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Microbial processes in the degradation of fibers

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1.1 Introduction

As society becomes more concerned about environmental quality, there are moves toward producing and using materials that will not accumulate in the environment. For example, many plastics that are made from petrochemicals persist indefinitely after being discarded. Similarly, synthetic fibers such as nylon or polypropylene are extremely stable in dark environments. Thus, there is a trend toward producing and using fibers that will break down after their disposal. Depending upon the fiber, their breakdown may occur by biotic or abiotic processes. Biotic processes involve biochemical reactions that are typically mediated by microorganisms such as bacteria and fungi. Abiotic processes include chemical oxidation and hydrolysis, and photodegradation. This chapter will focus only on biotic processes.

Of course, natural fibers like wool and cotton are broken down through biotic processes. Microorganisms have evolved enzymes that attack key bonds in these natural polymers, thereby releasing monomers that can be used as carbon and energy sources for microbial growth. In contrast, microorganisms lack enzymes to break down many synthetic fibers, thus these materials persist and accumulate in the environment.

This chapter provides an overview of microbial processes involved in the degradation of natural and synthetic fibers, starting with an introduction of relevant terminology. It discusses the methods used to assess the microbial breakdown of fibers and gives examples of the sources of microbial communities and the methods of incubation that are used in these studies. Finally, it provides examples of the types of bonds that are susceptible to microbial attack.

1.2 Background and terminology

Microbial processes, that change the structure or form of any material, always occur at the molecular level. In a general sense, microorganisms such as

bacteria and fungi can be considered 'bundles of enzymes' or sources of enzymes that catalyze a diverse array of chemical reactions that break down or modify substrates. Microorganisms carry out these activities to provide energy and suitable smaller molecules for the production of new cellular material, and ultimately new cells. Thus, when considering the microbial attack on any substance, it is important to remember that the size and physical and chemical characteristics of the substance influence how the microbes attack it. In addition, because microorganisms are living entities, environmental conditions must be suitable for their survival and growth. In this section, the general characteristics of fibers, microorganisms, and microbial processes will be discussed and some important terms will be defined.

1.2.1 Fibers, textiles and films

Fiber is the basic element of fabrics and other textile structure [1]. A fiber is typically defined as a material having a length at least 100 times its diameter. These can be natural, such as cellulose or wool, or synthetic, such as nylon. A textile is any product made from fibers [1]. This includes nonwoven fabrics such as felt, in which wool fibers are physically interlocked by a suitable combination of mechanical work, chemical action, moisture, and heat [1]; and woven fabrics in which yarns are interlaced perpendicular to each other. Yarns are made of fibers twisted together in a continuous strand.

Many of the so-called thermoplastic, biodegradable natural polymers, known as poly(hydroxyalkanoates), and some synthetic polymers such as poly(lactides) can be made into fibers by cold drawing, melt spinning or thermal drawing [2, 3]. These polymers can also be extruded as sheets rather than fibers. These sheets are known as films which are not true textiles because they are not made of fibers [1]. Nonetheless, these films of poly(hydroxyalkanoates) or poly(lactides) have the same chemical properties as the corresponding fibers, so for convenience, films are often used in biodegradation studies.

Fibers are composed of polymeric molecules with different arrangements. These can be random or parallel [4]. Amorphous regions of a fiber are due to random or unorganized arrangement of the polymers. In contrast, a parallel, highly ordered arrangement of the polymers is referred to as a crystalline region [4]. Fibers generally contain both types of polymer arrangements (for example, cotton is about 30% amorphous and 70% crystalline) and, typically, fibers of a particular type display greater strength with an increasing proportion of crystalline regions. These different arrangements also affect the biodegradability of fiber; the amorphous regions are more susceptible to biodegradation than the crystalline regions (for example, amorphous cellulose is biodegraded more rapidly than crystalline cellulose [5]).

Hydrogen bonding between chains of polymers is the major force that

contributes to crystallinity [4]; the sum of the myriad of weak hydrogen bonds between adjacent polymers yields a strong, tight structure in the crystalline region of a fiber. Covalent cross-linking also occurs in some fibers, most notable are wool and silk in which disulfide bonds form between amino acid residues; the greater the number of disulfide bonds, the tighter the fiber structure. 'Tightness' imparted by hydrogen bonding and cross-linking reduces the susceptibility of the fiber to biodegradation.

1.2.2 Biodegradation, mineralization and biomass formation

The term biodegradation may have different connotations for people in different situations. In the broadest sense, biodegradation is the biologically catalyzed reduction in the complexity of chemicals [6]. A simple example is the conversion of glucose to ethanol during yeast fermentation; ethanol is a less complex molecule than glucose. Mineralization can be considered complete biodegradation, leading to the conversion of organic forms of elements to inorganic forms, as shown in Table 1.1. In some cases, two products are shown Table 1.1, for example, under aerobic conditions, organic-N may be converted to NH_3 or NO_3^-, depending upon the environmental conditions and the structure of the microbial community, that is, if conditions are favorable for nitrifying bacteria, NH_3 (the first product of mineralization) may be oxidized to NO_3^-. An anomaly in Table 1.1 is the appearance of CH_4 (an organic compound) as a mineralization product of organic-C. This occurs in methanogenic environments (see Section 1.3.2), where CH_4 production occurs along with CO_2 production; however, it is generally accepted that CH_4 is a product of mineralization in these environments. In non-methanogenic environments, CO_2 is the product of mineralization (Table 1.1).

Table 1.1 Major products of microbial mineralization under aerobic or anaerobic conditions

Conditions	Substrate	Inorganic products
Aerobic	Organic-C	CO_2
	Organic-H	H_2O
	Organic-N	NH_3, NO_3^-
	Organic-S	SO_4^{2-}
Anaerobic	Organic-C	CO_2, CH_4
	Organic-H	H_2, H_2O
	Organic-N	NH_3
	Organic-S	H_2S

An important product of microbial metabolism and biodegradation is biomass or new cell material. In heterotrophic microorganisms, new cell

material is formed by the incorporation of some of the carbon from the biodegradable organic substrates, while a portion of the organic carbon is mineralized to yield energy for biosynthesis of biomass. More energy is produced from the oxidation of an organic substrate in the presence of O_2, than in the absence of O_2 (see Section 1.2.5), thus, under aerobic conditions, more energy is available for biosynthesis and more substrate carbon is incorporated into biomass in the presence of O_2. In general, about 50% of substrate carbon is assimilated into new biomass under aerobic conditions, whereas only about 10% of substrate carbon is assimilated into new biomass under anaerobic conditions.

1.2.3 Microorganisms

The two major groups of microorganisms associated with the breakdown of organic matter are bacteria and fungi, both groups are extremely diverse in form, habitat and activity. Bacteria are typically simple, unicellular, prokaryotic (having no nucleus) organisms. They are commonly 1 to 5 μm in size, and are invisible to the naked eye. Some bacteria grow in filamentous forms, and these masses of cells can be seen without a microscope. Some bacteria, known as strict aerobes, require O_2 for growth, whereas others, known as strict anaerobes, are killed in the presence of O_2. Others, known as facultative anaerobes, can grow in the presence or absence of O_2. Many bacteria are heterotrophs that derive energy and carbon from organic matter and these play a major role in nutrient cycling in the environment.

Fungi are more complex microorganisms. They are eukaryotic (having a nucleus) and are typically multicellular, making them much larger than bacteria. Their filamentous growth of hyphae yields structures that are often large enough to be visible to the naked eye (for example, mold growing on bread). However, soils can contain kilometers of fungal hyphae that are not readily visible. Many fungi produce spores that are spread by wind, and upon landing, these spores can remain dormant until conditions are favorable for their germination and growth. This form of dispersal can often be observed as mildew growth on plants or other materials. Fungi are heterotrophs and most are strict aerobes. The ability of fungal hyphae to rapidly penetrate into tissues and other organic materials makes these organisms important decomposers. This invasion puts the hyphae into intimate contact with potential food sources.

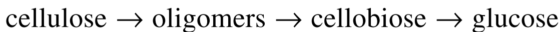
1.2.4 Growth requirements for heterotrophic microorganisms

Although the habitats, activities, and metabolism of bacteria and fungi are extremely diverse, there are certain requirements and conditions needed by

all of these microorganisms for growth and reproduction. Water is essential for all life; the typical microbial cell consists of about 70 to 85% water. The availability of water is expressed as water activity (a_w), defined as the ratio of the vapor pressure over the substance or medium to the vapor pressure of pure water (at a given temperature) [7], thus, the a_w of pure water is 1.00. The presence of salts or other solutes in the medium reduces the a_w, as does the dryness (lack of water) of the environment. Most bacteria and fungi require $a_w > 0.90$ for growth; however, some xerophiles (organisms that can grow under relatively dry conditions) are active at $a_w < 0.85$. Xerophiles are typically molds and yeasts [7].

On a dry weight basis, a typical microbial cell contains about 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% phosphorus, and 1% sulfur [8]. These elements, along with potassium, magnesium, sodium, calcium and iron are considered macronutrients required for cell growth [9]. In addition, micronutrients are required by some microorganisms, and these include cobalt, copper, manganese, molybdenum, nickel, selenium, tungsten, vanadium and zinc. These metals are typically present in cofactors or specific enzymes.

