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7. Microbial biodegradation of polyurethane

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Abstract. Polyurethane (PU) is a general term used for a class of polymers derived from the condensation of polyisocyanate and polyol. Polyurethanes are an important and versatile class of man-made polymers used in a wide variety of products in the medical, automotive and industrial fields. Depending on the chemical structures of the polyisocyanates and polyols, PU can adopt various forms ranging from flexible to rigid and from low density to solid elastomer. Over three-fourths of the global consumption of PU is in the form of foams amounting to approximately 5 % of the total amount of plastic produced.

Despite its xenobiotic origin, PU has been found susceptible to biodegradation by naturally occurring microorganisms. Microbial degradation of PU is dependent on the many properties of the polymer such as molecular orientation, crystallinity, cross-linking and chemical groups present in the molecular chains which determine the accessibility to degrading-enzyme systems.

Several reports have appeared in the literature on the susceptibility of PU by fungal attack. Results varied from fungal isolates utilizing colloidal polyester PU as the sole carbon and energy source and displacing esterase activity to their inability to grow solely on PU and the enzymes having to be induced. The majority

of data in the literature concerning bacterial biodegradation of PU have concentrated on *Comamonas acidovorans*, *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*. These soil isolates are capable of utilizing PU as the sole carbon and energy source. The soluble extra cellular polyurethane-degrading enzymes that these microbes produce have a high similarity to Group I lipases, which contain a G-X-S-X-G serine hydrolase motif. One membrane-bound enzyme expressed by *C. acidovorans* also contains a serine hydrolase motif; however, the highest degree of similarity for this enzyme is with an acetylcholinesterase.

This chapter describes the microorganisms, their enzymes and genes involved in PU degradation. A basic understanding of the biological processes that include the role of polyurethane-degrading enzymes will enhance in the development of new bioremediation techniques of polyurethane waste and the creation of strains for this purpose.

1. Introduction

Polyurethanes are present in many aspects of modern life. They represent a class of polymers that have found a widespread use in the medical, automotive and industrial fields. Polyurethanes can be found in products such as furniture, coatings, adhesives, constructional materials, fibers, paddings, paints, elastomers and synthetic skins. Polyurethane should be abbreviated to PUR in compliance with official German and International standards. However, the abbreviation PU is more common in English texts.

Advantages of polyurethanes are that they have increased tensile strength and melting points making them more durable [1]. Their resistance to degradation by water, oils, and solvents make them excellent for the replacement of plastics [2]. As coatings, they exhibit excellent adhesion to many substances, abrasion resistance, electrical properties and weather resistance for industrial purposes [2-4]. Depending on the chemical structures of the polyisocyanates and polyols, PU can adopt various forms ranging from flexible to rigid and from low density to solid elastomer.

The chemical composition of PU precludes them from being classified as pure plastics but rather as a mixed polymer. The urethane group, which is the basis of this class of plastics, represents a small part of the macromolecule and some PU products do not contain a urethane group. Despite the lack of this base unit, all PU are based on the composition of polyisocyanates. The polyisocyanate polyaddition is distinct from polymerization and polycondensation for the production of synthetic polymers and this feature explains their versatility.

The global plastic consumption in 1997 totaled about 145 million tons with polyurethanes comprising a 5 % share resulting in PU being fifth in global plastic consumption [5]. Over three-fourths of the global consumption

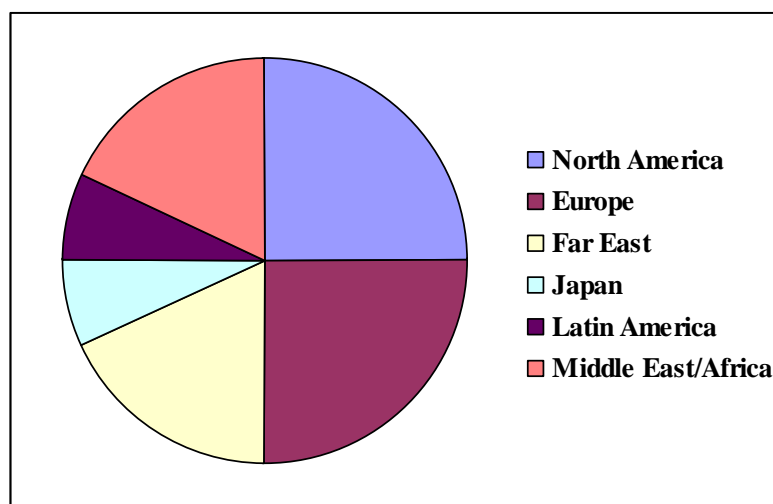
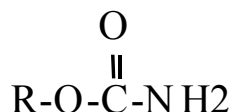


Figure 1. The main global consumers for polyurethane are North America (25 %), Europe (25 %), the Far East (18 %), Japan (7 %), Latin America (7 %), and the remaining split between the Middle East and Africa [5].

of PU is in the form of foams. In the United States alone, the production of PU increased from 45,000 tons in 1960 to 2,722,000 tons in 2004. The main global consumers of polyurethane are summarize in Figure 1.

2. Physical and chemical properties

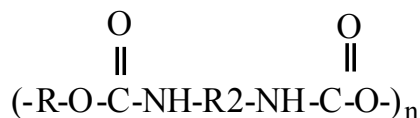
Polyurethanes were first produced and investigated by Dr. Otto Bayer in 1937. Polyurethane is a polymer in which the repeating unit contains a urethane moiety. Urethanes are derivatives of carbamic acids which exist only in the form of their esters [6]. This structure can be represented by the following, generalized amide-ester of carbonic acid:



Variations in the R group and substitutions of the amide hydrogen produce multiple urethanes. Although PU may contain urethane groups, other moieties such as urea, ester, ether or an aromatic may be included [2]. The addition of these functional groups may result in fewer urethane moieties in the polymer than functional groups.

The urethane linkage results most readily through the reaction of an isocyanate with an alcohol [6, 7]. The hydrogen atom of the hydroxyl group

is transferred to the nitrogen atom of the isocyanate [1]. The major advantage of PU is that the chain is not composed exclusively of carbon atoms but rather of heteroatoms, oxygen, carbon and nitrogen [1]. The simplest formula for PU is linear and represented by:



R represents a hydrocarbon containing the alcohol group, R₂ is a hydrocarbon chain and n is the number of repetitions. Diisocyanates are employed in PU production reactions because they will react with any compound containing active hydrogen [6].

Table 1. Raw materials for synthesis of polyurethane.

