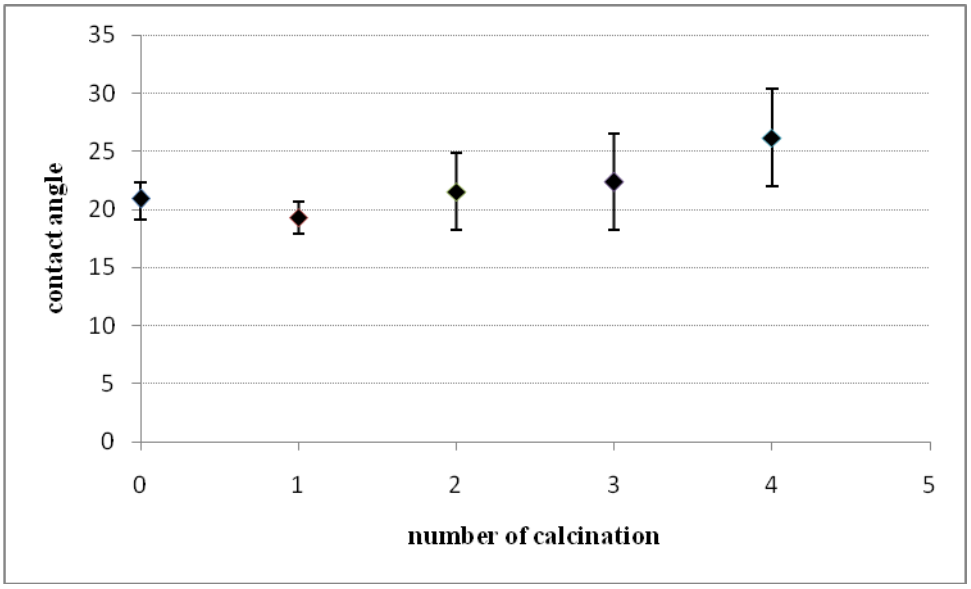


Figure A.6 Contact angle measurements for sample 2 after 5 repeated calcinations.

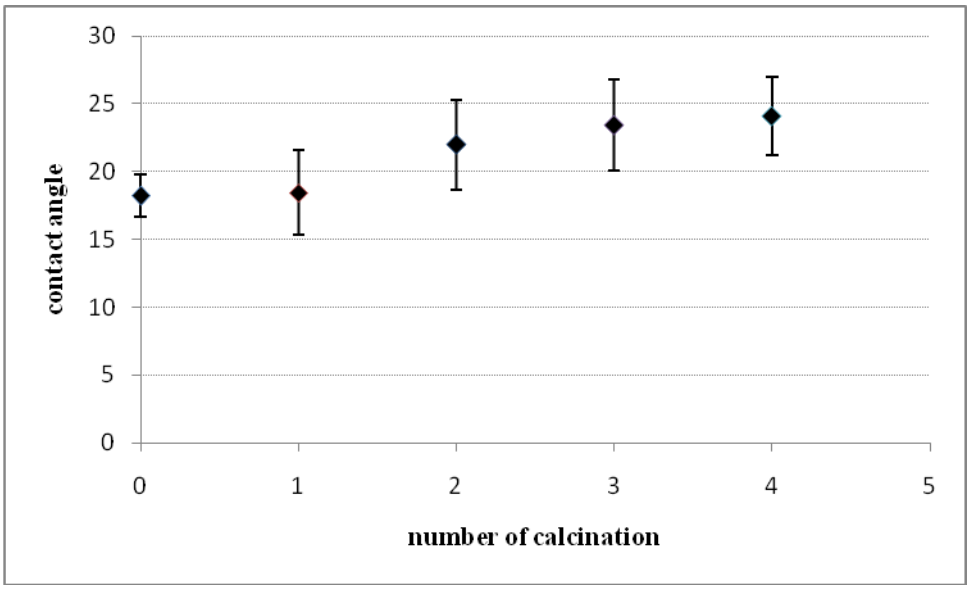


Figure A.7 Contact angle measurements for sample 3 after 5 repeated calcinations.

Following the photocatalytic antimicrobial activity tests, the photocatalytic effect was observed to be reduced. The results are given in Appendix A, section A.2.1.1, Tables A.2 and A.3.

The decrease in the photocatalytic activity against *E. coli* cells may be due the corrosion of the glass substrates during heat treatment processes. Moreover, the porous structure of the films, obtained by *E. coli* doping improving the number of attainable surface activations sites may be deteriorated leading to the reduction of the photocatalytic antimicrobial effect. Further studies are required in order to investigate the effect of successive heat treatments on the photocatalytic antimicrobial activities of the coated glass substrates.

Therefore, one can conclude that;

- Samples prepared by Koç, 2009) have significant amount of antimicrobial activity rates ranging from 72±4% to 80±3% against *E. coli* cells after 4 h black light illumination.
- The highest amount of *E. coli* cells doped sample has the highest photocatalytic activity with 80±3% of inactivation rate.
- Samples have a retardation effect on the germination of *A. niger* spores and the longest retardation was achieved at the highest concentration of *E. coli* cells doped sample.
- There is a decrease in antimicrobial activity and in hydrophilicity after repeated heat treatments. It was thought that the decrease in hydrophilicity caused a decrease in the photocatalytic antimicrobial activity.