Microorganisms absorb their nutrients, that is, in order to be taken into the cell, the nutrients must be soluble and small enough to pass though the cell wall and cytoplasmic membrane. The utilization of macromolecules presents a special problem because these molecules are far too large to be taken into the cell. Large polymeric molecules such as proteins and polysaccharides must be broken down outside of the cell, and then small subunits or monomers can be taken into the cell. Many fungi and bacteria produce extracellular enzymes that hydrolyze soluble or insoluble polymers, for example, cellulose (the most abundant organic compound in the biosphere) is made of glucose subunits joined by β-(1 → 4)-linkages. This insoluble polymer is the major component of cotton. Although the sizes of cellulose molecules differ, it has been estimated that there are about 1000 to 1500 glucose subunits in each cellulose molecule [10]. Extracellular cellulases hydrolyze the β-(1 → 4)-linkages and ultimately produce glucose monomers, summarized as follows:



cellulose → oligomers → cellobiose → glucose

Glucose is readily transported into microbial cells, and its subsequent metabolism provides energy and carbon for microbial growth and reproduction.

Similarly, the peptide bonds in proteins (the major constituents of wool and silk) are hydrolyzed by proteolytic enzymes leading to the following sequence of products:

protein → polypeptides → simple peptides → amino acids

Amino acids are easily transported into microbial cells, and they can serve

as sources of energy, carbon, nitrogen and sulfur, depending on the amino acid structure, the metabolism of the microorganisms, and the environment in which the microorganisms are growing.

In a microbial community, only a few microbes may actually produce the extracellular enzymes needed to hydrolyze insoluble polymers in fibers. Clearly, the microbes that produce these hydrolytic enzymes play a key role in the biodegradation of fibers. However, they do not have an exclusive role in the compete biodegradation of the hydrolysis products. Once a polymer is broken into soluble subunits, other microorganisms in the community can transport these small molecules into their cells and use them as sources of energy and substrates for growth. Thus, in diverse microbial communities, the microorganisms that produce the extracellular hydrolytic enzymes do not consume all of the organic constituents derived from the fibers.

1.2.5 Terminal electron acceptors

Monomers and other small molecules are oxidized by heterotrophic microorganisms to produce energy for biosynthesis and other cellular activities. During these oxidations, the electrons that are removed from a substrate serving as an energy source must be ultimately passed to a terminal electron acceptor. The diversity of microorganisms, and particularly bacteria, allows a variety of compounds or ions to serve as terminal electron acceptors. Table 1.2 summarizes several energy yielding reactions with acetate serving as the electron donor coupled to various terminal electron acceptors. The free energy is expressed as kJ mol^{-1} of acetate that is oxidized.

Table 1.2 Energy yielding processes used by microorganisms to oxidize acetate in the presence of different terminal electron acceptors (after [11] and [12])

Microbial process	Reaction with acetate as the electron donor (energy source)	$\Delta G^{\circ\prime}$ (kJ mol^{-1} of acetate)
Aerobic respiration	$CH_3COO^- + 2O_2 \rightarrow CO_2 + HCO_3^- + H_2O$	–849
Mn(IV) reduction	$CH_3COO^- + 4MnO_2 + 2HCO_3^- + 3H^+ \rightarrow 4MnCO_3 + 4H_2O$	–737
Denitrification	$5CH_3COO^- + 8NO_3^- \rightarrow 4N_2 + 5CO_2 + 5HCO_3^- + 8OH^- + H_2O$	–733
Fe(III) reduction	$CH_3COO^- + 24Fe(OH)_3 \rightarrow 8Fe_3O_4 + CO_2 + HCO_3^- + 37H_2O$	–712
Sulfate reduction	$CH_3COO^- + SO_4^{2-} + H^+ \rightarrow HS^- + CO_2 + HCO_3^- + H_2O$	–52
Methanogenesis	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	–31

Typically, O_2 serves as the terminal electron acceptor in aerobic environments. Aerobic respiration gives the greatest energy yield per mol of acetate (–849 kJ mol^{-1}, Table 1.2). Under anaerobic conditions, Mn(IV), nitrate, Fe(III), sulfate, and carbon dioxide are commonly used terminal electron acceptors. Based on the equations in Table 1.2, Mn(IV) reduction and nitrate reduction to N_2 (known as denitrification) yield approximately the same amount of energy per mol of acetate. There is slightly less energy gained when Fe(III) is used as a terminal electron acceptor. Much less energy is available under sulfate-reducing or methanogenic conditions.

If two or more potential terminal electron acceptors are available, the one that yields more energy for the microbial population or community will typically be used first. For example, *Pseudomonas stutzeri* is able to grow with either O_2 or nitrate as a terminal electron acceptor. If *P. stutzeri* is incubated aerobically in the presence of nitrate, it will preferentially use O_2 as its terminal electron acceptor because of the greater energy yield. As the concentration of O_2 is depleted, *P. stutzeri* will synthesize the enzymes required to use nitrate as its terminal electron acceptor [13]. Similarly, if nitrate and sulfate are present in an environment that is devoid of O_2, the activities of the microbial community are dominated by the nitrate reduction (denitrification). In this case, there is a competition between two groups of bacteria in the community, these are the nitrate-reducing bacteria and the sulfate-reducing bacteria. There are several reasons why the nitrate-reducing bacteria out-compete the sulfate-reducing bacteria [14], and one of these is the greater energy yield when nitrate is used as the terminal electron acceptor (Table 1.2).

In anaerobic, dark environments in which Mn(IV), Fe(III), nitrate, and sulfate are scarce, methanogenesis becomes the dominant microbial process [15]. This often occurs in landfills and methanogenesis is exploited in the anaerobic digestion of sewage sludges. Methanogens have a very limited range of substrates that can be used as energy sources, including acetate (Table 1.2), formate, and H_2 (other substrates used by methanogens are listed in Zinder [16]). Because of their limited range of substrates, methanogens occupy the terminal position in the decomposition of organic matter in anaerobic environments, and they depend on three other microbial processes to degrade complex polymers such as cellulose or protein. These include: (a) hydrolysis that yields monomers from polymers; (b) fermentative acidogenesis that produces acids (e.g. lactate, propionate, butyrate) and alcohols (e.g. ethanol, propanol, butanol) from the monomers; and (c) acetogenesis that forms acetate, H_2 and CO_2 from the products of acidogenesis. H_2 is an important energy source for methanogens; using CO_2 as a terminal electron acceptor, hydrogenotrophic methanogens carry out the reaction in equation 1.1:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^{\circ\prime} = -136 \text{ kJ mol}^{-1} \text{ methane} \quad 1.1$$

This is a greater energy yield than derived from acetate (–31 kJ mol^{-1} methane, Table 1.2), and hydrogenotrophic methanogens grow much more quickly that acetate-utilizing methanogens.

The passage of H_2 from other microbial processes to methanogens is known as interspecies H_2 transfer, and this process plays a key role in the overall thermodynamics of the anaerobic process. For example, the acetogenic conversion of butyrate to acetate and H_2 is thermodynamically unfavorable ($\Delta G^{\circ\prime}$ is positive). However, this reaction can occur only if H_2 is removed by a hydrogenotrophic methanogen [17] because the overall reaction carrier out by the syntrophic association of the acetogen and a methanogen is thermodynamically favorable ($\Delta G^{\circ\prime}$ is negative).

1.3 Incubation conditions used for studying biodegradation of fibers and films

In the environment, fibers or textiles can find their way into aerobic or anaerobic environments. Many aquatic environments and the top few centimeters of soil contain sufficient O_2 to be aerobic. In contrast, anaerobic conditions exist in the deeper soils, water-logged soils, aquatic sediments, and landfills. Thus, biodegradation studies have assessed the fates of fibers and textiles under aerobic and anaerobic conditions.

1.3.1 Aerobic incubations

Experimentally, aerobic incubations are much easier to set up and maintain than anaerobic conditions. Accordingly, many of the studies assessing the biodegradation of fibers and films have been done under aerobic conditions. In addition, fungi often play an important role in the breakdown of polymers, and many of these are strict aerobes. Of course, O_2 serves as the terminal electron acceptor under aerobic conditions. The major requirements of these aerobic studies are to ensure that the fibers are accessible to the microorganisms, and that there is an ample supply of O_2. To fully evaluate the progress of the biodegradation, the experimental method must also allow the collection of the residual polymers or biodegradation products, which may include collection of carbon dioxide that is liberated during the mineralization of the fiber or film (Table 1.1).

One of the simplest methods for aerobic culturing (if carbon dioxide is not collected) is to add the fiber or film and the microbial inoculum to a container with liquid medium. This is covered in some manner to allow air to get into the container while preventing foreign microorganisms from contaminating the culture system; this is typically achieved by means of a sterile foam plug in the neck of the container. The container may be placed on a rotary shaker to increase the rate of aeration. However, if the depth of

the liquid is low and the surface area of the liquid is high, there will be adequate O_2 diffusion without shaking.

The formulation of the medium depends on the goal of the aerobic experiment. Often the medium contains only inorganic salts (including phosphate and ammonium or nitrate), and the polymer is the sole source of carbon for the heterotrophic microorganisms. This type of medium was used by Modelli *et al.* [18] in their investigations of the biodegradation of flax fiber by the bacterium *Cellvibrio fibrovorans*; similarly, Wiegand *et al.* [19] used ammonium nitrate-containing medium to isolate bacteria that degrade polyester amide BAK 1095.