Polyisocyanate	2,4-Tolylene diisocyanate 4,4'-Diphenylmethane diisocyanate 1,3-Xylylene diisocyanate Hexamethylene diisocyanate 1,5-Naphthalene diisocyanate
Polyol	
Polyester-type	Poly(butylene adipate) Poly(ethylene butylene adipate) Poly(ethylene adipate) Polycaprolactone Poly(propylene adipate) Poly(ethylene propylene adipate)
Polyether-type	Poly(oxytetramethylene) glycol Poly(oxypropylene) glycol Poly(oxypropylene)-poly(oxyethylene) glycol
Chain extension/crosslinking agent	1,4-Butanediol Ethylene glycol 1,3-Butanediol 2,2-Dimethyl-1,3-propanediol Trimethylpropane Glycerol 1,2,6-Hexanetriol

For industrial applications, a polyhydroxyl compound can be used. Similarly, polyfunctional nitrogen compounds can be used at the amide linkages. By changing and varying the polyhydroxyl and polyfunctional nitrogen compounds, different PU can be synthesized [6]. Polyester or polyether resins containing hydroxyl groups are used to produce polyester- or polyether-PU respectively [3]. Examples of the raw materials used in the synthesis of PU are summarized in Table 1.

Variations in the number of substitutions and the spacing between and within branch chains produce PU ranging from linear to branched and flexible to rigid. Linear PU is used for the manufacture of fibers and molding [3]. Flexible PU is used in the production of binding agents and coatings [2]. Flexible and rigid foamed plastics, which make up the majority of PU produced, can be found in various forms in industry [4]. Using low molecular mass pre-polymers, various block copolymers can be produced. The terminal hydroxyl group allows for alternating blocks, called segments, to be inserted into the PU chain. Variation in these segments results in varying degrees of tensile strength and elasticity. Blocks providing rigid crystalline phase and containing the chain extender are referred to as hard segments [4]. Those yielding an amorphous rubbery phase and containing the polyester/polyether are called soft segments. Commercially, these block polymers are known as segmented PU [8].

3. Polyurethane degradation

Research has been initiated to elucidate whether additives to the chemical structure of PU could decrease biodegradation. Kanavel *et al* [9] observed that sulfur-cured polyester and polyether PU had some fungal inertness. However, they noted that even with fungicides added to the sulfur- and peroxide-cured PU, fungal growth still occurred on the polyester PU and most fungicides had adverse effects on the formulations. Kanavel *et al.* [9] also recognized the need for physical testing of the PU after extended exposure to the activity of fungi.

Santerre *et al* [10] varied the amount of degradation products released by varying the physical makeup of the polyester PU, as coatings on glass tubes or as films. This implied that while urethane and urea groups are susceptible to hydrolysis, they are not always accessible to the enzyme and degradation may never proceed past the polymer surface. Although the polyether PU showed no significant degradation, they consistently showed higher radiolabel products release from soft-segment-labeled, enzyme-incubated samples than controls. The authors attributed these results to the shielding of ester sites by secondary structures and hydrogen bonding within the hard segment.

Santerre and Labow [11] tested the effect of hard segment size on the stability of PU against cleavage. Analysis was performed with polyether PU and their susceptibility to cholesterol esterase. Three polyether PU were synthesized with varying molar ratios of [^{14}C]-diisocyanate to chain extender and constant polyether makeup. A 10-fold increase in enzyme concentration of cholesterol esterase previously used [10] was used to approach plateau values for polyether PU hydrolysis. Upon treatment with cholesterol esterase, Santerre and Labow [11] observed that radiolabel release was significantly dependent on the amount of hard segment contained within the polymer. In the polymer with the lowest concentration of hard segment, higher numbers of carbonyl groups are exposed to the surface. With increased hard segment size, a greater number of carbonyl groups are integrated into secondary hard segment structures through hydrogen bonding. The investigators also concluded that an increase in hard segment size does lead to restrictions in polymer chain mobility.

In the medical field PU show resistance to macromolecular oxidation, hydrolysis and calcification [12]. Polyurethane elastomers are being used in place of other elastomers due to higher elasticity and toughness, and resistance to tear, oxidation and humidity [2, 6, 13]. In addition, polyether derivatives are inexpensive to produce as prepolymers, which can lower the overall cost of polymer production.

Huang and Roby [14] tested the biodegradability of polyamide-urethanes for medical purposes. They synthesized PU with long repeating units and alternating amide and urethane groups from 2-aminoethanol. The resulting partial crystalline fibers were observed to undergo hydrolysis by subtilisin less readily than polyamideesters with degradation proceeding in a selective manner. The amorphous regions on the PU were being degraded prior to the crystalline regions. These fibers showed promise as absorbable sutures and implants where *in vivo* degradation is needed. The investigators also noted that PU with long repeating units and hydrophilic groups would less likely to pack into high crystalline regions as normal PU, and these polymers were more accessible to biodegradation.

Tang *et al* [15] added surface-modifying macromolecules (SMM) containing fluorinated end groups to the base PU to reduce the material's susceptibility to hydrolysis by lysosomal enzymes. Synthesized polyester urea-urethanes were radiolabeled with [^{14}C] and coated onto small hollow tubes. Biodegradation experiments were carried out using methods previously established by Santerre *et al* [10]. Results indicated that degradation was inhibited by the SMM surface. Different SMM formulations provided varying degrees of enzyme resistance. It was noted that some SMM formulations were incompatible with the PU and led to increased

biodeterioration. The mechanism of inhibition was not deduced and will be the subject of further study.

In an attempt to increase biocompatibility and reduce bacterial adhesion on PU surfaces, Baumgartner *et al* [16] synthesized phosphonated PU. They used glycerophosphorylcholine (GPC) as the chain extender, which incorporated phosphorylcholine head groups into the PU backbone. This gave the PU surface some characteristics of a red blood cell surface. Physical tests on the PU showed a small decrease in tensile strength and transition temperature with increasing GPC concentration. Water absorption by the PU was increased with increased GPC content. To test bacterial adhesion to the PU, Baumgartner *et al* [16] used a radial flow chamber. They passed a culture of *Staphylococcus aureus* across phosphonated and unphosphonated PU at a rate of 8 ml/min. The phosphonated PU showed a decrease in bacterial adhesion with increased GPC content.

Lack of degradability and increasing depletion of landfill sites as well as growing water and land problems have led to concern about plastics [17]. As more and more raw materials (e.g. crude oil) become in short supply for the synthesis of plastics, recycling of waste plastics is becoming important [18]. Degradability problems promoted researchers to investigate modification or productions that led to either chemically degradable or biodegradable PU.

Huang *et al* [19] derived polyester PU from polycaprolactonediol in an effort to produce biodegradable PU for use in the medical field. Several different PU were made containing polyester subunits of various lengths. The polymers were subjected to degradation by the enzyme axion and two species of fungi. The enzyme and fungi degraded each PU. In addition, it was also noted that there was an increase in the biodegradability of the polyester PU with increase in the chain length of the polyesters.