APPENDIX B

MEDIUM AND AGAR BASES

1. Luria-Bertani (LB) Broth:

Product Company: Merck

Basis: 1L (in distilled water)

Tryptic soy broth, 10g

Yeast extract, 5g

NaCl, 10g

2. Luria-Bertani (LB) Agar:

Product Company: Sigma-Aldrich

Basis: 1L (in distilled water)

Agar, 15g

Tryptic soy broth, 10g

Yeast extract, 5g

NaCl, 10g

3. Potato Dextrose Agar:

Product Company: Sigma-Aldrich

Basis: 1L (in distilled water)

Agar, 15g

Dextrose, 20g

Potato extract, 4g

APPENDIX C

BACTERIAL CELL CALIBRATION CURVE

Calibration Curve for *Escherichia coli* XL-1 Blue

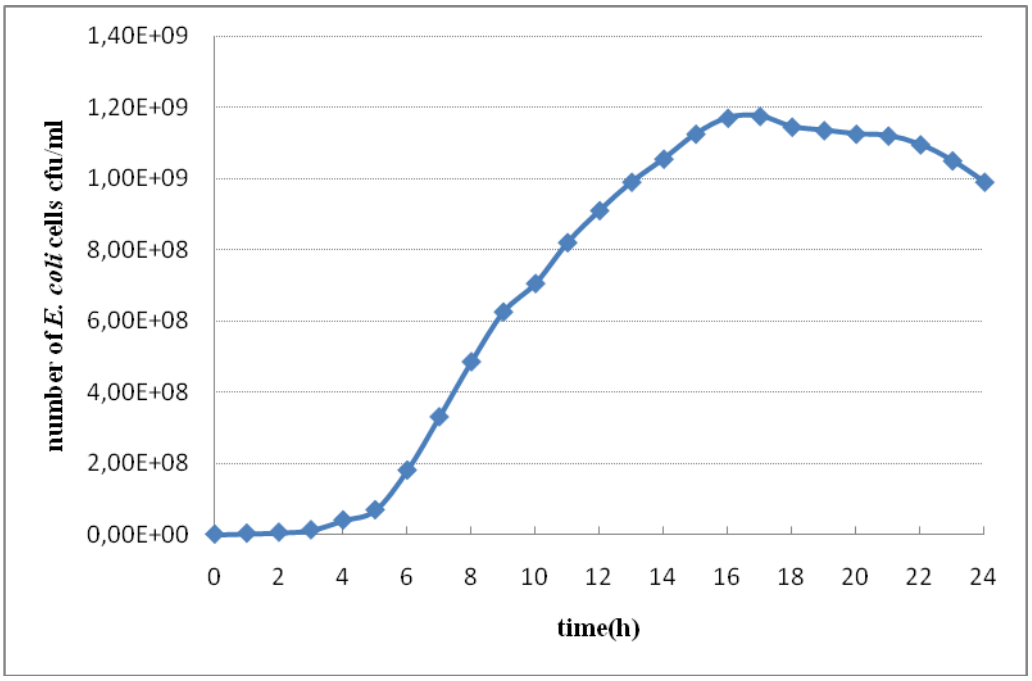


Figure C.1 Calibration curve for *Escherichia coli* XL-1 Blue

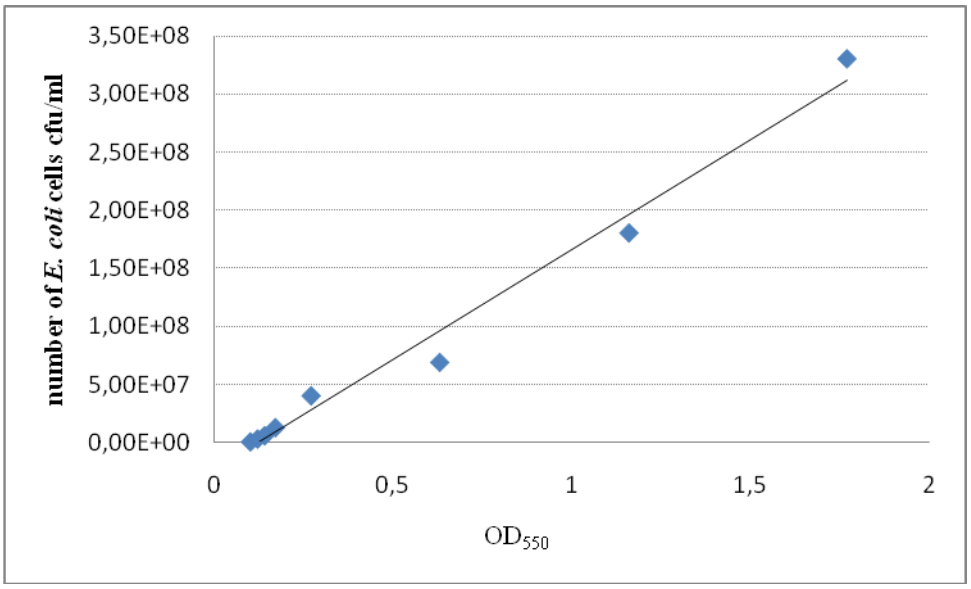


Figure C.2 Calibration curve for *Escherichia coli* XL-1 Blue