If the fiber contains nitrogen and sulfur, such as in wool and silk, the inorganic nitrogen and sulfur sources may be omitted, so the fiber serves as the carbon, nitrogen and sulfur source. This approach is illustrated by the work of Stahl *et al.* [20, 21] who incubated the fungus *Microsporum gypseum* in nitrogen-free and sulfur-free medium with wool as the sole carbon, nitrogen and sulfur source. They observed mineralization of organic nitrogen and organic sulfur to ammonium and sulfate, respectively. Similarly, media devoid of nitrogen salts were used in study of chitosan-gallen and poly(L-lysine)-gellan fibers by filamentous fungi [22], and these two nitrogen-containing polymers served as the carbon and nitrogen sources for growth. Some studies that investigate fungal biodegradation of fibers use growth medium that is supplemented with a readily utilizable carbon source, such as glucose. For example, wool biodegradation studies using the fungi *Trichophyton simii* and *Aspergillus niger* used nitrogen-free, glucose-containing liquid medium [23].

1.3.2 Anaerobic incubations

The methods required to create and maintain conditions that are suitable for growing anaerobic cultures are more difficult than those required for culturing aerobic cultures. Nonetheless, use of methods such as the serum bottle modification of the Hungate technique [24] is now routine in many laboratories. As discussed in the previous section, the formulation of the medium depends on whether the fiber or fabric is to serve as the sole source of carbon, nitrogen or sulfur. In addition, the formulation of the anaerobic culture medium depends upon which terminal electron acceptor is to be considered in the study. As shown in Table 1.2, the list of terminal electron acceptors includes Mn(IV), nitrate, Fe(III), and sulfate. In addition, bicarbonate (carbon dioxide) serves as the terminal electron acceptor for methanogenesis (equation 1.1). Supplementing the medium with an abundant supply of one of the terminal electron acceptors prescribes the microbial process that occurs in the cultures. Fermentation, in which some oxidized organic compound serves as the terminal electron acceptor, also occurs under anaerobic conditions.

It is well documented that microbial activities in landfills lead to the production of methane [25, 26]. However, lack of moisture in the landfill often slows this process [25, 27]. Given sufficient moisture, anaerobic microbial process, other than methanogenesis, can occur in landfills. The analysis of groundwater samples upgradient to and downgradient from a landfill indicated that the alkalinity was higher downgradient (consistent with the mineralization of organics to bicarbonate in the landfill), and that sulfate was depleted, suggesting that sulfate-reduction occurred in the landfill [28]. In addition, higher concentrations of soluble Fe(II) were found downgradient, likely a product of Fe(III) reduction in the landfill. From the examination of materials dug from a landfill, Gurijala and Suflita [27] reported that textiles appear to absorb and concentrate sulfate, thus, they would exist in a microenvironment that is rich in this terminal electron acceptor. Despite the variety of potential electron acceptors, most studies of fiber or film biodegradation have focused on methanogenic reactions, with a few investigations of nitrate reduction, fermentation and sulfate reduction.

Poly(3-hydroxybutyrate) is a thermoplastic that can be spun or filmed by conventional processes [29], and the anaerobic biodegradation of this polymer has been studied extensively. Under certain growth conditions, poly(3-hydroxybutyrate) accumulates as inclusion bodies that are bacterial carbon storage reservoirs; because poly(3-hydroxybutyrate) and related poly(hydroxyalkanoates) are simply bacterial carbon reserves, it is not surprising that they are readily biodegradable. In one investigation, Budwill *et al.* [30] supplemented anoxic activated sludge from a domestic sewage treatment plant and demonstrated that poly(3-hydroxybutyrate) is mineralized with nitrate as the terminal electron acceptor.

The biodegradation of poly(hydroxyalkanoates) under sulfate-reducing conditions has also been demonstrated [31, 32]. A mixture of a sulfate-rich lake sediment and anoxic lake water served as the growth medium and source of microorganisms. This mixture was supplemented with different amounts of poly(hydroxyalkanoates) as the carbon and energy source [32]. Sulfide was produced during incubation, and the rate of sulfide production increased with the amount of poly(hydroxyalkanoate) added to the cultures. The addition of molybdate, and inhibitor of sulfate-reduction, decreased the rates of sulfide production and poly(hydroxyalkanoate) degradation, indicating that this biodegradation process was driven by sulfate reduction.

In a pure-culture study, Janssen and Schink [33] elucidated the pathway for the depolymerization and subsequent fermentation of poly(3-hydroxybutyrate) by *Ilyobacter delafieldii*; the products of fermentation were acetate, butyrate, and H_2, with a molar ratio of acetate:butyrate of 2.32:1. In a co-culture of *I. delafieldii* and the H_2-consuming, sulfate reducer *Desulfovibrio vulgaris*, the molar ratio of acetate:butyrate changed to 4.02:1. Accordingly, the amount of H_2 detected in the co-culture was much lower in the presence

of the sulfate reducer, and sulfide production was observed [33]. This illustrates that even a simple mixture of microorganisms can alter the flow of carbon and electrons under anaerobic conditions.

Biodegradation of poly(hydroxyalkanoates) under methanogenic conditions has been the focus of several studies [34, 35, 36, 37]; using medium in which carbon dioxide was the sole terminal electron acceptor, Budwill *et al.* [34] demonstrated rapid methanogenesis from three different poly (hydroxyalkanoates). Methane production from these substrates was observed after 3 to 4 days of incubation. Gartiser *et al.* [35] examined a variety of test methods to assess the methanogenic biodegradability of a poly(hydroxyalkanoate) co-polymer and several other polymers. They concluded that the use of media formulations with inadequate amounts of bicarbonate or carbon dioxide resulted in poor methanogenic biodegradation.

Anaerobic degradation of other fibers has also been studied: when the anaerobic rumen fungus *Piromonas communis* was cultured on cotton fibers as its sole carbon source, it produced an extracellular cellulase that rapidly solubilized these fibers [38]. The cellulase enzyme system of *P. communis* was similar to that of another crystalline cellulose-degrading, anaerobic rumen fungus, *Neocallimastix frontalis* [39], and Nakashimada *et al.* [40] cultivated *N. frontalis* on cellulose in co-culture with two methanogens, *Methanobacterium formicicum* and *Methanosaeta conncilii*. Cellulose depolymerization and fermentation by the fungus yielded substrates, including acetate, formate, H_2 and CO_2, for the methanogens. *M. formicicum* produced methane from formate, H_2 and CO_2, whereas M. *conncilii* produced methane from acetate. These experiments demonstrated the breakdown of cellulose from cotton to produce methane in a defined microbial co-culture.

The anaerobic breakdown of cotton has also been considered as a method to produce volatile fatty acids and methane [41]. When the pH of a growth medium that contained cotton fibers as the carbon and energy source for a methanogenic consortium was adjusted to different initial values, Tükenmez *et al.* [41] observed that the amounts of fermentation products were different. The molar ratios of acetic, propionic, and butyric and methane were altered in this manner, suggesting that processes for producing specific fermentation products from cotton could be developed.

Using anaerobic, thermophilic incubation conditions, with wool as the major source of carbon in the growth medium, Riessen and Antranikian [42] isolated a new bacterium that was named *Thermoanaerobacter keratinophilus*. This isolate grows optimally at 70°C, and produces an extracellular protease that is responsible for the degradation of native keratin in the wool. One of the goals of this work was to isolate a thermostable keratinolytic enzyme that might be used to modify wool fibers in the textile industry.

1.4 Sources of microorganisms and enzymes for laboratory incubations

There are many different types of studies that are done to assess the biodegradation of fibers, textiles and films. These range from experiments that use undefined, natural microbial communities, to pure cultures of microorganisms, to highly purified enzymes that depolymerize macromolecules. Table 1.3 summarizes some of the different types of studies, beginning with the broad diversity of microbial activities in communities from natural sources, to and ending with work using purified enzymes.