In a later study, Phua *et al* [20] observed that two proteolytic enzymes, papain and urease degraded a medical polyester PU. The PU they tested was Biomer®, segmented, cross-linked polyester PU. Although cross linking was previously described as a way of inhibiting degradation [7], papain (molecular weight 20.7 kDa) had little difficulty in diffusing into the film and causing breaks in the structural integrity. Urease activity, because of its size (molecular weight 473 kDa), was limited to the PU surface and therefore was not significant. Phua *et al* [20] also proposed that papain degraded the polymer by hydrolyzing the urethane and urea linkages producing free amine and hydroxyl groups. The effect of papain on polyether PU was assessed by Marchant *et al* [21]. Comparison of papain activity to aqueous hydrolysis resulted in both releasing degradation products. Ether linkages were non-enzymatic ally hydrolyzed by water while degradation of the urethane groups was dependent on the presence of the proteolytic enzyme.

Labrow *et al* [22] treated polyester PU and polyether PU with human neutrophil elastase and porcine pancreatic elastase. The polyester PU was readily degraded by porcine pancreatic elastase at a rate ten times higher than by human neutrophil elastase. The rate of polyester PU degradation by porcine pancreatic elastase was also ten times higher than its activity against the polyether PU. Human neutrophil elastase had no significant activity against the polyether PU. These results indicate a distinct similarity to the degradation of PU by cholesterol esterase [10, 11, 23]. Inhibition of porcine pancreatic elastase was achieved with the elastase specific inhibitor NMSAAPVCMK.

4. Fungal biodegradation

After years of production of PUs, manufacturer's found them susceptible to degradation. Variations in the degradation patterns of different samples of PUs were attributed to the many properties of PUs such as molecular orientation, crystallinity, cross-linking, and chemical groups presented in the molecular chains which determine the accessibility to degrading-enzyme systems [24]. The regularity in synthetic polymers allows the polymer chains to pack easily, resulting in the formation of crystalline regions. This limits accessibility of the polymer chains to degradation whereas; amorphous regions on the PU can degrade more readily. Huang and Roby [14] observed PU degradation proceeded in a selective manner, with the amorphous regions being degraded prior to the crystalline regions. Also, it was observed that PUs with long repeating units and hydrolytic groups would be less likely to pack into high crystalline regions as normal polyurethanes, and these polymers were more accessible to biodegradation. Several investigators have suggested microbial attack on PUs could be through enzymatic action of hydrolases such as ureases, proteases and esterases [25-28].

Several reports have appeared in the literature on the susceptibility of PUs to fungal attack [7, 29, 30]. These studies revealed that polyester-type PUs are more susceptible to fungal attack than other forms. In addition, polyether PUs were noted to be moderately too highly resistant. Boubendir [31] isolated enzymes with esterase and urethane hydrolase activities from the fungi *Chaetomium globosum* and *Aspergillus terreus*. These organisms did not grow solely on PU and the enzymes had to be induced. Induction of the enzymes was accomplished by addition of liquid polyester PU to the growth media. Activity of the enzymes was determined by assays based on ethyl carbamate (urethane) as artificial substrate.

Four species of fungi, *Curvularia senegalensis*, *Fusarium solani*, *Aureobasidium pullulans*, and *Cladosporium* sp. were isolated based on their

ability to utilize a colloidal polyester PU (Impranil DLNTM) as the sole carbon and energy source [32]. *Curvularia senegalensis* was observed to have a higher PU-degrading activity and therefore subsequent analysis of this fungal isolate was carried out. An extracellular polyurethanase (PUase) displaying esterase activity was purified from this organism. The protein has a molecular mass of 28 kDa, is heat stable at 100 °C for 10 minutes and inhibited by phenylmethylsulphonylfluoride (PMSF).

Wales and Sagar [33] proposed a mechanism for the degradation of polyester PUs by extracellular esterases. Polyurethane degradation is the result of synergistic activity between endopolyurethanases and exopolyurethanases. Endoenzymes hydrolyze the PU molecule at random locations throughout the polymer chain leading to loss of tensile strength. Exoenzymes remove successive monomer units from the chain ends however, show little loss of tensile strength.

5. Bacterial biodegradation

In a large-scale test of bacterial activity against PUs, Kay *et al* [34] investigated the ability of 16 bacterial isolates to degrade polyester-PU. Seven of the isolates tested degraded PU when the media was supplemented with yeast extract. Two isolates, *Corynebacterium* sp. and *Pseudomonas aeruginosa*, could degrade PU in the presence of basal media. However, none of the isolates grew on PU alone. Physical tests of the degraded polyester PU revealed different but significant decreases in tensile strength and elongation for each isolate. In a further study [35] tested the chemical and physical changes in degraded polyester PU. Polyurethanes taken from *Corynebacterium* sp. cultures had significant reductions in both tensile strength and elongation after three days of incubation. Infra-red spectrophotometer analysis revealed the ester segment of the polymer to be the main site of attack. The investigators noted that supplementing the media with glucose inhibited esterase production. However, addition of PU did not increase esterase activity.

Halim *et al* [36] tested the growth of several species of bacteria on PU military aircraft paint. The investigators isolated *Acinetobacter calcoaceticus*, two *Pseudomonas* sp., *Pseudomonas cepacia*, and *Arthrobacter globiformis*. In addition, the U.S. Navy supplied two strains of *A. calcoaceticus*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. All species were capable of utilizing the polyurethane paint as a sole carbon and energy source with the exception of *P. cepacia*. Using fluorescein diacetate as an esterase substrate, the remaining species showed esterase activity in the absence of PU. This data indicated that the PUases were constitutively expressed.

5.1. Polyurethane degradation by *Bacillus*

Blake and Howard [37] reported bacterial degradation of a polyester PU (Impranil DLN) by a species of *Bacillus*. The pattern of degradation involved the binding of cells to the polymer with subsequent floc formation, and the degradation of substrate. The growth of the *Bacillus* sp. on a solid medium resulted in the visual disappearance of the polyurethane. The complexity of the bacteria-polyurethane interaction was more apparent when grown on a polyurethane liquid medium. Incubation of the *Bacillus* sp. in media supplemented with polyurethane resulted in the appearance of a chalky precipitate that appeared to be resistant to further degradation.

Electrophoretic mobility, electrical impedance, and dynamic light diffraction measurements were performed on the *Bacillus*-polyurethane system. *Bacillus* cells had a relatively weak net negative charge corresponding to a zeta potential of -6 mV. Colloidal polyurethane had a strongly negative charge with a zeta potential of -42mV. Complex formation between the PU and cells results in a zeta potential of -20 mV.

Electrical impedance data showed that on average the *Bacillus* cell had a volume of around 3.9 mm³ corresponding to a spherical equivalent diameter of just over 2 mm. The majority of the polyurethane particles were sufficiently small to be below the detection limit, 0.6 mm, for electrical impedance. The relative volumes as a function of size for polyurethane and *Bacillus* were determined by static light diffraction methods. The results from the static light diffraction methods verified that of the electrical impedance results.

The above methods were then used to examine the formation of a complex between *Bacillus* and polyurethane. The electrophoretic mobility data showed that the peaks that were associated with the free polyurethane and the free *Bacillus* were replaced by a single peak that possessed the size and charge properties anticipated for a complex of the large *Bacillus* with the strongly negatively charge polyurethane. This evidence was corroborated with electrical impedance measurements that showed there was an increase in the total volume of the *Bacillus* cells as a function of time as they were mixed with an excess of polyurethane. Evidence that the increase in cell size occurred at the expense of the polyurethane came from light diffraction measurements. Further evidence that the *Bacillus* cell forms a complex with polyurethane was obtained through microscopic observations. These observations showed that the majority of the cells in the presence of polyurethane were coated with small particles of various dimensions (Figure 2).

This evidence indicates that two populations exist in polyurethane cultures: one that is coated with polyurethane and one that is not. At lower

concentrations of polyurethane, it may be that the two populations of bacteria are dependent on different sources of nutrition. The first population is coated with polyurethane and the polyurethane is metabolized into small, soluble metabolites, which are released into the medium. The second population, which is not covered in polyurethane, uses the small, soluble metabolites produced by the first population to grow. At higher concentrations of polyurethane all the cells present in the media may be coated with polyurethane. The more cells coated with polyurethane the more polyurethane that is degraded and the more metabolites available for growth. This would result in polyurethane-coated cells, which are not free in solution and therefore not detectable.

A follow up study [38] revealed that when grown on 1 % Impranil DLNTM YES medium, a lag phase growth was noted for the first 5 h which was followed by logarithmic growth for 8 h, reaching a cell density of $2.60 \times 10^8 \pm 1.17 \times 10^7$. The Monod plot for all concentrations of polyurethane tested did not follow simple Monod kinetics. At higher concentration (9.0 mg/ml to 3.0 mg/ml) of Impranil DLNTM Monod kinetics were not observed. The μ values dramatically decreased at a concentration of 3.0 mg/ml from 1.5 mg/ml to 0.466 doublings/h from 0.721 doublings/h. The μ continued to drop at higher concentrations from 0.466 doublings/h at 3.0 mg/ml to 0.369 doublings/h at 9.0 mg/ml. This dramatic decrease in μ may be explained by observations in a previous study by Blake and Howard [37] that polyurethane was observed to accumulate on the cell surface of a *Bacillus* sp.

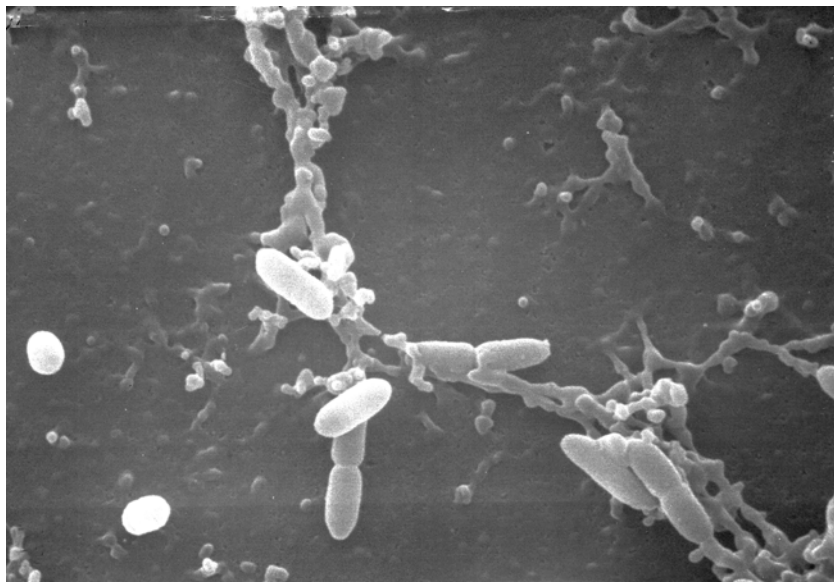


Figure 2. Scanning Electron Micrograph of complex formed between *Bacillus* cells and polyurethane after a 4 hour exposure.

5.2. Polyurethane degradation by *Pseudomonas*

Three Pseudomonads have been isolated for their ability to utilize a polyester PU as the sole carbon and energy source. Interestingly, the three species of bacteria produce different PUase activities but are inhibited by serine hydrolase inhibitors. These data suggest that either esterase and/or protease activities are involved in the degradation of Impranil.

Growth of *Comamonas acidovorans* on colloidal polyester-polyurethane resulted in growth parameters for K_S and μ_{max} of 0.3 mg/ml and 0.7 doublings/h respectively [39]. A 42kDa PUase enzyme displaying esterase/protease activity has been purified and characterized [39]. Nakajima-Kambe *et al* [40, 41] reported a strain of *C. acidovorans* that could utilize solid polyester PU as the sole carbon and nitrogen source. These authors indicated the role of an extracellular membrane bound esterase activity in PU degradation. Purification of the membrane bound esterase revealed a thermally labile protein having a 62 kDa molecular mass [42]. *C. acidovorans* strain TB-35 was isolated from soil samples by its ability to degrade polyester PU [40]. Solid cubes of polyester PU were synthesized with various polyester segments. The cubes were completely degraded after 7 days incubation when they were supplied as the sole carbon source and degraded 48 % when they were the sole carbon and nitrogen source. Analysis of the breakdown products of the PU revealed that the main metabolites were derived from the polyester segment of the polymer. Gas chromatographic analysis revealed the metabolites produced were diethylene glycol, trimethylolpropane, and dimethyladipic acid. In agreement with these findings, a later study [43] examined the biodegradation of polyester-polyurethane foam by *P. chlororaphis* ATCC 55729. Concentrations of ammonia and diethylene glycol increased over time with an increase of bacterial growth and a decrease in PU mass. A possible biodegradative pathway of PU is shown schematically (Figure 3). Further analysis of strain TB-35 revealed that the degradation products from the polyester PU were produced by an esterase activity [41]. Strain TB-35 possesses two esterase enzymes, a soluble, extracellular and one membrane-bound. The membrane-bound enzyme was found to catalyze the majority of the polyester PU degradation. The membrane-bound PUase enzyme was purified and characterized [42]. The protein has a molecular mass of 62 kDa, heat stable up to 65 °C and inhibited by PMSF. The structural gene, *pudA*, for the PU esterase was cloned in *Escherichia coli*. Upon nucleotide sequencing of the open reading frame (ORF), the predicted amino acid sequence contained a Gly-X-Ser-X-Gly motif characteristic of serine hydrolases. The highest degree of homology was detected with the *Torpedo californica* acetylcholinesterase