Table 1.3 An overview of different types of studies that are done to assess the biodegradation of fibers, textiles and films

Types or sources of microorganisms or depolymerizing materials	Type or purpose of experiment	Examples and comments
Entire microbial communities	To assess biodegradation in soil	Passive soil burial tests simply involve burying fibers or textiles in soil for some period of time and then examining them for signs of biodegradation. Peacock [43] used this method to study the changes in linen, cotton, silk and wool over a 32-week incubation time. Modelli *et al.* [18] buried flax fibers in soil amended with ammonium phosphate to provide inorganic nutrients and CO_2 production was used to monitor the biodegradation of these fibers.
	To assess *in vitro* biodegradation	These types of experiments provide conditions that would help stimulate microbial activities. Keller *et al.* [44] provided a continuous stream of oxygen through a column of soil that was supplemented with nitrate to stimulate biodegradation of fiber composites. Kasuya *et al.* [45] used various natural water samples supplemented with many inorganic nutrients and an ample supply of O_2 to test the biodegradation of poly(3-hydroxybutyrate) and related co-polymers. The methanogenic degradation of these polymers by microbes in anaerobic sewage sludge was studied by Budwill *et al.* [34] and Abou-Zeid *et al.* [37] in culture

Table 1.3 Continued

Types or sources of microorganisms or depolymerizing materials	Type or purpose of experiment	Examples and comments
		systems that provided strict anaerobic conditions and all macro- and micronutrients.
Enrichment or selective culture	To isolate microbes that degrade a specific fiber or film	An insoluble polymer is used as the sole carbon source to select microorganisms that will use the polymer for growth. Using strict anaerobic methods and serial transfers, Janssen and Harfoot [47] isolated the poly(3-hydroxybutyrate) fermenting bacterium *Ilyobacter delafielkii*. Chang *et al.* [48] isolated the bacterium, *Chitinimonas taiwanensis*, which depolymerizes and grows on chitin. Two strains of poly(L-lactic acid)-degrading *Amycolatopsis* sp were isolated by detecting clear zones on the mineral agar plates containing poly(L-lactic acid) [49].
Pure cultures	To study the capability of a single species to degrade fibers or films	Pure cultures are obtained from a commercial or private culture collection for these studies. Shrivastava *et al.* [23] surveyed 10 fungal isolates to see which would degrade wool. Pranamuda and Tokiwa [50] obtained 25 strains of *Amycolatopsis* and tested their abilities to degrade poly(L-lactide). Ohkawa *et al.* [22] used seven fungal species to study the biodegradation of chitosan-gellan and poly(L-lysine)-gellan.
Crude preparations of depolymerizing material	To carry out biodegradation studies without viable micro-organisms or purified enzymes	Mixtures or unpurified extracellular enzymes from various sources are used for degradation studies. Using cotton fibers and other substrates, Wilson and Wood [39] detected three different types of enzyme present in culture filtrates of *Neocallimastix frontalis* RK21. Haga *et al.* [51] used commercially available crude cellulases from the fungus *Trichoderma viride* to test the extents of biodegradation of fibers that had

Table 1.3 Continued

Types or sources of microorganisms or depolymerizing materials	Type or purpose of experiment	Examples and comments
		different mercerization treatments. Christeller [52] used extracts from the midgut of the larva of the brown house moth to study the degradation of wool.
Purified enzymes	To understand the specificity of enzymes and the mechanisms of depolymerization, pure extracellular enzymes are studied	Enzymes are obtained by purifying from microbial cultures, or by purchasing from commercial sources. Ignatova *et al.* [53] purified a monomeric keratinase from a thermophilic actinomycete strain of *Thermoactinomyces candidus* that degrades wool. Pranamuda *et al.* [54] isolated a poly(L-lactide)-degrading enzyme produced by a *Amycolatopsis* sp. This enzyme also degrades silk powder. Wang *et al.* [55] purified a low molecular weight peptide from a cellulolytic fungus and studied its effects on cotton fibers. Arai *et al.* [56] purchased three proteolytic enzymes to study their degradation of silk fibers and films.

Passive soil burial experiments (Table 1.3) are often done under laboratory conditions, with soil and fibers incubated in closed containers. Typically, the soil and the fibers are moistened and the incubation container is sealed to ensure an adequate supply of water for microbial activity. For example, Peacock [43] maintained 65% relative humidity in incubation containers that contained linen, cotton, silk and wool in two different soil types. The goal of this work was to simulate wet archaeological burial environments to assess the decay of textile fabrics. The order of susceptibility to biodegradation was found to be linen > cotton > wool > silk. No nutrients were added to these soils [43]. In contrast, Modelli *et al.* [18] used the soil burial method (Table 1.3), but added ammonium and phosphate to stimulate microbial activity to ensure these essential nutrients were not limiting in the moistened soil. These were incubated under aerobic conditions for up to 180 days.

Experiments to test *in vitro* biodegradation of fibers or films are very common, and these are often the first tests done to assess the biodegradability

of a polymer. These take advantage of the diversity of microbial communities from various environments and provide conditions to increase the likelihood of observing biodegradation. As shown in Table 1.3, experiments are typically done by providing important macro- and micronutrients and an ample supply of O_2 for aerobic studies, or strict anaerobic conditions for anaerobic studies. The variety of sources of inocula can include soil [44], sediments [32], natural waters [45], aerobic sewage [46], and anaerobic sewage sludge [34, 37].

To gain a better understanding of biodegradation processes, researchers often isolate pure cultures of microorganisms for these studies. The selective or enrichment techniques used usually involve incubating a mixed microbial community from soil, or some other environment, in liquid culture with the selected fiber or film as the sole carbons sources (Table 1.3). After suitable incubation times, serial transfers are made into fresh medium with the same fiber or film as the growth substrate. After a few of these transfers, the culture will be enriched with those microorganisms that can degrade the provided carbons source. This method was used to isolate the poly(3-hydroxybutyrate)-fermenting bacterium *Ilyobacter delafielkii* [47] and the chitin-degrading bacterium *Chitinimonas taiwanensis* [48]. Nakamura *et al.* [49] used a modification of the enrichment procedure in which insoluble poly(L-lactic acid) was added to mineral agar plates, and those microorganisms that grew and hydrolyzed the polymer to soluble products produced clear zones around the colonies (Table 1.3). This method allows easy selection of isolates that degrade an insoluble polymer.

After pure cultures of bacteria or fungi have been isolated, they are typically stored in a researchers' private culture collections or deposited in a commercial culture collection such as the American Type Culture Collection or the United Kingdom National Culture Collection. Some investigations that used pure cultures are summarized in Table 1.3. As an example, Shrivastava *et al.* [23] tested the abilities of 10 fungal isolates to degrade wool. Four of these isolates were *Trichophyton simii* strains, and they all degraded keratin from wool; six of the isolates were *Aspergillus niger* strains, and only two of these degraded keratin. Pranamuda and Tokiwa [50] obtained 25 strains of *Amycolatopsis* from various commercial culture collections and tested their abilities to degrade poly(L-lactide). Seven pure strains of fungi were used by Ohkawa *et al.* [22] to study the biodegradation of fibers composed of chitosan-gellan and poly(L-lysine)-gellan. Both fibers were biodegradable under aerobic conditions, with the greatest amount of degradation observed with *Penicillium caseicolun* and *Aspergillus oryzae*. In addition to monocultures studies, pure cultures can be combined to monitor the effects of a simply, defined microbial community. For instance, Nakashimada *et al.* [40] incubated the cellulose-degrading fungus, *N. frontalis*, with two methanogens and observed methane production.

The biodegradation of fibers and other polymers is generally initiated by extracellular enzymes, thus, if a microbial culture is degrading a polymer, the enzyme or enzymes that attack the complex substrate can often be detected in the culture supernatant, after the cells have been removed by centrifugation or ultrafiltration. Two examples of this are given in Table 1.3, including the work of Wilson and Wood [39] who detected three different types of enzymes in the culture filtrates of the fungus *Neocallimastix frontalis* strain RK21 grown on cotton fibers. Some crude enzyme preparations are commercially available, and Haga *et al*. [51] used one of these preparations of cellulases to examine how different mercerization treatments affected cotton biodegradation. Mercerization is the treatment of cotton fibers to improve their strength, uniformity, luster and affinity for dyes [1]. In general, the untreated cotton was less susceptible to enzymatic attack than the cotton that was treated with ammonia and/or sodium hydroxide, due to the increase in amorphous regions in the fiber as a result of the mercerization treatment. Extracts from sources other than microbial cultures can also be tested for fiber depolymerizing activity. This is illustrated by the work of Christeller [52] who used extracts from the midgut of the larva of *Hofmannophila pseudospretella* (brown house moth) to study the degradation of wool. These experiments were done under highly anaerobic conditions, and they demonstrated that the degradation of this fiber consisted of reduction and solubilization, followed by proteolysis of the wool protein.

Finally, purified enzymes can be used to study their characteristics, specificity and the mechanisms of depolymerization of macromolecules (Table 1.3). Ignatova *et al.* [53] grew a thermophilic actinomycete strain of *Thermoactinomyces candidus* that produced a keratinase in liquid medium with wool as the sole source of carbon and nitrogen. The monomeric enzyme was purified 62-fold and it was found to have a molecular weight of 30 kDa, with the pH and temperature optima of 8.6 and 70°C, respectively. The purified enzyme catalyzes the hydrolysis of a variety of proteins and the keratins in hair, feathers and horns. Pranamuda *et al*. [54] isolated a poly(L-lactide)-degrading enzyme produced in a liquid culture of *Amycolatopsis* sp. (strain 41), the molecular weight of the enzyme was estimated to be between 40 and 42 kDa, with pH and temperature optima of 6.0 and 37 to 45°C, respectively; this enzyme also degrades silk powder. The culture supernatant of the cellulolytic fungus *Trichoderma pseudokoningii* was the source of a low molecular weight peptide isolated by Wang *et al*. [55]; this peptide chelates Fe(III) and reduces it to Fe(II) in the presence of O_2, generating a free radical that leads to the destruction of hydrogen bonds and cleavage of glycosidic bonds; these mechanisms produce short fibers of cellulose. Rather than isolating enzymes, Arai *et al*. [56] purchased three proteolytic enzymes to study their degradation of silk fibers and films (Table 1.3); these included collagenase type F, α-chymotrypsin type I-S, and protease type XXI. All three enzymes hydrolyzed silk, but the protease was the most active.

1.5 Analytical methods used to assess biodegradation of fibers and films

Microbial metabolism of a substrate causes chemical changes to that substrate. If the substrate has a microscopic or macroscopic form, the chemical changes brought about by the microbial metabolism can change the physical form or structure of the substrate. A variety of physical and chemical methods are available to follow the changes caused by microbial metabolism of a substrate; the methods used depend to a large extent on the size of the substrate. For example, if the substrate is a simple soluble molecule, such as an amino acid, in an aqueous growth medium, physical methods would have very limited applicability. However, chemical methods would provide ample evidence of biodegradation of the amino acid. A variety of chemical analyses could be used in this case, including monitoring the decrease in concentration of the substrate, measuring the release of ammonium or carbon dioxide from the biodegradation of the amino acid.

On the other hand, if the substrate is large and insoluble in water, such as a fiber or a film of biodegradable material, physical measurements are commonly used to assess the initial microbial attack on the substrate. These methods include microscopic examination, measure of weight loss, or measure of the loss of mechanical strength. As the biodegradation of the fiber continues, individual small molecules are released and chemical analyses of monomers and products of mineralization can be detected. Often, several of these methods are used in a single study to confirm and characterize the biodegradation of the test material.

1.5.1 Detecting subtle changes in fiber structure or composition

Some of the initial reactions during the biodegradation of a fiber may produce only small changes in its structure or composition. These are typically observed using very sophisticated instrumental methods. Amass *et al.* [57] reviewed the use of many of these methods, so only a few examples are given here.

Infrared spectroscopy or Fourier transformed infrared spectroscopy (FT-IR) is often used to detect changes in crystallinity or minor chemical changes in a fiber. Wang *et al.* [55] used the 'finger-print region' of 1400 to 900 cm^{-1} to detect changes in the crystallinity of cotton fibers treated with a peptide that shortened the fibers. Their infrared data also indicated that the intermolecular hydrogen bonds were disrupted. Increases in specific IR absorbances can be used to indicate changes in fibers. For example, Khabbaz *et al.* [58] observed that microbial activity on poly(L-lactide) produced a new absorbance at 1600 cm^{-1}, which they interpreted as being due to an increased number of carboxylate ions in the residual polymer. Similarly, Frisoni *et al.*

[59] observed increased absorbance at 1540 and 1650 cm^{-1}, when acetylated cellulose fibers were examined after 13 days' incubation with a cellulolytic bacterial strain. These absorbances are characteristic of the amide group, and Frisoni *et al.* [59] surmised that proteins were bound to the residual fibers.

X-ray diffraction can be used to study changes in fabrics during biodegradation. Park *et al.* [60] contacted cellulose fabrics with soil microorganisms and measured changes in crystallinity by X-ray diffraction measurements. They observed that the crystallinity increased, during incubation; this was attributed to the initial preferential biodegradation of the amorphous regions of the fibers, leaving the more resistant crystalline regions.

Solid-state cross-polarization/magic-angle-spinning (CP/MAS) ^{13}C-nuclear magnetic resonance (NMR) spectrometry can also be used to detect change in crystallinity of polymers. Vishu Kumar *et al.* [61] treated chitosan with a serine protease producing low molecular weight, depolymerized chitosans. The native chitosan gave CP/MAS ^{13}C-NMR peaks with narrower widths than those from the low molecular weight chitosans, indicating that depolymerization gave products with lower crystallinity than the native fibers.

1.5.2 Visual observations and microscopy

An early indication of biodegradation of a fiber or film is a change in its appearance. This may occur on a macroscopic scale, so the changes can be observed with the naked eye. Figure 1.1 shows the obvious changes in the appearance of a film of poly(3-hydroxybutyrate) after incubation in an anaerobic culture for 10 days [62]; the top strip was incubated in sterile medium, whereas the bottom strip was incubated in the viable culture.

The depolymerization of fibers or films leads to the formation of water-soluble small molecules; this results in the dissolution of the fiber or film, accounting for the 'holes' in the film in Fig. 1.1. This type of biodegradation can also be observed by placing a polymer on the surface of an aqueous medium or in an agar plate. As an example, Ratajska *et al.* [46] floated chitosan (*N*-deacetylated chitin comprised of chains of D-glucosamine) in liquid medium containing mixed microbial community from a wastewater. Initially, the polymer covered essentially the entire surface of the liquid. Over a 5-week incubation period, dissolution caused the diameter of the polymer mat to decrease until it was about one-fifth of its original size.

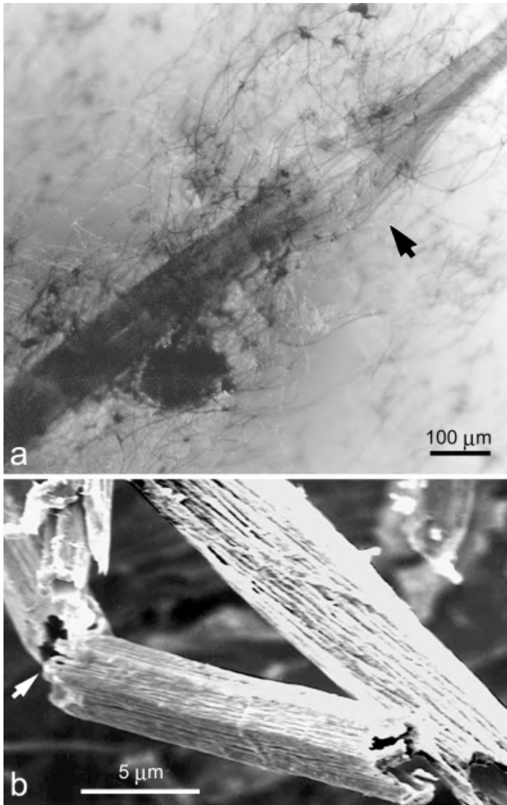
To isolate silk-degrading microorganisms, Tokiwa *et al.* [63] incorporated washed silk powder in agar plates. After inoculation and incubation, those microorganisms that utilized the silk by depolymerizing and solubilizing it were readily detected because they produced clear zones around the colonies. Subsequently, a silk-degrading, filamentous bacterium (an *Amycolatopsis* sp.) was shown to produce clear zones in a plate that contained poly(L-lactide).

1.1 Biodegradation of poly(3-hydroxybutyrate) films under methanogenic conditions. The top strip was incubated in sterile medium and the bottom strip was incubated in anaerobic cultures for 10 days at 35°C. Initially, the strips were 0.016 mm thick and 1 cm × 7 cm. Reprinted with permission from Budwil [62]. Copyright 1995 K. Budwill.

Microscopy is often used to observe microbial colonization and physical changes in fibers. Figure 1.2a shows the colonization of a poly(L-lysine)-gellan fiber by the fungus *Curvalaria* sp. [22]. Fibers (1.5 to 2 cm in length) were incubated with this fungus in aqueous medium for 40 days prior to microscopic examination. Biodegradation led to the collapse of the fiber at the location shown by the arrow in Fig. 1.2a. Scanning electron microscopy is also used to view the effects of biodegradation; this provides much higher magnification than light microscopy. Scanning electron microscopy was used to observe the damage to flax fiber (Fig. 1.2b) incubated for 13 days with the cellulolytic bacterium *Cellvibrio fibrivorans* [18]. This photo shows that the fibers remained cylindrical but they were shortened by the microbial activity.

1.5.3 Measuring weight loss

Biodegradation of fibers or films result in the dissolution of part or all of the material, resulting in an overall weight loss of the material. Indeed, measuring weight loss is the common method for detecting biodegradation of a variety of insoluble fibers or films. For example, Shrivastava *et al.* [23] used weight loss as one means of detecting the biodegradation of wool by the fungal species *Trichophyton simmi* and *Aspergillus niger*. Each culture received



1.2 (a) Examination of a poly(L-lysine)-gellan fiber by light microscopy showing growth of the fungus *Curvalaria* sp. After 40 days' incubation, biodegradation led to the fracture of the fiber shown by the arrow. Reprinted from Ohkawa *et al.* [22] with kind permission of the authors and Springer Science and Business Media (copyright 2000). (b) Scanning electron micrograph of flax fibers after 13 days' incubation with *Cellvibrio fibrivorans* at 28°C. The arrow shows the fracture of the fiber. Reprinted with permission from Modelli *et al.* [18]. Copyright 2004 American Chemical Society.

250 mg of wool, and after 4 weeks of incubation in unicultures, the weight losses were 58% and 22% in the *T. simmi* and *A. niger* cultures, respectively.

Weight loss measurement was one of several methods used to study the biodegradation of cellulose fibers from flax by two strains of *Cellvibrio* [59]. These aerobic cultures initially contained 0.1 g of natural fiber or fibers with different degrees of acetylation. After 13 days of incubation, weight losses of 20% to 76% were recorded; these data showed that more highly acetylated fibers were more resistant to biodegradation.

Measuring weight loss of polymers has also been used to follow biodegradation in anaerobic culture systems. Abou-Zeid *et al.* [37] incubated

individual films of poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-co-3-hydroxyvalerate), and poly(ε-caprolactone) in methanogenic cultures. Over a 10-week incubation time, the weight loss of the first two polymers was much greater than that of the latter polymer.