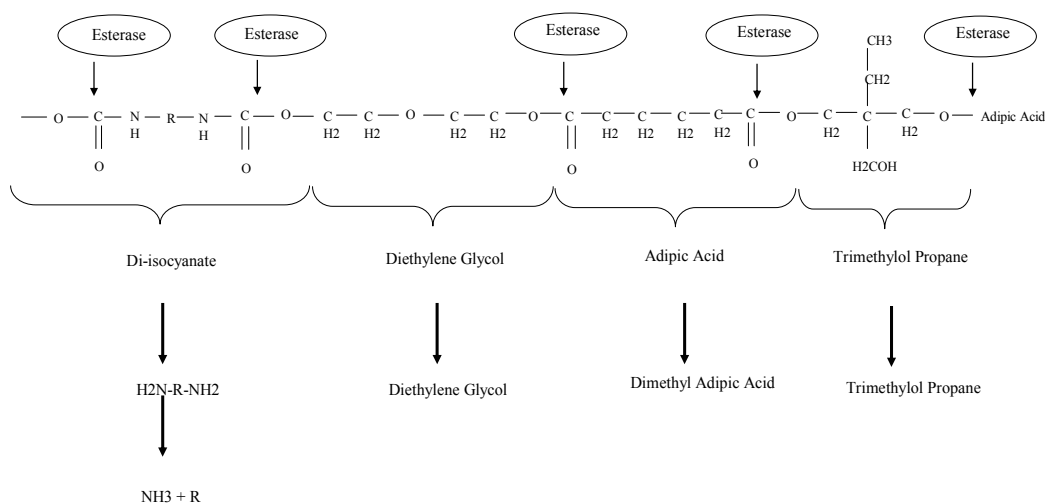


Figure 3. Theoretical degradative pathway of polyester-polyurethane by esterase activity of *Pseudomonas*.

(T ACh E), possessing the Ser-His-Glu catalytic triad, with the glutamate residue replacing the usual aspartate residue. Similarity in the number and positions of cysteine and salt bonds was very apparent between Puda and T AchE, as were also identities of sequences and their positions in the α -helix and β -strand regions between the two. In the neighborhood of the glutamate residue of the Ser¹⁹⁹-His⁴³³-Glu³²⁴ catalytic domain of Puda, there were three hydrophobic domains, one of which constituted the surface-binding domain, which occurred in the C-terminus of most bacterial poly(hydroxyalkanoate) (PHA) depolymerases.

Growth of *Pseudomonas fluorescens* on PU resulted in values of 0.9mg/ml and 1.6 doublings/h for K_S and μ_{max} respectively [44]. Two PUase enzymes have been purified and characterized from this bacterial isolate, a 29kDa protease [44] and a 48kDa esterase [45]. In addition, to the enzymology of the PUases the gene encoding a 48 kDa protein has been cloned and expressed in *E. coli* [45]. The gene encoding Pula has been sequenced (Genbank, Accession AF144089). The deduced amino acid sequence has 461 amino acid residues and a molecular mass of 49 kDa. The Pula amino acid sequence showed high identity with Group I lipases (58 to 75 %).

Growth of *Pseudomonas chlororaphis* on polyurethane resulted in values of 0.9 mg/ml and 1.3 doublings/h for K_S and μ_{max} respectively [46]. Two PUase enzymes have been purified and characterized, a 65 kDa esterase/protease and a 31 kDa esterase [47]. A third PUase enzyme, 60 kDa esterase, has been partially purified and characterized [46]. Two genes

encoding polyurethanase activity from *P. chlororaphis* have been cloned in *E. coli* [48, 49]. Both genes can be expressed in *E. coli*. However, the PueA enzyme is secreted in the recombinant *E. coli* and displays a beta-zone of clearing on polyurethane agar plates while PueB is not secreted in the recombinant *E. coli* and displays an alpha-zone of clearing on polyurethane agar plates. In addition, PueB has been noted to display esterase activity towards ρ -nitrophenylacetate, ρ -nitrophenylpropionate, ρ -nitrophenylbutyrate, ρ -nitrophenylcaproate, and ρ -nitrophenylcaprylate while PueA has been reported to display esterase activity only towards ρ -nitrophenylacetate and ρ -nitrophenylpropionate.

Upon cloning PueA [48] and PueB [49] from *P. chlororaphis* in *Escherichia coli*, the recombinant proteins were noted to have a high homology to Group I lipases. This family of lipases and other serine hydrolases, are characterized by an active serine residue that forms a catalytic triad in which an aspartate or glutamate and a histidine also participate [50-52]. Sequence analysis of the two-polyurethanase genes revealed that both encoded proteins contain serine hydrolase-like active site residues (G-H-S-L-G) and a C-terminal nonapeptide tandem called repeat in toxin (RTX), (G-G-X-G-X-D-X-X-X) repeated three times. Group I lipases lack an N-terminal signal peptide but instead contain a C-terminal secretion signal. The secretion of these enzymes occurs in one step through a three-component, ATP-binding cassette (ABC) transporter, Type I secretion system [53]. Proteins secreted by Type I systems typically exhibit two features: (i) an extreme C-terminal hydrophobic secretion signal located within the last 60 amino acids that is not cleaved as part of the secretion process and (ii) α -roll structure stabilized by glycine-rich RTX motifs. The RTX repeats form a Ca^{2+} -roll. These ions coordinated between adjacent coils of the motifs are thought to be important for proper presentation of the secretion signal to the secretion machinery, but their exact role is controversial.

Table 2. Identity comparison of PueB and other serine hydrolases.

Protein	Length (aa/nt)	% Identity (aa/nt) ^a	Strain	Accession number
PueB	567/1704	100/100	<i>Pseudomonas chlororaphis</i>	EF175556
PueA	617/1801	42/59	<i>Pseudomonas chlororaphis</i>	EF175556
Pu1A	451/1353	24/41	<i>Pseudomonas fluorescens</i>	AF144089
PudA	548/1644	11/31	<i>Comamonas acidovorans</i>	AB009606
TliA	476/1428	26/40	<i>Pseudomonas fluorescens</i> B52	AF083061
LipA	613/1789	36/53	<i>Serratia marcescens</i> SM6	BAA02519
Lipase	617/1801	39/55	<i>Pseudomonas</i> sp. MIS38	BAA84997
LipApf33	476/1428	27/41	<i>Pseudomonas fluorescens</i> 33	BAA36468
Lipase	449/1338	25/39	<i>Pseudomonas fluorescens</i> SIK W1	JQ1227

^a Amino acid and nucleotide identities were determined with Bioedit version 4.8.8 program.