1.5.4 Tensile properties (breaking load)

Hydrolysis of chemical bonds during microbial depolymerization of macromolecules weakens fibers, and this can be detected by measuring tensile properties, as illustrated in a study by Seves *et al.* [64]. These researchers buried pieces of silk fabric (1 cm × 1 cm) in soil and these were incubated for up to 2 months. Yarns were removed from fabric and subjected to a breaking load test. Prior to burial, the mean breaking load of the warp yarns was 297 grams-force, whereas after 2 months' incubation, this was drastically reduced to only 2 grams-force.

Similarly, Arai *et al.* [56] studied the degradation of silk fibroin fibers by several proteolytic enzymes. They monitored loss of weight, tensile strength, changes in the infrared spectra, changes in the molecular weight distribution of fibroin, and in the amounts of soluble polypeptides released by the actions of the proteolytic enzyme. Of all the methods used, the tensile strength measurement was the most sensitive for detecting the onset of the silk fiber biodegradation.

1.5.5 Detecting the products of mineralization

Table 1.1 summarized the most common products of microbial mineralization. Heterotrophic metabolism leads to oxidation of some of the substrate carbon to carbon dioxide, if this activity occurs under methanogenic conditions; both carbon dioxide and methane are mineralization products. Thus, monitoring increases in the amounts of one or both of these products in a closed culture system provides sound evidence to demonstrate mineralization of the substrate being tested for its biodegradability. Although most studies monitor the products of organic carbon mineralization, detecting the products of the mineralization of organic sulfur and organic nitrogen from substrates also provides excellent evidence of microbial degradation of these compounds, for example, the fungal metabolism of wool led to the release of ammonium and sulfate as the mineralization products of the nitrogen and sulfur, respectively [20, 21].

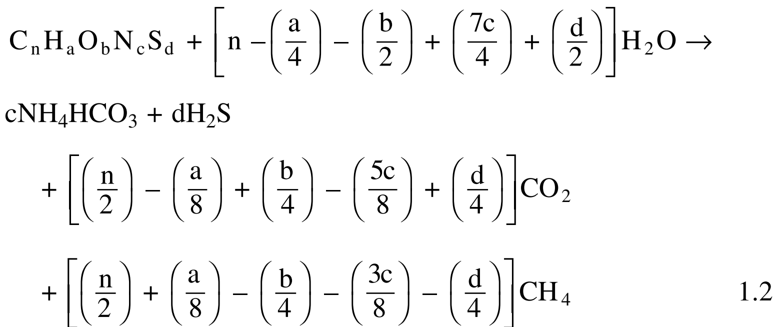
Carbon dioxide formation was measured by Keller *et al.* [44] who studied the kinetics of aerobic biodegradation of fiber composites comprised of degummed hemp and a polyesteramide in a column that contained 300 g of soil mixed with a fiber composite. A constant stream of oxygen was passed through the column and the effluent gas was passed through a sodium hydroxide solution to trap the carbon dioxide resulting from mineralization of the fiber

composite. The amount of trapped carbon dioxide was determined by titration with hydrochloric acid.

Biochemical oxygen demand tests with aqueous culture systems [45] can be used to measure the volume of carbon dioxide liberated from the mineralization of a substrate. Ohkawa *et al.* [22] used this method to follow the mineralization of fibers of the polyion complexes of chitosan-gellan and poly(L-lysine)-gellan. Filamentous fungi were used in this investigation and carbon dioxide release was observed.

The methanogenic biodegradability of several polymers, including cotton, cellulose acetate fibers, poly(lactide), and poly(hydroxyalkanoates), was studied by Gartiser *et al.* [35]. The increase in gas volume (estimated from excess pressure in the culture headspace or collected in graduated collecting tubes) was used as the measure of biogas production, which is the sum of methane and carbon dioxide formed. Gartiser *et al.* [35] stated that the theoretical yield of biogas was 2.23 mL for each milligram of organic carbon in the growth medium. Most of the polymers yielded biogas, but widely different yields were obtained depending on the growth medium used and the polymer. Incubation times ranged from about 35 to 80 days. Methanogenic degradation of cotton typically yielded 40 to 75% of the theoretical biogas yield. In sharp contrast, the biogas yield from poly(lactide) was less than 10% of theoretical yield [35].

The amounts of methane and carbon dioxide produced from a substrate can be estimated by Buswell's equation. A form of this equation for substrates that contain carbon, hydrogen, oxygen, nitrogen and sulfur [65] is shown in equation 1.2:



$$C_nH_aO_bN_cS_d + \left[n - \left(\frac{a}{4}\right) - \left(\frac{b}{2}\right) + \left(\frac{7c}{4}\right) + \left(\frac{d}{2}\right)\right] H_2O \rightarrow$$

$$cNH_4HCO_3 + dH_2S$$

$$+ \left[\left(\frac{n}{2}\right) - \left(\frac{a}{8}\right) + \left(\frac{b}{4}\right) - \left(\frac{5c}{8}\right) + \left(\frac{d}{4}\right)\right] CO_2$$

$$+ \left[\left(\frac{n}{2}\right) + \left(\frac{a}{8}\right) - \left(\frac{b}{4}\right) - \left(\frac{3c}{8}\right) - \left(\frac{d}{4}\right)\right] CH_4 \qquad 1.2$$

Of course, if the substrate contains no nitrogen or sulfur, the values of 'c' and 'd' are zero, and the number of moles of NH_4HCO_3 and H_2S expected are zero. Similarly, the 'c' and 'd' terms in the calculation of the expected CO_2 and CH_4 are zero.

Budwill *et al.* [34] studied the methanogenic degradation of poly(3-hydroxyalkanoates). The poly(3-hydroxybutyrate) used in that study had a molecular weight of about 10^6 daltons, giving an empirical formula of

$C_{48000}H_{72000}O_{24000}$. Based on Buswell's equation, total gas volume measurements, and gas chromatography analyses, the yields of methane and carbon dioxide were 108% and 60%, respectively, of the predicted yields. The overall biogas recovery was 87% of predicted amount [34]. Of course, equation 1.2 does not account for carbon incorporation into biomass, and typically about 10% of the organic carbon in the substrate is converted to cellular material under methanogenic conditions. Thirteen percent of the polymer carbon was not found as biogas [34], and this proportion is consistent with the expected amount of carbon that would be incorporated into biomass under the growth conditions used in that study.

1.5.6 Detection of intermediates of biodegradation of fibers and films

Finding the products of mineralization demonstrates extensive or complete biodegradation of fibers or films, and the methods to detect these products are quite simple. However, partial degradation through depolymerization leads to the reduction in molecular weight of the polymer, and to the production of intermediates with various molecular sizes. These products are often more difficult to detect than those of mineralization, but several different methods can be used for this. The choice of the method is dictated by the size of the intermediates; when the intermediates are still polymeric, they are usually characterized by size exclusion (gel permeation) chromatography, often using high performance liquid chromatography (HPLC) methods.

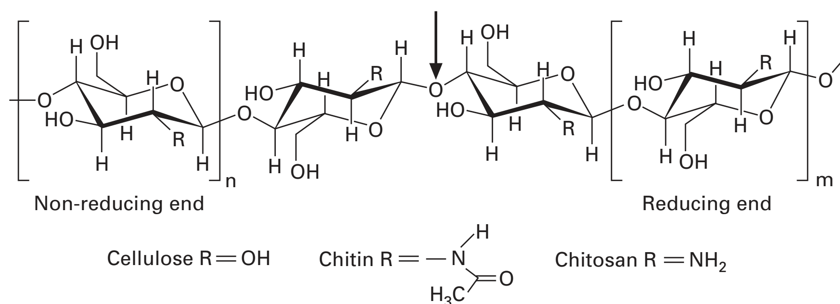
Size exclusion chromatographic analyses showed that a microbial community incubated with chitosan at 20°C yielded polymers with a weight average molecular weight (M_w) of 7.8 kDa [46]; this was a sharp decrease from the initial M_w of the chitosan that was 261 kD. Based on size exclusion chromatographic analyses, Hakkarainen *et al.* [66] showed that the biodegradation of poly(L-lactide) led to lower molecular weight polymers; the initial M_w of the substrate was about 550 kDa, and after 4 weeks of incubation with compost microorganisms, the M_w decreased to about 350 kDa. In another study, Arai *et al.* [56] tested the abilities of three different commercial enzymes to degrade silk fibroin with M_w of 119.8 kDa. After 17 days' incubation with collagenase type F, α-chymotrypsin type I-S, or protease type XXI, the M_w decreased to 94.5, 53.7, and 102.4 kDa respectively. Clearly, size exclusion chromatography can provide sound evidence of depolymerization.

Further biodegradation of polymers yields oligomers and monomers. A variety of different analytical methods are used to detect these low molecular weight compounds. For example, Chang *et al.* [48] isolated a Gram-negative bacterium, *Chitinimonas taiwanensis*, that depolymerizes and grows on chitin (a homopolymer of β-(1 → 4)-linked *N*-acetyl-β-D-glucosamine). HPLC

analysis of a culture supernatant showed that the major intermediate was a chitotriose, consisting of three *N*-acetyl-β-D-glucosamine moieties. In another study, Wiegand *et al.* [19] used HPLC-mass spectrometry to detect and identify biodegradation products of polyester amide BAK 1095. They found monomers of adipic acid and aminocaproic acid, oligomers of these two acids in various proportions and more complex oligomers. Nakamura *et al.* [49] isolated and purified a poly(L-lactate)-degrading enzyme from an actinomycete. Mixtures of the purified enzyme and the polymer yielded the monomer, lactate, which was detected by thin layer chromatography.

1.6 Examples of types of bonds that are susceptible to enzymatic attack

Cellulose is a major component of some natural fibers; cotton contains about 94% cellulose [67] and the flax fibers contain about 60–80% cellulose [68]. The enzymatic hydrolysis of cellulose has been reviewed by Mansfield *et al.* [5] and Leschine [69]. Figure 1.3 shows the structure of cellulose (R = OH), which is a homopolymer of glucose moieties jointed via β-(1 → 4)-linkages. Depolymerization occurs at these glycoside bonds (shown by the arrow in Fig. 1.3). The cellulases that catalyze these hydrolyses fall into three groups: (a) endoglucanases; (b) exoglucanases; and (c) β-glucosidases. The endoglucanases randomly hydrolyze internal β-(1 → 4)-glycosidic bonds (where n and m are large integers) thereby quickly decreasing the polymer length but slowing increasing the concentration of reducing sugars [70]. In contrast, the concentration of reducing sugars increases rapidly through the activity of exoglucanases. These enzymes remove cellobiose from the non-reducing end of cellulose (n = 1, and m is a large integer). As a result, the polymer length decreases slowly, because only two glucose moieties are removed with each hydrolysis. β-Glucosidases hydrolyze cellobiose (n = m = 0) and short oligosaccharides (n = 0, and m is a small integer) to release

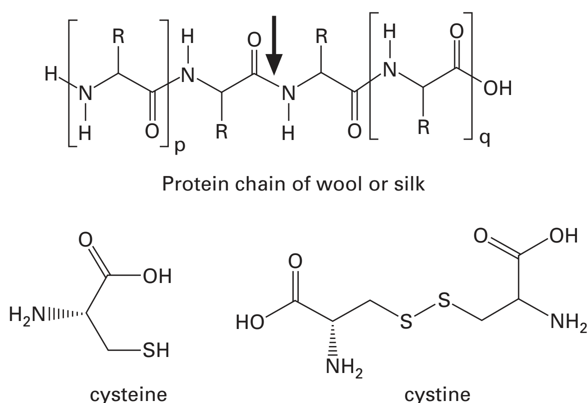


1.3 Structures of cellulose, chitin and chitosan. The arrows indicate the location of enzymatic hydrolysis.

glucose. Some, but not all, cellulose-degrading microorganisms produce all three types of cellulases, and these are usually three different enzymes [70]. However, some cellulases can exhibit more than one activity as illustrated by the cellulase isolated and characterized by Han *et al.* [70]. This enzyme has both endo and exo activities.

Figure 1.3 also shows the structure of chitin [R = NHC(O)CH_3]. The hydrolysis of this polymer is similar to that of cellulose, involving chitinase that randomly hydrolyzes β-(1 → 4)-glycosidic bonds (where n and m are large integers), chitobiase that carries out the hydrolysis of non-reducing sugar, and chitobiohydrolase that removed dimeric units from the non-reducing end (n = 1, and m is a large integer) [71]. Chitin deacetylase hydrolyzes the *N*-acetamido bonds in chitin to yield chitosan (Fig. 1.3, R = NH_2). Chitosan is depolymerized by chitosanases in a manner similar to the depolymerization of chitin. Type XXV serine protease from *Streptomyces griseus* also depolymerizes chitosan yielding low molecular weight chitosan (dimer to hexamer) and the monomer [61].

Protein is the major component of wool, comprising about 97%, with about 2% being lipids and 1% being mineral salts, carbohydrates and nucleic acid residues [72]. Hydrolysis of the peptide bonds in the wool protein yields 18 amino acids [73], including sulfur-containing amino acids, most notably cysteine (Fig. 1.4). Keratins (including wool) are distinguished by their high cystine content, which is approximately 500 μmol g^{-1} of wool. A disulfide bridge between two cysteine residues forms cystine (Fig. 1.4). Adjacent protein chains are often cross-linked through the disulfide bonds of the numerous cystine moieties [73]. Thus, the biodegradation of wool required the hydrolysis of peptide bonds (Fig. 1.4) and cleavage of disulfide bonds.



1.4 General structure of a protein (different R groups represent various amino acids) and the structures of cysteine and cystine. The arrow indicates the location of enzymatic hydrolysis.

The biodegradation of keratins is hampered by the cross-linking of disulfide bonds because they hinder the accessibility of peptide bonds to protein-hydrolyzing enzymes. Under the low redox conditions in an anaerobic environment, it appears that the disulfide bonds are reduced to loosen the peptide chains ($chain_1–S–S–chain_2 + 2H \rightarrow chain_1–SH + HS–chain_2$). Christeller [52] proposed a model for keratinolysis in the midgut of *H. pseudospretella* which included reduction of disulfide bonds in this manner. *In vitro* the addition of the reducing agent 1,4-dithiothreitol to split disulfide bonds stimulated the lysis of keratin by an extracellular keratinase isolated from a wool-degrading actinomycete [53].

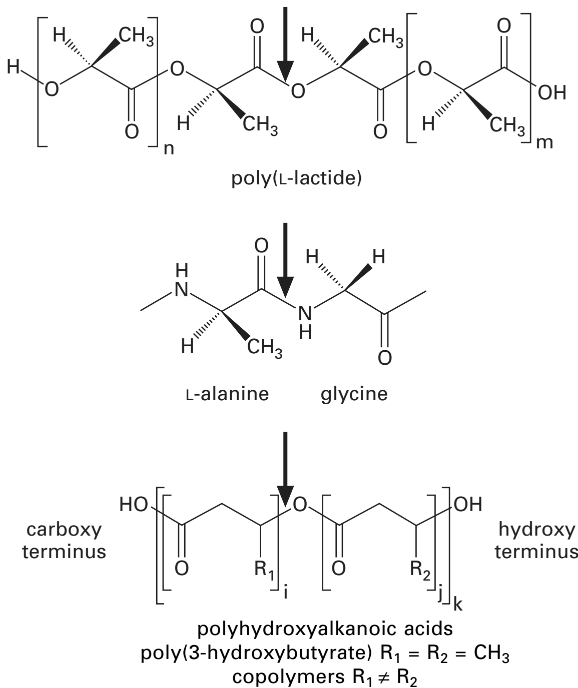
Sulfitolysis is another means by which the disulfide bond is broken by some fungi. This reaction occurs in the presence of sulfite and under alkaline conditions. It cleaves the disulfide in cystine to S-sulfocysteine and cysteine [74, 75]. This causes the keratin chains to denature by releasing them from one another ($chain_1–S–S–chain_2 + SO_3^{2-} \rightarrow chain_1–S–SO_3^- + {}^-S–chain_2$). The fungal oxidation of thiols in the cysteine residues yields sulfide for this reaction, and the liberation of ammonium from the deamination of the amino acids creates the alkaline conditions to help drive this reaction [76].

After the structure of keratin in the wool is loosened by breaking the disulfide bonds, extracellular proteolytic enzymes hydrolyze the peptide bonds as shown in Fig. 1.4. The hydrolysis releases soluble peptides that are further hydrolyzed to amino acids.

Silk is also a proteinaceous fiber. Raw silk from the silkworm *Bombyx mori* is made primarily of a filamentous protein called fibroin held together by a gum-like protein called sericin. Before weaving, the raw silk is degummed to remove sericin [64]. Fibroin comprises heavy-chain and light-chain proteins held together by disulfide bonds. Tanaka *et al.* [77] provide evidence that there is only one disulfide bond between each heavy-chain and light-chain molecule in silk from *B. mori*.

Not surprisingly, the biodegradation of silk is similar to the biodegradation of wool, except that, because there are many few disulfide bonds in silk, they do not hinder proteases to any great extent. For example, Arai *et al.* [56] demonstrated the breakdown of silk by three commercially available proteases, without having to add a reducing agent to break the disulfide linkages. These proteases hydrolyze the peptide bonds shown in Fig. 1.4. Silk also contains a few small peptides and two of these have been shown to inhibit some bacterial and fungal proteinases [78]. This is a clever adaptation that the silk-producing insects have evolved to increase the longevity of their silk by inhibiting depolymerization.

The structure of poly(L-lactide) is shown in Fig. 1.5. The L-lactic acid unit of this polymer is structurally very similar to L-alanine and glycine (Fig. 1.5). Silk from *B. mori* is rich in glycine (44.6 mol%) and L-alanine (29.5 mol%) [63]. Because of these structural similarities, Tokiwa *et al.* [63]



1.5 The structure of poly(L-lactide) compared to the structures of L-alanine and glycine moieties, and the general structure of poly(hydroxyalkanoic) acids. The arrows indicate the location of enzymatic hydrolysis.

postulated that silk-degrading microorganisms might be able to degrade poly(L-lactide). They verified this by isolating a silk-degrading actinomycete that also degraded poly(L-lactide). Pranamuda and Tokiwa [50] surveyed 25 strains of *Amycolatopsis*, and found that 13 of these degraded silk. Of these, 12 also degraded poly(L-lactide).