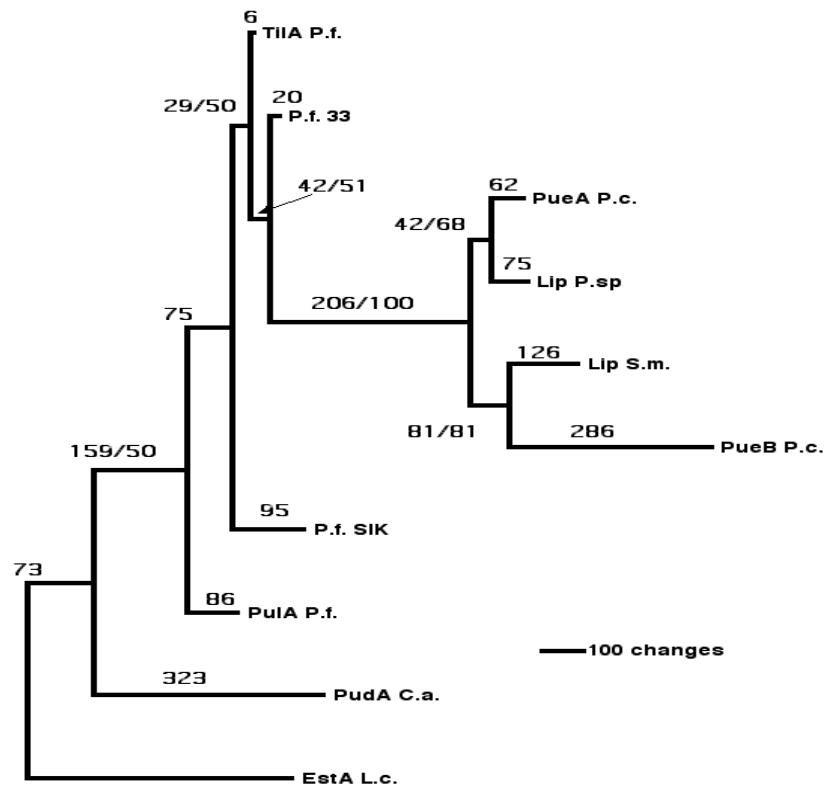


Figure 4. Single most parsimonious tree inferred from the phylogenetic analysis of polyurethanases and lipases. The numbers above the branches depict total character support/bootstrap support for each branch and node. Branch lengths reflect number of changes estimated along each branch.

Comparison between the amino acid and nucleotide sequences of these two genes revealed that they share 42 % and 59 % identity respectively (Table 2). Parsimony analysis of the predicted amino acid sequences for the PueA, PueB, PudA, and Pula polyurethanase enzymes and similar lipase enzymes was also performed (Figure 4). Interestingly the polyurethanase enzymes do not form a single cluster, but appear to be distributed among multiple lineages [49]. These analyses suggest that the polyurethanase enzymes thus far studied have evolved from lipases, and are not derived from a single source.

Howard *et al* [54] identified a gene cluster resembling a binding-protein-dependent ABC transport system in *Pseudomonas chlororaphis* in connection with PueA and PueB (Figure 5). The identified ABC transport system consists of three components: an ATPase- binding protein (ABC), an integral membrane protein (MFP), and an outer membrane protein (OMP). The ABC

pathway has been shown to mediate translocation of an alkaline protease in *Pseudomonas aeruginosa* [55]. Also, the ABC pathway has been shown to be involved in secretion of a lipase from *Serratia marcescens* [56], which is located separately from the lipase gene on the chromosome and secretes protease, lipase and S-layer proteins [58]. A gene cluster (accession number AF083061) was identified for an ABC transporter specific for a lipase in *Pseudomonas fluorescens* SIK W1 [59] and a similar gene cluster (accession number AB015053) was identified in *Pseudomonas fluorescens* 33 for a lipase gene and two serine proteases [57]. Interestingly, when the two ABC exporter gene clusters of *Pseudomonas fluorescens* are compared to the ABC exporter gene cluster of the one found in *Pseudomonas chlororaphis*, a unique gene arrangement is observed (Figure 5). It appears that the novel gene arrangement observed is a combination of the two *P. fluorescens* gene clusters, and may have resulted through either a rearrangement or an insertional event between the two ABC gene clusters observed in *P. fluorescens*.

Further investigation of the gene cluster involved growth studies to compare the effects of a PueA deficient strain and a PueB deficient strain with the wild type strain in polyurethane utilization (Table 3). *Pseudomonas chlororaphis* wild type and its PueA derivatives when grown on 1 % Impranal DLN™ YES medium exhibited a lag phase growth for the first 3 h then was followed by logarithmic growth for 6 h. The wild type reached a cell density of $2.31 \times 10^8 \pm 0.87$. The PueA mutant, *P. chlororaphis pueA::Kan^r*, had an 80 %

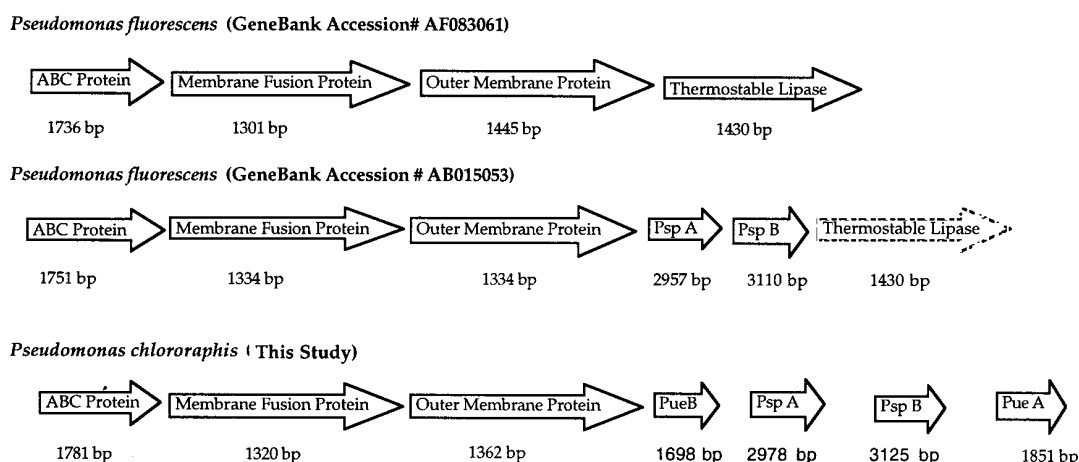


Figure 5. Comparison of the gene clusters from two strains of *Pseudomonas fluorescens* and the PUase gene cluster from *Pseudomonas chlororaphis*. The ABC Reporter Protein, Membrane Fusion Protein and Outer Membrane Protein are involved in Type I translocation of the extracellular protein. The PspA and PspB proteins are serine protease homologues.

Table 3. Growth kinetic analysis of *P. chlororaphis* and its derivatives using polyurethane as the sole carbon source^a.