Tokiwa and Jarerat [79] reviewed the biodegradation of poly(L-lactide). They wrote that proteinase K and alkaline proteases from *Bacillus* are able to degrade poly(L-lactide). Studies also showed that a poly(L-lactide)-degrading enzyme isolated from a strain of *Amycolatopsis* was a serine type protease that also degrades silk fibroin; thus, there is considerable evidence that many enzymes that can hydrolyze peptide bonds (as illustrated by the bond between L-alanine and glycine in Fig. 1.5) are also able to hydrolyze the ester bond in poly(L-lactide) (Fig. 1.5). This hydrolysis ultimately releases lactic acid, which is readily metabolized by many microorganisms. Surprisingly, lipases do not cleave the ester bonds in poly(L-lactide) [79], but will hydrolyze the amorphous polymer poly(DL-lactide).

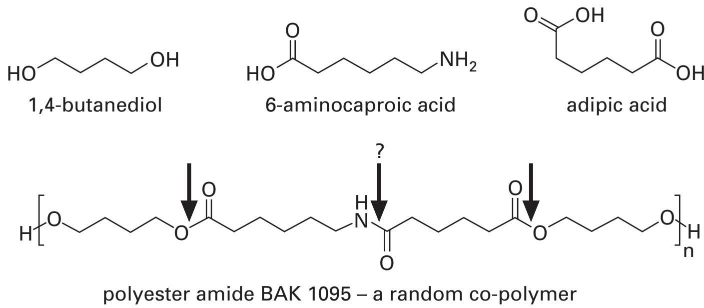
Like poly(lactides), the monomers of poly(hydroxyalkanoates) are linked by ester bonds, as illustrated in Fig. 1.5. Among the most common

poly(hydroxyalkanoates) are poly(3-hydroxybutyrate) (shown in Fig. 1.5 where $R_1 = R_2 = CH_3$) and simple co-polymers such as poly(3-hydroxybutyrate-3-hydroxyvalerate) where $R_1 = CH_3$ and $R_2 = CH_2CH_3$. In the latter case, the proportions of the 3-hydroxybutyrate and 3-hydroxyvalerate monomers incorporated into the co-polymer can vary, giving different values of the subscripts 'i' and 'j' in the general formula in Fig. 1.5. However, there are a wide variety of monomers (e.g. R_1 and R_2 in Fig. 1.5) that can be incorporated into different poly(hydroxyalkanoates). For example, Steinbüchel and Valentin [80] reported 91 different hydroxyalkanoic acids that are found in natural poly(hydroxyalkanoates).

Poly(hydroxyalkanoates) are polyesters that exist inside of many bacterial cells as storage compounds of carbon and energy, and they also exist outside of the cells. Enzymes that degrade the intracellular (native) polyesters are different from those that degrade the extracellular polyesters [81]. This discussion will focus on the enzymes that depolymerize the extracellular poly(hydroxyalkanoates), which are more crystalline than the intracellular polyesters. Of course, man-made fibers and films made of poly(hydroxyalkanoates) exist outside of microbial cells, and therefore extracellular depolymerases are required for their biodegradation.

Most, if not all, extracellular depolymerases have endo- and exohydrolase activities on poly(hydroxyalkanoates) [81]. The endohydrolase activity randomly cleaves ester bonds some distance from the terminus of the molecule, yielding smaller polymers. The exohydrolase activity cleaves ester bond near the terminus of the polymer, yielding monomers, monomers and dimers, or a mixture of oligomers. Shirakura *et al.* [82] demonstrated that the purified depolymerase from *Alcaligenes faecalis* strain T_1 hydrolyzed insoluble poly(3-hydroxybutyrate) and soluble trimer and larger oligomers of D-(–)-3-hydroxybutyrate. This enzyme cleaved only the second and third ester linkages from the hydroxy terminus (Fig. 1.5, $R_1 = R_2 = CH_3$ and $j = 2$ or 3) of the trimer and tetramer, but it acted as a endohydrolyase with the pentamer or higher oligomers.

The biodegradable polymers shown in Figs 1.3 and 1.5 are all of biological origin. In contrast, the polyester amide BAK 1095, shown in Fig. 1.6, is chemically synthesized as a random co-polymer of 1,4-butanediol, 6-aminocaproic acid and adipic acid. BAK 1095 contains 40% ester bonds and 60% amide bonds. Wiegand *et al.* [19] demonstrated that the ester bonds are hydrolyzed by a commercial esterase, and they isolated 15 bacterial strains that grew on this co-polymer. All of the isolates depolymerized BAK 1095 by hydrolyzing the ester bonds, but based on the analyses of accumulated products, there was no evidence to indicate that the isolates cleaved the amide bonds (indicated by a question mark in Fig. 1.6). Thus, oligoamides accumulated in the growth medium that contained ammonium nitrate as the nitrogen source. However, Wiegand *et al.* [19] cited several studies that have



1.6 The general structure of the random polyester amide BAK1095. The molecule shown contains two moieties from 1,4-butanediol, one moiety from 6-aminocaproic acid and one moiety from adipic acid. The arrows show enzymatic cleavage sites. The question mark indicates that cleavage of the amide bond was not clearly established (Weigand *et al.*) [19].

demonstrated cleavage of amide bonds, so it is very likely that each of the bond types shown in Fig. 1.6 is broken. The presence of the readily available ammonium nitrate in the medium used by Wiegand *et al.* [19] would have precluded selection of microorganisms that split the amide bonds and extract the organic nitrogen for cell growth.

1.7 Future trends

The microbial processes involved in hydrolysis of biodegradable fibers are now very well understood, including environmental conditions under which biodegradation can occur and the types of chemical bonds that are amenable to microbial attack. In addition, a wide variety of procedures and analytical methods have been established to follow biodegradation of fibers, films and textiles. Thus, evaluation of the biodegradability of novel fibers introduced in the future will be straightforward. With the knowledge and experience gained from studying biodegradation and the enzymology associated with these processes, it is now possible to apply these to improve the treatments of natural materials to yield usable fibers with less impact on the environment, and to improve the qualities of the fibers and textiles. A few examples are given below.

Many raw fibers require degumming before they can be used. Conventional processes that remove plant gum from ramie and hemp use hot alkaline solutions, and these methods have high energy demands, of raising concerns about disposal methods. As an alternative, Kapoor *et al.* [83] evaluated the use of a polygalacturonase from a bacterium (*Bacillus* sp. strain MG-cp-2) to degum ramie and hemp to produce cellulosic fibers. In experiments with ramie, a chemical treatment with 2% NaOH at 90°C for 8 h released 4.1

μmol of reducing sugar per cm^3, whereas an enzyme treatment at 50°C for 9 h released 7.6 μmol of reducing sugar per cm^3. In contrast, the enzyme treatment of hemp released less reducing sugar than did the chemical treatment. Kapoor *et al.* [83] concluded that sequential chemical and enzyme treatments provided a better fiber.

Degumming is also applied to raw silk to remove the protein sericin from the fibroin filaments. The typical method for sericin removal involves dissolving this protein by boiling the raw silk in aqueous solutions containing alkali and detergents; this process consumes large amounts of water and energy. Seeking a 'greener' treatment, Freddi *et al.* [84] tested the abilities of four proteases (three from microorganisms and one from a plant) to act as degumming agents. The standard condition for chemical degumming was treating silk at 98°C for 1 h in a solution of soap (10 g dm^{-3}), and sodium carbonate (1 g dm^{-3}). In contrast, no detergent was used with the enzyme treatments and the temperatures of the treatments were between 50 and 65°C, with a pH of 3 to 10, depending on the enzyme. The alkaline and neutral proteases effectively degummed the silk, giving nearly complete removal of the sericin.

The use of microbial and mammalian transglutaminases to modify wool textiles has recently been reported [85]. These enzymes cross-link proteins leading to increased protein stability and increased resistance to proteolytic and chemical degradation. Treating wool with these transglutaminases increased the tensile strength of yarns and reduced felting shrinkage in the fabric. The enzymatic treatment is more environmentally friendly than the conventional treatment of wool with chlorine to prevent felting shrinkage [85].

Tzanov *et al.* [86] investigated the preparation of cotton fabrics using a series of enzymatic treatments. To replace conventional alkaline boiling as a means of scouring (e.g. 0.2 M NaOH in 1 g dm^{-3} surfactant), they evaluated the use of commercial bacterial and fungal pectinases for 'bio-scouring' cotton. The presence of a surfactant improved the scouring with both enzymes, and these treatments at 40°C and 55°C for 2 h resulted in an absorbency and removal of fiber cuticle comparable to the conventional alkaline boiling.

Hydrogen peroxide is commonly used to remove the natural gray color from cotton. Tzanov *et al.* [86] also explored the enzymatic production of hydrogen peroxide using glucose oxidase with glucose and oxygen as substrates. In this 'bio-bleaching' process, the rate of aeration was key to the success of the method because glucose and protein appeared to stabilize the hydrogen peroxide. Tzanov *et al.* [86] extended this work and established a closed loop process of scouring, bleaching and reusing starch contained in desizing baths.

Advances made over the past few decades have improved our understanding of the biodegradation of fibers, films, and fabrics. Experience has shown which types of polymers are susceptible to biodegradation, and which types are not susceptible. Applying this solid background of information, new

fibers and composites can be developed that will be biodegradable and will not remain in the environment indefinitely. Building on our knowledge of microbial and enzymatic activities, new environmentally-friendly processes can be developed to provide more sustainable fiber and fabric production.

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