Strain	μ_{\max}	doubling time, min	K_s , mg/ml	Cell density, cells/ml
<i>P. chlororaphis</i> (wild type)	1.32	31.5	0.800	$2.31 \times 10^8 \pm 0.87$
<i>P. chlororaphis pueA::Kan^r</i>	1.09	38.2	0.917	$4.66 \times 10^7 \pm 0.13$
<i>P. chlororaphis pueA::Kan^r</i> (pPueA-1)	1.41	29.5	0.710	$2.86 \times 10^8 \pm 0.09$
<i>P. chlororaphis</i> (pPueA-1)	1.54	27.0	0.649	$3.85 \times 10^8 \pm 0.98$
<i>P. chlororaphis pueB::Kan^r</i>	1.19	34.9	0.893	$2.35 \times 10^8 \pm 0.148$
<i>P. chlororaphis pueB::Kan^r</i> (pPueB-1)	1.37	30.4	0.735	$3.59 \times 10^8 \pm 0.187$
<i>P. chlororaphis</i> (pPueB-1)	1.41	29.5	0.781	$3.99 \times 10^8 \pm 0.813$

^a The concentrations of Impranil DLNTM used were: 9.0 mg/ml, 6.0 mg/ml, 3.0 mg/ml, 1.5 mg/ml, 0.75 mg/ml, 0.54 mg/ml, 0.375 mg/ml, and 0.18 mg/ml. Each concentration was prepared in triplicate.

decrease in cell number ($4.66 \times 10^7 \pm 0.13$) whereas, both the complement, *P. chlororaphis pueA::Kan^r* pPueA-1 and *P. chlororaphis* pPueA-1 had an increase in cell densities, $2.86 \times 10^8 \pm 0.09$ (25 % increase) and $3.85 \times 10^8 \pm 0.98$ (65 % increase) respectively. The results obtained from the cell densities of each strain were reflected in the growth kinetic studies. Values for K_s and μ_{\max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0.18 mg/ml to 9.0 mg/ml. *P. chlororaphis*, wild type exhibited a μ_{\max} of 1.32 whereas, the PueA insert ional mutant, *P. chlororaphis pueA::Kan^r*, exhibited a μ_{\max} of 1.09. It would be hypothesized that a deletion of the *pueA* gene would result in a decrease in growth rate. However, the large decrease in growth obtained from the insert ional mutant may indicate that PueA plays a more major role as compared to PueB in polyurethane degradation by *P. chlororaphis*. When multiple copies of the *pueA* gene were introduced into either the wild type, *P. chlororaphis* pPueA-1, a μ_{\max} value of 1.54, or the mutant, *P. chlororaphis pueB::Kan^r*, pPueA-1, a μ_{\max} value of 1.41, was obtained. An increase in growth rate seems plausible since more PueA produced from the added plasmid would reflect more polyurethane degraded, resulting in an increase in the amount of nutrients available to the cells.

The PueB mutant, *P. chlororaphis pueB::Kan^r*, had an 18 % decrease in cell number ($2.35 \times 10^8 \pm 0.148$) whereas, both the complement, *P. chlororaphis pueB::Kan^r* pPueB-1 and *P. chlororaphis* pPueB-1 had an increase in cell densities, $3.59 \times 10^8 \pm 0.187$ and $3.99 \times 10^8 \pm 0.813$ respectively. The results obtained from the cell densities of each strain were reflected in the growth kinetic studies. Values for K_s and μ_{\max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0.18 mg/ml to 9.0 mg/ml.

P. chlororaphis wild type exhibited a μ_{\max} of 1.31. When multiple copies of the *pueB* gene were introduced into the wild type, *P. chlororaphis* pPueB-1, a μ_{\max} value of 1.41 was obtained which was similar to the complement, *P. chlororaphis* *pueB*::Kan^r pPueB-1, μ_{\max} value of 1.37. An increase in growth rate seems plausible since more PueB produced would reflect more polyurethane degraded resulting in an increase in the amount of nutrients available to the cells. However, these values are small and may indicate that PueB plays a minor role as compared to PueA in polyurethane degradation by *P. chlororaphis*. The insertional mutant, *P. chlororaphis* *pueB*::Kan^r, displayed a μ_{\max} value of 1.19. Again, it would be hypothesized that the deletion of the *pueB* gene would result in a decrease in growth rate however; this small variation compared to the wild type suggests that degradation of polyurethane by *P. chlororaphis* may be more dependent on PueA.

5.3. Binding of polyurethane by polyurethanase enzymes

Enzyme molecules can easily come in contact with water-soluble substrates thus allowing the enzymatic reaction to proceed rapidly. However, the enzyme molecules are thought to have an extremely inefficient contact with insoluble substrates (e.g. PU). In order to overcome this obstacle, enzymes that degrade insoluble substrates possess some characteristic that allows them to adhere onto the surface of the insoluble substrate [59-61].

The observations made by Akutsu *et al* [42] for the polyurethanase Puda indicate that this enzyme degrades PU in a two-step reaction: hydrophobic adsorption onto the PU surface followed by the hydrolysis of the ester bonds of PU. The PU esterase was considered to have a hydrophobic-PU-surface binding domain (SBD) and a catalytic domain. The SBD was shown to be essential for PU degradation. This structure observed in Puda has also been reported in poly(hydroxyalkanoate) (PHA) depolymerase, which degrades PHA. PHA is insoluble polyester synthesized as a food reserve in bacteria. In PHA depolymerase enzymes, the hydrophobic SBD has been determined by amino acid sequence analysis and its various physicochemical and biological properties [60, 62]. Another class of enzymes that contain a SBD is cellulases. Several cellulase enzymes have been observed to contain three main structural elements: the hydrolytic domain, a flexible hinge region, and a C-terminus tail region involved in substrate binding [63-65].

Thus far, only two types of PUase enzymes have been isolated and characterized: a cell associated, membrane bound PU-esterase [42] and soluble, extracellular PU-esterases [39, 45, 46]. The two types of PUases seem to have separate roles in PU degradation. The membrane bound PU-esterase would allow cell-mediated contact with the insoluble PU substrate

while, the cell-free extracellular PU-esterases would bind to the surface of the PU substrate and subsequent hydrolysis. Both enzyme actions would be advantageous for the PU-degrading bacteria. The adherence of the bacteria cell to the PU substrate via the PUase would allow for the hydrolysis of the substrate to soluble metabolites which would then be metabolized by the cell. This mechanism of PU degradation would decrease competition between the PU-degrading cell with other cells and also allow for more adequate access to the metabolites. The soluble, extracellular PU-esterase would in turn hydrolyze the polymer into smaller units allowing for metabolism of soluble products and easier access for enzymes to the partially degraded polymer.

Studies addressing binding of PUase to soluble PU have been performed. The equilibrium binding of Impranil DLN (polyester-polyurethane) to purified PueA from *Pseudomonas chlororaphis* was studied by kinetic exclusion assays conducted on a KinExA flow fluorimeter. Briefly, the KinExA comprises an immunoassay instrument that exploits an immobilized form of the polyurethane substrate to separate and quantify the fraction of unoccupied binding sites that remain in solution reaction mixtures of PueA and soluble polyurethane. In this case, the immobilized polyurethane was Bayhydrol 110 adsorption coated onto polystyrene beads, while the soluble polyurethane was Impranil DLN. The results of these binding studies are summarized in Figure 6. Kinetic exclusion assays conducted with 6.6 $\mu\text{g/ml}$ PueA in the absence of soluble polyurethane produced fluorescence signals of greater than 2.2 volts with mvolt noise. In the presence of increasing concentrations of soluble Impranil DLN, the fluorescence signal attributed to PueA with unoccupied binding sites decreased to an extrapolated constant value at an infinitely high concentration of the soluble polyurethane that represented nonspecific binding to the beads. The fraction of soluble PueA that contained unoccupied polyurethane binding sites was calculated as the ratio of the difference between the fluorescence signal observed in the absence of Impranil DLN minus that observed in its presence, divided by the difference in fluorescence signals between zero and an infinitely high concentration of the soluble polyurethane.

The binding data in Figure 6 were fit to a one-site homogeneous binding model with an apparent equilibrium dissociation constant of 220 ± 30 mg/ml Impranil DLN. Since both the soluble Impranil DLN and the immobilized Bayhydrol 110 are hydrolysable substrates for the active PueA enzyme, care was taken to perform individual measurements in such a manner as to minimize the time of exposure of the polyurethane substrates to the active PueA. Thus the PueA–Impranil DLN mixtures were assayed within two minutes of mixing, while the PueA captured on the immobilized Bayhydrol was exposed to the fluorescent labeling reagents and wash buffer within 4

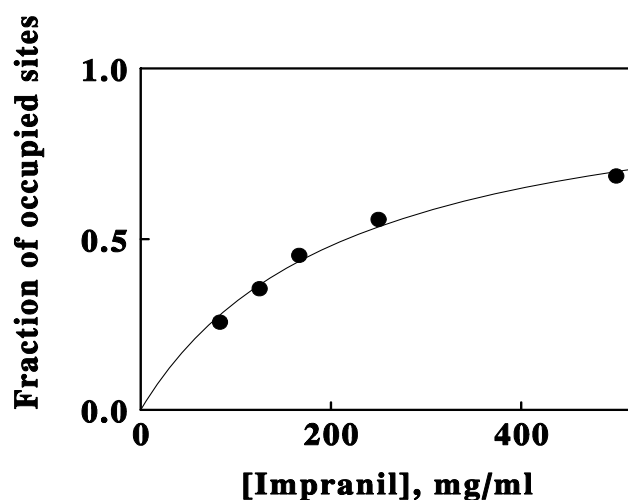


Figure 6. Equilibrium binding of Impranil DLN to PueA. The concentration of occupied polyurethane binding sites present in different reaction mixtures of PueA and soluble Impranil DLN were determined by kinetic exclusion assays on a flow fluorimeter as described in the text. Each determination was expressed as a fraction of the total PueA in solution and plotted vs. the concentration of free soluble polyurethane. Each datum represents the average of at least two determinations. The parameters for the curve drawn through the data were determined by nonlinear regression analysis using a one-site homogeneous binding model.

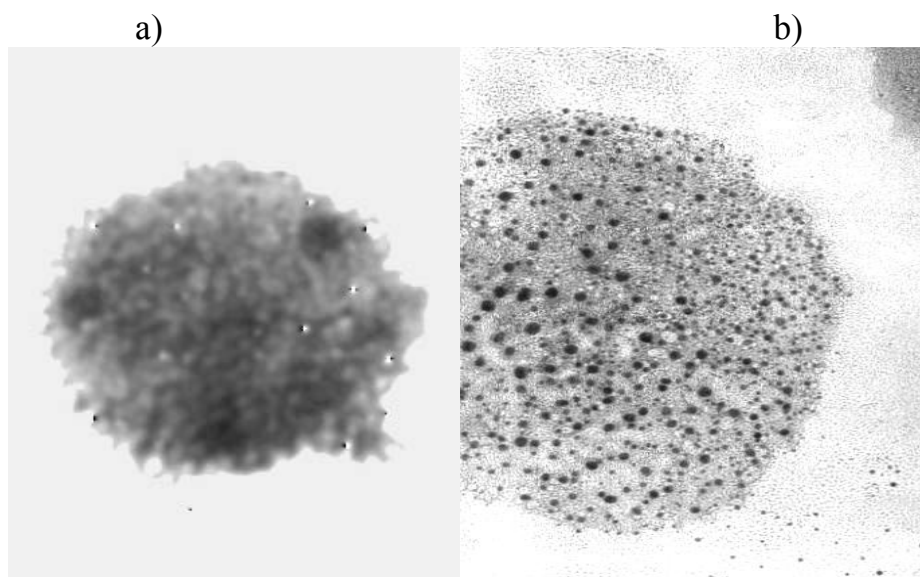


Figure 7. Electron micrographs of embedded Bayhydrol 110™ polyurethane particles. **a)** Electron micrograph of polyurethane particles taken at a magnification of 15,000 x. **b)** Electron micrographs of Immunogold-labeled PueA (1:5,000,000,000 dilution of 0.83 mg/ml PueA) bound to embedded polyurethane particle at 15,000x magnification.

minutes of the initial exposure of the hydrolase to the immobilized substrate. Control experiments demonstrated that much longer exposure times (at least 3-fold longer) were required before a time-dependent deterioration in individual fluorescence signals could be detected.

Electron micrographs were used in conjunction with the analysis of binding via the KinExA 3000, Kinetic Exclusion Assay unit. Grids were analyzed at high magnification and electron micrographs were produced from sections incubated in 1:5,000,000 PU and 1:5,000,000 PueA (Figure 7). The TEM analysis of PueA, showed PueA to have a high affinity for the polyurethane substrate. Binding was found to be so extensive, that only the most dilute concentrations of PueA could be used, to allow for visualization of areas with individual immunogold labeling.

Conclusions

The regularity in synthetic polymers allows polymer chains to pack easily, resulting in the formation of crystalline regions. Crystallinity limits accessibility of polymer chains to degradation whereas; amorphous regions within PU can degrade more readily. In addition, polyester-type PU is considered to be more susceptible to microbial attack than polyether-type PU. The hydrolysis of ester bonds in the polyester segments of PU has been shown to occur through esterase activity. Little information has been reported on the degradation of the isocyanate segment of PU however; the production of ammonia indicates this attack does occur.

A diverse group of microorganisms including fungi and bacteria capable of PU degradation can be isolated from soil. The majority of information available to date concerning the mechanisms that bacteria use in biodegradation of PU is from the Pseudomonad group. The esterase enzymes responsible for PU degradation were noted to have a high homology to Group I lipases. Upon nucleotide sequencing of these ORFs, the predicted amino acid sequence all contained a Gly-X-Ser-X-Gly motif characteristic of serine hydrolases. Parsimony analysis of the predicted amino acid sequences for the PueA, PueB, PudA, and PulA polyurethanase enzymes and similar lipase enzymes have been performed. Interestingly the polyurethanase enzymes do not form a single cluster, but appear to be distributed among multiple lineages. These analyses suggest that polyurethanase enzymes thus far studied have evolved from lipases, and are not derived from a single source. Learning more about the pathways for degradation and the genes involved in PU degradation is essential in developing either recombinant derivatives or enriching for indigenous PU-degrading microorganisms for bioremediation.

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